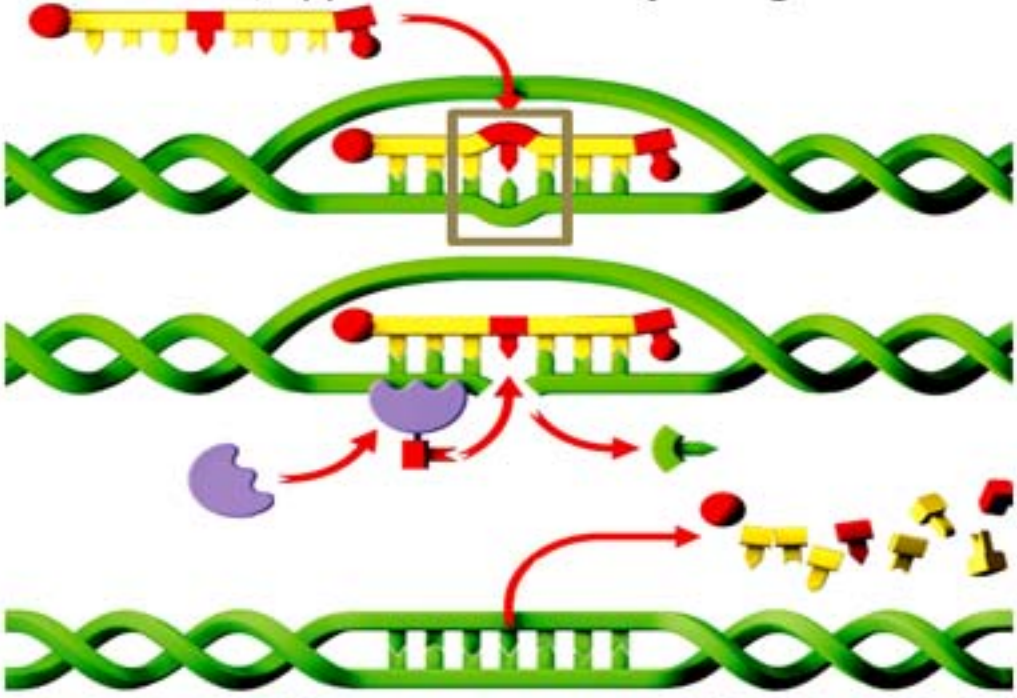
NABC Report 26

New DNA-Editing Approaches

Methods, Applications & Policy for Agriculture



Edited by

Allan Eaglesham & Ralph W.F. Hardy

2014

North American Agricultural Biotechnology Council Report

The cover illustration shows a non-transgenic breeding technology that uses the natural or inherent mismatch-repair system to effect a change. In eukaryotic cells, the gene-repair oligonucleotide (GRON) is delivered into the cell, traverses the cytoplasm to the nucleus, locates and binds selectively by homology pairing to its target sequence and effects (a) specific sequence change(s) in its target gene. Nucleases and other degrading enzymes in the cells then break down the GRON.

Courtesy of Greg Gocal (Cibus).

An annotated version is on page 102.

NORTH AMERICAN AGRICULTURAL BIOTECHNOLOGY COUNCIL

Boyce Thompson Institute, Tower Road, Ithaca, NY 14853

607-254-4856 Fax-254-8680 NABC@cornell.edu

http://nabc.cals.cornell.edu

Providing an open forum for exploring issues in agricultural biotechnology

NABC Report 26

New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture

Proceedings of the twenty-sixth annual conference of the North American Agricultural Biotechnology Council, hosted by Cornell University and Boyce Thompson Institute, October 8–9, 2014

Edited by
Allan Eaglesham and Ralph W.F. Hardy

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North American Agricultural Biotechnology Council
Boyce Thompson Institute B15
533 Tower Road
Ithaca, NY 14853

607-254-4856 fax-254-8680
nabc@cornell.edu
http://nabc.cals.cornell.edu

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* * *

On behalf of NABC, we thank Abel Ponce de León (University of Minnesota) for exemplary leadership as NABC's chair for 2013–2014.

Ralph W.F. Hardy
President
NABC

Allan Eaglesham
Executive Director
NABC

February 2015

[1]RWFH and AE also served on the planning committee.

PREFACE

Low rates of targeted gene deletion and editing in crop plants and livestock have limited advances in research and the application of these techniques to agriculture. Within the last few years, new technologies, such as zinc finger nucleases (ZFNs) and meganucleases, have been developed that have made targeted gene modifications feasible for several plant and animal species. Furthermore, the recent advent of two breakthrough gene-editing technologies, transcription activator-like effector nucleases (TALENs) and CRISPRs (clustered regularly interspaced short palindromic repeats)/Cas9, offer highly efficient and accurate means of gene editing that are being rapidly adopted by researchers. These technologies promise to greatly speed progress toward introduction of crop and livestock genotypes with valuable new traits not achievable in reasonable timeframes using conventional breeding techniques. Importantly, the ZFN, meganuclease, TALEN and CRISPR/Cas9 genes responsible for creation both of targeted gene deletions and improved "replacement" genes can, themselves, be eliminated by conventional breeding to yield plants and livestock that potentially will not be classified as genetically modified organisms (GMOs).

The twenty-sixth annual conference of the North American Agricultural Biotechnology Council (NABC), *New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture*

- Offered a primer of the science behind the new methods for gene modification,
- Provided specific examples of improved crops and livestock that may soon enter the marketplace, and
- Addressed key policy issues underpinning oversight of these potential non-GMOs in the United States, Canada and beyond.

NABC 26—hosted by Cornell University and Boyce Thompson Institute in Ithaca, New York, October 8–9, 2014—brought together academic researchers, industry leaders, and government officials. Presentations were grouped under five topics:

- Keynotes,
- Technologies,
- Uses,
- Non-governmental regulatory aspects, and
- Governmental regulatory aspects.

For the final session, a panel of speakers responded to questions from each other and from the participants. A poster session was held on the evening of the first day, during a reception.

Participants in the *Student Voice at NABC* program[1] attended the keynote and plenary sessions and met as a group after the reception to discuss issues that emerged from the first day's proceedings.

This volume contains an overview of chief points that emerged from the conference, manuscripts provided by some of the speakers, transcripts of verbal presentations by other speakers, transcripts of Q&A sessions, which included attendee participation, a report from the *Student Voice* delegates, and poster abstracts.

NABC's twenty-seventh annual conference—*Stewardship for the Sustainability of Genetically Engineered Crops: The Way Forward in Pest Management, Coexistence and Trade*—will be hosted by the Pennsylvania State University on the State College campus, June 2–3, 2015.

Allan Eaglesham
Executive Director
NABC

Ralph W.F. Hardy
President
NABC

[1]The *Student Voice at NABC* program provides grants of up to $750 to graduate students at NABC-member institutions (one student per institution) to offset travel and lodging expenses. Also, registration fees are waived for grant winners. Information on the *Student Voice at NABC 27* will be available at http://nabc.cals.cornell.edu/StudentVoice.htm.

CONTENTS

Overview and Summary of NABC 26
New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture

Allan Eaglesham[1] and Ralph W.F. Hardy
North American Agricultural Biotechnology Council (NABC)
Ithaca, New York

aeaglesh@twcny.rr.com

OVERVIEW

Recently developed technologies—zinc finger nucleases (ZFNs), meganucleases (MNs), transcription activator-like effector nucleases (TALENs) and CRISPRs (clustered regularly interspaced short palindromic repeats)/Cas9—offer highly efficient and accurate means of DNA/gene editing that are being rapidly adopted by researchers. These technologies—the focus of the twenty-sixth annual conference of the North American Agricultural Biotechnology Council (NABC 26)—promise to greatly speed progress toward introduction of crop and livestock genotypes with valuable new traits not achievable in reasonable timeframes using conventional breeding techniques. Importantly, these technologies, responsible for creation both of targeted gene deletions and improved replacement genes, can be eliminated by conventional breeding to yield plants and livestock that potentially will not be classified as genetically engineered/modified organisms (GMOs).

The presentations at NABC 26 were grouped in six areas:

- Keynote addresses,
- Technology descriptions,
- Uses of the technologies,
- Non-governmental regulatory aspects,
- Governmental regulatory aspects, and
- *Student Voice*.

[1]We are grateful to Don Weeks (University of Nebraska-Lincoln) for his suggestions for improvement in this chapter.

As editors, we have selected—based on our judgment—significant statements made by speakers in the keynote and plenary sessions, as well as in the Q&A and the "tie-up" sessions and the *Student Voice* report. As such, this overview includes inputs from many of the attendees.

Since regulations in the United States, Canada, and Europe differ substantially, we have grouped statements on regulatory aspects accordingly; also, we include a global grouping with broad relevance. This volume—*NABC Report 26*—provides full reports for the interested reader. The quality and quantity of these articles are excellent and cutting-edge, underpinning the development of new tools and their applications for plant and animal agriculture and for appropriate science-based risk/benefit oversight to ensure safety for food, feed and the environment.

Although the nominal focus of the conference was on DNA editing, the dialogue frequently turned to genetically engineered plants and animals (GMOs). The discussions touched upon in this chapter are presented under these two headings, in broad categories, with the objective of providing a relatively brief overview of the conference proceedings:

- **DNA Editing of Plants and Animals**
  - — Technology
  - — Uses
  - — Regulatory Issues
  - — General Considerations
- **Genetically Engineered (GM) Plants and Animals**
  - — Uses
  - — Regulatory Issues
  - — Labeling
  - — General Considerations.

SUMMARY

DNA Editing of Plants and Animals

Technology

- Zinc finger nucleases (ZFNs) were the first widely used gene-editing tools.
- ZFNs were made up of two separate zinc fingers (designed to bind specifically to two separate, but closely spaced, DNA sequences) with each ZF carrying a nonspecific nuclease domain that was designed to dimerize and cut the DNA sequence between the two zinc fingers.
- The next major step was the demonstration of ZFN activity in whole cells and organisms— where DNA cleavage by ZFNs (or other nucleases) can lead to gene knockout or gene replacement.
- Additional work showed efficacy in cultured human cells, in plants and in nematodes. ZFNs and other nucleases have now been used successfully to modify the genomes of more than fifty distinct organisms.

- A drawback to the use of ZFNs was the difficulty of deriving reliable designs for new targets.
- The discovery of a simpler DNA-recognition code in transcription activator-like effectors (TALEs) in *Xanthomonas* bacteria led to a new platform for targeted cleavage.
  - TALENs were rapidly adopted in preference to ZFNs, particularly in research labs, for the simplicity of their design, for the higher rate of success of new designs, and for the apparent sequence specificity of the ultimate reagents.
- The latest additions to the tool kit are the CRISPR/Cas RNA-guided nucleases. In this case, recognition is mediated by a guide RNA.
  - In addition, only a single protein, Cas9, is required for DNA cleavage. It doesn't need to be modified when the target is changed; only the guide RNA needs to be changed. These features have led to rapid adoption of CRISPR nucleases.
- Repetitive patterns in the genome of *Escherichia coli*, discovered over 25 years ago, have been found to be a defense system against viruses. Modification of this natural defense mechanism led to the development of the CRISPR/Cas9 system now used for gene editing.
- Ultimately, these targetable nucleases make breaks only at the desired DNA targets. Everything that happens afterward depends on cellular DNA-repair activities. This means that the outcomes of nuclease-mediated targeting events may be somewhat different, but usually result in desired gene knockouts or gene-replacement events.
- The majority of repairs proceed by a process called non-homologous end-joining (NHEJ) DNA repair—an error-prone process that often results in loss of gene activity (*i.e.* gene knockout).
- If extra copies of a modified gene are present at the time of DNA cleavage, homologous recombination (HR) can take place at the cut site. This results in the highly accurate installation of a new gene with new and potentially favorable activity.
- Genome engineering, as typically practiced, uses sequence-specific nucleases that recognize unique sites in the plant or animal genome and introduces targeted DNA double-strand breaks (DSBs).
- There are four classes of sequence-specific nucleases: Transcription activator-like (TAL) effector nucleases, zinc-finger nucleases, homing endonucleases or mega-nucleases and CRISPR/Cas9.
- Because no protein engineering is required and targeting is achieved simply through base pairing, CRISPR/Cas9 has emerged as the reagent of choice for making targeted chromosome breaks.

- The most striking feature of TAL effectors is the central repeats that are mainly 34 amino acids in length. The repeats are nearly identical except for the two amino acids at positions 12 and 13, the so-called variable di-amino acids. The di-amino acids actually determine the specificity of DNA binding for each repeat.
- Importantly, after TALENs have been introduced into plants and cause the desired genetic changes, the DNA sequences that contain the transgenic TALEN genes and the associated selectable-marker genes can be segregated out in progeny through standard genetic crossing. This results in plants that contain only the desired mutations and valuable new agronomic traits, but not the transgenes.
- The CRISPR/Cas9 system, is more affordable, remarkably easier to use, and well-suited for multiplex gene targeting and high-throughput genome-wide gene editing at similar or even higher efficiencies than ZFNs and TALENs.
- For many species, delivering the gene-editing tool into the cell remains a challenge.
- We have multiple competing platforms. If it is too expensive to use a TALEN to modify a crop, then a CRISPR approach may be a valid alternative.
- Synthetic biology is seen as an important new means of constructing modified organisms to produce products of value, with TALENs and CRISPERs as enabling tools.
- One prospect that scientists are currently excited about is applying Cas9 towards *in vivo* somatic cell editing.
- We are continuing to better understand the subtle properties of TAL effectors in part by studying them in their native context, but we are far from having fully exploited them in DNA-targeting applications.
- The promise of non-transgenic breeding technologies is their extraordinary breadth.
- EXZACT™ Precision Technology is based on ZFNs and has been used commercially to successfully modify field crops that soon will be in the marketplace.
- Using ZFNs, it is possible to target multiple genes to the same location, which decreases the number of loci involved in breeding and facilitates multi-trait product development.
- EXZACT™ Precision Technology is available and accessible both to the public and private sectors through a Dow AgroSciences' licensing agreement.
- Targeted gene deletion, gene editing and gene replacement have been demonstrated in tobacco, maize, canola, tomato and wheat.
- Enzymes and transcription factors from different organisms have different efficiencies. HR technologies allow for production of closely related enzymes and transcription factors, differing by one or two amino acids at the active site to be precisely modified, effectively mutagenizing a low-efficiency enzyme or transcription factor for greater efficiency.

— We can go in and make pretty much any modification we want in any gene we want whether it encodes an enzyme, a structural protein or a transcription factor. That capacity is currently there. As we look towards the future, designing for desired variations *in vivo* is certainly a possibility.

- Anyone setting out to achieve genetic modification with these gene-editing technologies must start with "a" genome of "a" plant for which the sequence is already known.
- In regard to regulatory oversight, one reason for needing a definition of gene editing is to "anchor" regulation and policy decisions. If you break or lose a DNA sequence—both forms of gene editing—regulatory guidance from the USDA says that this is not a regulated article. But nucleotide addition or replacement is more tricky. How many nucleotides over what span of DNA constitute an edit versus creation of a transgene?
  — Some scientist are uncomfortable saying what number of nucleotides for what span of DNA it would be, but perhaps such a definition will be needed.

Uses

- Whereas multiple strategies must be deployed to achieve food security, it is clear that amongst these is the need to accelerate the rate of crop improvement. Recent advances in genome engineering promise to make this possible. From targeted mutagenesis to targeted gene insertion, genome editing is transforming plant science, making it possible to create genetic diversity with precision, efficiency and control.
- Targeted mutagenesis is particularly valuable for altering gene activity or function.
  — Removing toxins, such as ricin from castor oil, or anti-nutritionals, such as trypsin inhibitors from soybean, are potential traits of value. Similarly, antigenic determinants that cause allergic reactions could be removed from nut or grain proteins.
- The real advantage of mutagenesis with sequence-specific nucleases is their precision. Traditional methods of mutagenesis that use chemicals, X- or gamma-rays, transposons or T-DNA provide virtually no control over where in the genome mutations are created.
- Sequence-specific nucleases rarely cleave at unintended or off-target sites and, thus, typically create mutations only at the intended sites.
- The high level of control afforded by DNA repair through HR makes it possible to create plant varieties with complex traits, such as tolerance of biotic or abiotic stress or that more efficiently use inputs such as fertilizer and water.
- With gene targeting, late-blight resistance in potato can be achieved in a much shorter timeframe than with traditional breeding and with only subtle alterations to the genome.
- The promise of gene editing in livestock is enormous.

- Gene editing—which allows geneticists to introduce (introgress) any natural trait into any breed without the use of recombinant DNA—has the potential of improving animal genetics for meeting increasing agricultural and biomedical needs with minimal environmental impact.
- With precision inactivation (knockout, KO) of specific genes required for organ development *in utero*, pigs could be used as bioreactors for production of donor-specific organs/tissues by *blastocyst complementation* or *exogenic organ production*.
- Gene editing saves about eight generations of backcrossing and the entire attendant screening for alleles desirable to industry.
- Gene editing is not limited to single changes. Because of the high efficiency of the procedure, multiple selected mutations can be simultaneously introduced into genomes.
- If a genetic alteration is not detrimental to an animal, it is highly unlikely it would be to humans.
- An important goal in developing TALEN technology for rice is to apply it for our basic scientific research and for breeding disease-resistant rice varieties.
- Collectively mutating all three disease-susceptibility-gene (S-gene) promoters causes plants to become durably and broadly resistant to bacterial blight disease.
- We have been using TALEN technology also to generate genetic materials of rice to gain basic understanding of the roles of rice SWEET (sucrose-transporter) genes in plant growth, development and production in addition to disease susceptibility.
- TALENs were successfully applied to edit the promoters of two disease-susceptibility SWEET genes to render the otherwise susceptible rice resistant to a broad range of bacterial-blight pathogen field isolates.
- CRISPR/Cas9 is highly efficient for genome editing in rice.
- In the United States, Cibus' commercial herbicide-resistance canola (***SU Canola***) is now in the launch phase. In Canada, in late 2013, Cibus and its partner BASF received PNT (plant with novel trait) approvals for herbicide-tolerant canola.
- An Expert Working Group on Novel Plant Breeding Techniques, appointed by the European Commission, concluded that Cibus' Rapid Trait Development System should be treated as mutagenesis and excluded from regulations applied to transgenes.
- Products like acrylamide-reduced potato and allergy-free peanut—with benefits that consumers can directly see—will bring links between the consumer and the science.
- The ARS is developing and refining genetic-engineering tools, including investigating technologies that utilize natural cellular mechanisms for genome repair that do not leave behind foreign DNA and precisely target genes of interest.

- There are two approaches for genetic engineering in farm animals:
  - — One is to modify the gene of interest in the genome of a somatic cell with either TALENs or CRISPRs.
  - — The second way is by direct modification of the gene of interest in the embryo genome, again using TALENs and CRISPRs.
- Recent reports indicate success in genetically engineering swine, cattle, and sheep by somatic cell nuclear transfer *(*SCNT) as well as direct embryo modification.
- To target the prion gene and, particularly, modify exon 3 in the prion gene, Cas9 nuclease was employed with a T7 promoter for *in vitro* transcription.
  - — Successful bi-allelic knock-outs, or modifications, were obtained at about 80 percent, by deletions as well as insertions.
  - — In some embryos, corrections were obtained by precise targeting and modification.
  - — Mono-allelic modifications were also effected.
- Insertion of a transgene into a particular locus has been achieved in a mammalian system.
  - — On the other hand, deletions occurred in some of the embryos.
- Putative knock-outs in swine embryos were achieved using NHEJ; this technology will be used to address animal-welfare issues.
- Pigs in particular have been modified with the CRISPR/Cas 9 system.
- There are possibilities of dual benefits where similar developmental issues in diseases apply to swine and humans.

Regulatory Issues–United States

- How will plant varieties created through gene targeting be regulated? Likely, each new variety will be considered on a case-by-case basis.
  - — The need for case-by-case evaluation of plants derived from gene targeting is warranted because of the range of modifications that can be created.
- Because genome engineering is a new approach to introduce genetic variation in plants, responsible regulation is required so that the technology can be best deployed for the public good.
- Policy issues associated with gene-editing in livestock and in biomedical research must be addressed for their real-world applications. Current deficiencies in regulatory oversight block enthusiasm for its adoption to agriculture.
- Regulatory pandering to public fears over food safety must change.
- Opinions rendered so far say that if the product does not contain pathogenic sequences, it should not be regulated.
- In the United States, it is encouraging to see that gene-editing of plants is being suggested as not requiring regulatory oversight. However, that concept remains under consideration *vis-à-vis* transgenic animals.

- It has been concluded by USDA-APHIS that the products of EXZACT™ Precision Technology fall outside their scope of regulation.
- The need to regulate plants developed through gene-editing techniques should be driven by the characteristics of the product (*i.e.* whether it is materially different from existing products present in food, feed or the environment) rather than by the method or process used to make that product.
- Because new plant-breeding technique (NPBT) approaches result in non-transgenic products, it is plausible that they may carry less perceived and actual risk, and that regulatory concerns will be minimal.
- It depends on whether you are looking at mutations versus addition of genes. One concern—especially with mutations—is harmonization in terms of what regulatory agencies are looking for.
- If regulatory oversight is developed for mutational products developed with the new technologies, it is to be hoped they will be harmonious and that required data will address questions of product and not process.
- Regulatory evaluation needs to be based on the safety of the product; if no risk is attendant on the product there should be little to no regulatory oversight.
- As of September 2014, APHIS has not been queried specifically about TALENS and CRISPRs/Cas9. However, in two letters—one on ZFN-1 and one on MN-1 breeding—APHIS stated that such plants were not subject to regulation because the techniques did not involve use of any plant pest at any stage.
- In light of these responses to letters of inquiry, USDA-APHIS appears poised to declare many—but not all—plants developed by the newer breeding techniques to be beyond its regulatory authority.
- In a recent scientific advisory panel (SAP) report, the SAP took a very precautionary approach to RNAi breeding and an affirmative view of the need for EPA to assert regulatory authority through FIFRA.
- FDA appears likely to assert that it will consider any animal modified by these newer breeding techniques also to be "new animal drugs."
- The basic message of a Venter report was that nothing in synthetic biology should avoid regulation.
- The advances in DNA editing are not yet changing the number or the type of applications for federal deregulation.
- Off-targeting is something we seek to avoid in genome editing.
- Fundamentally, we are interested in the phenotypes of these modified organisms, as opposed to what process was used in their production.
  - A paradigm shift from process to product would help to ensure a science-based evaluation of organisms developed through gene-editing technologies.

- It would be unwise to use the same regulatory process for DNA-edited crops as for GM crops. A better process should be adopted with correction of the problems caused by the current regulatory structure in the United States.
  - If there are potential food-safety risks, we should not adopt the FDA's voluntary process. A mandatory process is necessary to independently reassure the public regarding safety.

Regulatory Issues–Canada

- The Canadian Food Inspection Agency and Health Canada are committed to providing an efficient and appropriate level of regulatory oversight that encourages innovation while allowing Canadians to benefit from the advances brought by new technologies.
- The product-based approach allows the Canadian regulatory system to effectively adjust to any new developments in the science of plant breeding. Policy work is ongoing to help to ensure that guidance documents are available, as products of gene editing are brought forward for assessment.

Regulatory Issues–Europe

- There are several reasons to believe that the EU regulatory system will capture all newer breeding techniques.
- The European Food Safety Authority came to the conclusion that the aim of zinc-finger techniques is to integrate or exchange recombinant DNA and, therefore, it is comparable to transgenesis but more precise.
- The European Academies Science Advisory Council came to the following key conclusion and recommendation:
  - The trait and product not the technology in agriculture should be regulated, and the regulatory framework should be evidence-based.

Regulatory Issues–Global

- The Cartagena Protocol is likely to cover newer breeding techniques as regulated technologies.
- Without a paradigm change, poor and vulnerable populations will not have access to the new genetic-engineering technologies to enable them to raise their standards of living, improve their health and protect their environments.
- Differences in the regulation of new crops in different parts of the world will cause asynchrony in the approval of such crops. Consequently, global discussion concerning regulation of NPBTs is necessary to achieve synchronized and evidence-based governance.
- It is crucial for companies to be certain at the outset that their investments will not be in vain and that their future products will not be subject to the uncertain outcome of politicized regulatory procedures, as is the case with GMOs.

- When an organism does not contain recombinant DNA, it should not be risk assessed and regulated as a GMO.
- Flexibility is important—flexibility to learn and then to adjust as needs be and as new technologies come along.
  - Flexibility in the policy context is important also.
  - A universal, standardized set of definitions should be developed and utilized to mitigate confusion about the regulation, adoption, and legislation surrounding gene-editing technologies and their resulting products.
  - At issue is mutation versus gene addition. Part of the concern—especially with mutations—is harmonization in terms of what regulatory agencies are looking for.
  - If regulatory oversight is developed for mutational products developed with these technologies, it is to be hoped they will be harmonious and that required data will address questions of product and not process.
  - Regulatory evaluation needs to be based on the safety of the product; if no risk is attendant on the product there should be little to no regulatory oversight.

General Considerations

- The outcomes of our genetic modifications may be made even more precise, more controlled and more predictable so as to minimize concerns about off-target effects.
- Communication of science is essential, not only to the public but, as scientists and opinion leaders, to our communities as well as to the government.
- How should scientists address the public on the subject of gene-edited crops and livestock?
- Many people attending this conference can be thought leaders within their communities. Are there non-technical thought leaders who might be receptive to technical arguments?
- Gene editing is affected by the target sequence and reagent, and is not always precise. Appropriate standards are needed for determining whether or not there are off-target effects that affect the safety of the product:
  - In general, off-target effects are rather infrequent. Reasonable standards may be set for the types of products that are released. We have good genome sequences for most of the plants and animals with which we work. For a modest amount of money, we could determine the genome sequence in the plant or animal we wish to release to show that, in fact, the only mutation is the one actually wanted.
- Enzymes and transcription factors from different organisms have different efficiencies. Do these technologies allow for closely related enzymes and

transcription factors, differing by one or two amino acids at the active site, to be precisely modified, effectively mutagenizing a low-efficiency enzyme or transcription factor for greater efficiency?

— We can go in and make pretty much any modification we want in any gene we want whether it has a transcription factor or not. That capacity is currently there. As we look towards the future, screening *in vivo* for variations of interest is certainly a possibility.

- This conference is an attempt to assemble the available information in one place to see where we go from here into the future. Everybody wants the best outcome, For the public, the consumers, the industry, the researchers—everyone—the goal is the same.
- We have seen different sides of the issues from basic science to regulatory aspects to public acceptance or non-acceptance of this technology.
- We in the science community have to be available to speak about these issues and tell things as they are.
- Flexibility is important—flexibility to learn and then to adjust as needs be and as new technologies come along.
- By 2050, the demand for staple food crops alone will require yield increases of nearly 80%.
- Technologies that complement traditional management and breeding, but dramatically accelerate production and testing of improved crops, are in critical demand.
- Just as improved plant breeding and crop management spawned the Green Revolution in the 1960s, so too could these new technologies transform crop improvement in this generation.
- The mission of the Iowa State University Crop Bioengineering Consortium (CBC) is to deploy innovative, transformative genome-engineering technologies that identify, validate, and rapidly, but precisely, integrate strategically important traits and underlying genes into key crop plants.
- The CBC is establishing a platform comprising: active gene discovery and validation; incorporation of target gene modifications into crop plants using new NPBT approaches and novel delivery methods; trait verification and integration; and evaluation of regulatory, economic, environmental and societal impacts of the technology and the resulting traits.
- The time is ripe to launch a public-sector infrastructure for rapid, precise crop bioengineering.
- The CBC is developing high-throughput processes for all stages of the genome-engineering pathway, beginning with development of software for the prediction of CRISPR-editing targets for any gene in a variety of genomes.

- We could feed another 4 billion people if we could figure out how not to waste the food we produce, and a valid discussion is to be had about how much energy should go into production of various non-food agricultural products. However, there remains a need to produce more food.
  - There is no single solution to producing more food; the solutions include irrigation and pesticide application, and farming-equipment availability, along with improved breeding techniques.
  - Part of the solution is educational in terms of sharing science across the world.
- As we encounter new breeding technologies, especially site-directed gene-editing techniques, there would appear to be a window of opportunity to reframe public understanding of genetic engineering in agriculture.
- Properly answering *why are you doing this* is important for the public because many suspect that somebody is tinkering with something because they can do it rather than for a good reason.
  - If things are done by a multinational corporation, consumers are more hesitant than if they are done by a small company or by a public university.
- There is no need to regulate when there is no safety issue.
- A few articles have been published in scientific journals about gene-editing techniques and how they may be perceived and regulated. The consensus was that it would depend on whether exogenous DNA or endogenous DNA is involved. However, in practice, it may not be so simple.
- When consumers look at these new technologies, they are unlikely to appraise them simply on whether they contain introduced DNA. The situation is more complicated. They are going to consider, *inter alia*, the breeding method, the specifics of the trait, and the level of knowledge about the technique.
  - The factor that will influence consumer acceptance most is safety, and consumers will want to know who is ensuring safety.
  - A product in the public domain is a lot more acceptable to many consumers than if it's patent-protected.
- Consumers will need to know what scientists are doing and will need to have answers that are scientifically accurate and also understandable. The public does not want to be "dumbed down" to.
- Prevalent issues are:
  - What are the potential benefits and who benefits—who are the winners and who are the losers?
  - What is "natural"? We don't have a scientific definition, but, clearly, the public's perception of what is natural will come into play. The public may say that some things are natural that scientists would disagree with.

- If there are potential risks, then there should be oversight. Questions are:
  - What risks come from the process used?
  - What risks come from the products made from that process?
  - How does the risk profile compare to other agricultural breeding techniques and products?
- It is important to bear in mind that risk is not absolute, it's relative.

Genetically Engineered (GM) Plants and Animals

Uses

- Genetic engineering using recombinant-DNA vectors versus genome editing using site-specific DNAases: the gain in precision between the two methods is a factor of ten million.
- In the United States, not a single animal engineered for food production has been approved by the US Food and Drug Administration (FDA), which regulates GM animals.
- Transgenic technologies were greeted with more concern than enthusiasm by the general public and, especially, by several NGOs. The concerns focused on four areas:
  - Health effects due to the *un-naturalness* of products the modified genome might encode;
  - Environmental effects due to uncontrolled release of transgenes (*i.e.* GM animals) and reduced diversity of natural genomes;
  - Social concerns that huge corporations would have undue influence over diets; and
  - Moral concerns that were summed up by the phrase "playing God."

Regulatory Issues/United States

- The issue of huge corporations dominating the availability of GM products is, in large part, a direct consequence of the cumbersome regulatory processes.
- The mistake that was made in the United States, back in the 1980s, was to elect to use existing legislation. Although useful in the short term, this has been disastrous in the long term.
  - The United States did not adopt biotechnology-specific legislation. Rather, the US government developed a coordinated framework allowing the three primary administrative agencies [Department of Agriculture (USDA), Environmental Protection Agency (EPA), and Food and Drug Administration (FDA)] to develop policies under existing statutory authorities about regulating recombinant-DNA techniques.

- There is general agreement among regulatory agencies internationally that regulatory oversight should be reduced, but no one has proposed what the necessary data might be. The trend of requirement of more and more data should be reversed.
- The USDA Animal Plant Health Inspection Service (APHIS) created a category called a "regulated article" under the Plant Protection Act. EPA created a category called a "plant-incorporated protectant" (PIP) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). FDA created a voluntary consultation process for foods derived from biotechnology and later declared that all animals derived from biotechnology are "new drugs" under the Federal Food Drug & Cosmetic Act.
- USDA-APHIS has approved 96 petitions for non-regulated status.
  - USDA-APHIS now takes nearly 5 years to make a decision with a regulatory cost per trait of up to $34 million.
- Despite many successful and needed crop transformations by public-sector scientists, only one public-sector crop has ever achieved regulatory approval and commercial release: the virus-resistant papaya for Hawaii. APHIS has also approved a USDA-ARS virus-resistant plum, but EPA pesticide-labeling requirements have prevented its commercial release.
- EPA defined genetically-modified microorganisms (GMMs) as regulated "new chemicals" under the Toxic Substances Control Act (TSCA). Since then, EPA has approved one GMM for commercial use.
- FDA has not approved a single commercial release of animal agricultural biotechnology.
- The US regulatory system has not responded to the real-world evidence of benefits without novel harms. The US regulatory system could be improved through several efforts within the power of the regulatory agencies such as:
  - Adopting categorical exclusions;
  - Focusing anew on product, not process;
  - Exercising agency discretion to decline invoking new terms and new definitions that expand regulatory power; and
  - Creating a culture of facilitating innovation, science and technology.
- Regulatory officials at the three agencies regularly communicate and exchange information to ensure that any safety or regulatory issues that may arise are appropriately resolved.
- USDA-APHIS biotech regulations provide a petition process for the determination of non-regulated status.
- USDA-APHIS evaluates a variety of issues including the potential for plant-pest risk; disease and pest susceptibilities; the expression of gene products, new

enzymes, or changes to plant metabolism; weediness and impact on sexually compatible plants; agricultural or cultivation practices; effects on non-target organisms; and the potential for gene transfer to other types of organisms.

- The current regulations under 7 CFR 340[2] do not apply to all GM organisms or even all GM plants. For example, plants transformed by particle bombardment with DNA that is not derived from a plant pest do not trigger the regulations under 7 CFR 340.
- Although APHIS is not in a position to discuss agency deliberations on the proposed rule[3] until closeout, a document describes overarching principles for the regulation and oversight of the products of emerging technologies. These principles are described in a memo by the White House Office of Science and Technology Policy, partially as follows:
  - When no significant oversight issue—based on a sufficiently distinguishing attribute of the technology or the relevant application can be identified—agencies should consider the option not to regulate.
  - Decisions should be based on the best reasonably obtainable scientific, technical, economic, and other information, within the boundaries of the authorities and mandates of each agency.
  - Public participation is important for promoting accountability, for improving decisions, for increasing trust, and for ensuring that officials have access to widely dispersed information.
  - The federal government should actively communicate information to the public regarding the potential benefits and risks associated with new technologies.
  - The benefits of regulation should justify the costs (to the extent permitted by law and recognizing the relevance of uncertainty and the limits of quantification and monetary equivalents).
  - Federal regulation and oversight should provide sufficient flexibility to accommodate new evidence and learning and to take into account the evolving nature of information related to emerging technologies and their applications.
  - Risk assessment should be distinguished from risk management.
  - Federal agencies should seek to coordinate with one another, with state authorities, and with stakeholders to address the breadth of issues.
- Organisms engineered without plant-pest sequences may not fall under the 7 CFR 340 regulations.

[2]Regulation of organisms and products, altered or produced through genetic engineering, that are plant pests or for which there is reason to believe are plant pests.

[3]In early 2015, APHIS announced that it is abandoning the proposed rule, after several years' consideration, and will start over to produce a new rule.

- The USDA is not involved in oversight once the product has been deregulated. Development of insect resistance to *Bt* and/or weed resistance to herbicide, involves the EPA.
- Good stewardship requires monitoring for weeds that are becoming resistant and developing a rapid response to rectify that situation.
- Evaluators look for potential allergenic epitopes in proteins. If new information comes to light, they need to be told.
- USDA has a staff of about eighty on the regulatory side, about two-thirds of whom are scientists.

Regulatory Issues/Canada

- Canada takes a product-based approach to assessing plants with novel traits (PNTs) for use as food, as feed, and for release into the Canadian environment.
  - — The trigger for regulation in all cases is based on novelty.
  - — The regulatory trigger is not identical for novel foods, novel feeds, and PNTs. It is, therefore, necessary to consider whether a product may be novel under each relevant set of regulations.
- Completion of the regulatory process in Canada takes twenty months on average. Twelve to twenty-four months is typical.
- In Canada, only a half-dozen people are employed in regulatory agencies
- Once something is approved it is considered to be the same as any other cultivar that's out there.
  - — On the organic side, neighbors are encouraged to cooperate.

Regulatory Issues/Europe

- Requests to place a single event on the European market cost somewhere between 15 and 50 million euros.
- The European regulation provides consent for only 10 years. During these 10 years, monitoring is mandatory.

Regulatory Issues/Global

- Globally, regulatory oversight of biotech products is a time-consuming and expensive endeavor, estimated at $35 million per trait with an average of six years for regulatory approval/deregulation.
- At the international level, 168 countries have ratified the Cartagena Protocol on Biosafety, which governs the transboundary movement of "living modified organisms" (LMMs) from "modern biotechnology."
- Harmonization of GM regulations will increase international trade.
  - — In the international arena, harmonized regulations can result, detrimentally, in the lowest common denominator.

— Some scientists support, and strongly urge, international harmonization of requirements of scientific data and risk-analysis frameworks.

- There's a fairly large literature of poorly done anti-GM studies.

Labeling

- Why are GM crops examined so closely when food supplements are largely ignored?
- The information on food labels should have a bearing with regard to consumer safety.
- Labeling of GM foods has been reshaped by the protest industry over time, from a science issue to a choice issue.
- The protest industry is intent on using labeling to drive agricultural biotechnology out of the market
  - — It is allied with the organic industry, which sees this as a way of increasing their market share significantly.
- Those who are aligned against GM technology and foods derived from GM crops will use labeling as the next step in their campaign to denigrate and stigmatize this technology.
- In Europe, the advent of labeling meant that the processor simply stopped accessing any food that had an ingredient that required the food to be labeled, and it has had a tremendously detrimental impact.
- The purpose of a label is to provide effective, clear information to consumers so that they have safe foods.
- CSPI[4] does not support mandatory government-imposed labels except in situations where a safety or nutritional issue dictates it.
- The distinctions Europe makes between a food made *with* a GMO and a food made *from* a GMO should be dropped.
- The leading enzyme company, Novozymes of Denmark, produces many enzymes and if you were to label every food produced with those enzymes, it would entail almost 100% and become irrelevant.
- Across the country, numerous state and local governments have enacted, or are considering, laws that impact the cultivation, use, and labeling of GM plants. These laws are best described in three categories:
  - — Laws that ban the cultivation of GM plants;
  - — Laws that regulate the handling of GM plants; and
  - — Laws that impose disclosure requirements on the sale of GM plants, such as food labeling.

[4]Center for Science in the Public Interest.

- There are two types of labeling laws: those that apply to food and those that apply to seed.
- Only four states (to September 2014) have passed GM-food-labeling laws: Alaska, Connecticut, Maine and Vermont.
- Several states have enacted laws that require labeling of GM seed.
- In Vermont, a group of plaintiffs, led by the Grocery Manufacturers Association, is challenging the state's GM-food-labeling law alleging that the law violates:
  - The First Amendment's protection against forced commercial speech in requiring a label;
  - The First Amendment's protection against restricting commercial speech for preventing the use of the term "all natural" on food required to be labeled;
  - The Fifth Amendment's due process clause for containing vague terms regarding the restriction of using terms "similar" to "all natural";
  - The Commerce Clause for imposing unreasonable burdens on manufactures outside of Vermont; and
  - The Supremacy Clause on account of the fact that the law conflicts with federal law.
- If there is to be labeling *vis-à-vis* GM ingredients in foods, it should be in terms of stating their *absence*.

General Considerations

- Over the past 5 years, two obvious changes have been occurring: Of incoming freshmen, only 2 percent believe that they *don't* eat GM food; ninety-eight percent think either they are, or they might be, eating GM foods, and it doesn't concern them in the slightest. Secondly, most of them feel that regulatory policies fail to take advantage of recent developments, but they also feel that regulatory agencies can be trusted to save them.
- China needs fish for human consumption, but has deferred to the United States' approval of GM salmon, as have regulators in other countries who are waiting for the United States to do it the right way.
- The cost of DNA sequencing has decreased dramatically over the past two decades due to technological progress.
- Regarding transgenic techniques, the location at which the transgene lands is random.
- Biotechnology is largely in its infancy in terms of ability to modify algal genomes, especially in production-type algae.
- On the plant side, anti-GM rhetoric has been relatively quiet recently.
  - Greenpeace has been good at scaring the public about GM products; they can say anything on the Internet without proof.
  - There has been less resistance to *Bt* cotton because it isn't eaten.

- NABC's conference in 2013, on fruits and vegetables, focused in on three or four examples of consumer benefits. The non-browning Arctic apple was one of those and it seemed to be moving fairly quickly through the regulatory system; it's out for comment at the moment (October 2014).[5] Another example was *Bt* sweet corn, which doesn't require spraying with insecticide. Simplot's Innate™ potato technology is another good example, as is the (GM) means of tackling citrus-greening disease.
- The rapid and continuing global adoption of modern agricultural biotechnology has been encumbered by steadily increasing public anxiety, although scientists and regulators continually point to the weight of evidence that GM crops pose negligible risks to human health and the environment, *i.e.* GM crops are equivalent to their non-GM counterparts.
  - — Despite nearly two decades of safe use worldwide, large segments of the public continue to express concerns regarding foods derived from modern biotechnology.
  - — We must reframe genetic engineering using these new technologies in agriculture in a way that more effectively connects with the public.
- Traditional breeding, whether by farmers or by scientists, has been either unregulated or lightly regulated, primarily to assure seed purity and efficacy. Recombinant-DNA techniques have been carefully regulated domestically and internationally. The regulatory classifications of the newer techniques are still in debate and have much uncertainty.
- It's the retailer's job to understand customers' perceptions because their perceptions are their reality, whether or not they are based on fact.
  - — We have to listen to consumers and try to understand their concerns and their perceptions in order to gain their trust.
- Transparency without trust is useless, and trust doesn't happen overnight. You can't demand it. You have to earn it and you have to build it.
- The very large majority of customers' expressed concerns are focused on product recalls. By comparison, concerns over GMOs are few in number, and concerns over animal welfare are fewer still.
- At CSPI, they have looked at the data behind GM crops grown in the United States and concluded that foods made from those crops are safe to eat.
  - — There are benefits from those crops, to farmers and to the environment, although not necessarily any direct benefits to consumers. Those products need to be assessed on a case-by-case basis.
- Uses for agricultural biotechnology must be sustainable so that they are there for future generations of farmers.

[5]In early 2015, USDA approved the Arctic apple, which FDA is reviewing.

- Regarding consumers and food, the primary concerns are safety, healthfulness and nutrition. Taste is important, as are tradition and religious significance.
- Some consumers know a lot of about science, whereas some don't know much at all.
  - Consumers receive information from opinion leaders whose viewpoints they consider important and who may be with NGOs or universities.
  - However, for some consumers, if they believe something, scientific data and reasoned argument may not change their minds.
- International consensus indicates that GM crops are safe and beneficial.
- Selected breeding has been beneficial for the agricultural community with the production of superior animals with desirable production traits.
  - On the other hand, frequently, along with desirable traits, undesirable traits will segregate, such as susceptibility to diseases.
- A negative aspect of selective breeding is the length of time that it takes to achieve genotypic improvement; for cattle it can be about a quarter century.

Genome Engineering with Targetable Nucleases

Dana Carroll
University of Utah School of Medicine
Salt Lake City, Utah

dana@biochem.utah.edu

It is amazing what we can do with current tools of molecular biology. I often tell students we can answer questions today that we couldn't even phrase 30 years ago. For example, we can query whole genomes in a variety of ways, from sequencing to gene expression to chromatin status. In addition, for the last dozen years or so we have had the capability to modify genomes with great range, subtlety and specificity, and with striking efficiency. Part of this meeting is dedicated to describing the tools for making such modifications, and I will do my part. I will also offer a few thoughts about the applications of this technology in ways that have great potential for improving the human condition.

Zinc Fingers

First, a little history. As implied by their "nuclease" designations, the genome-engineering tools act by making breaks in DNA. Many years of research on many different organisms and in many laboratories led to an understanding by the mid-1990s that double-strand breaks are repaired by cellular mechanisms that rely on homologous recombination or error-prone end joining. This suggested that the very low frequency of "classical" gene targeting could be improved by making targeted double-strand breaks. Meanwhile, Chandrasegaran and colleagues discovered that the recognition and cleavage activities of the Type IIS restriction endonuclease, FokI, could be physically separated (Kim *et al.*, 1996). They replaced the natural recognition domain with alternatives from other sources and showed that cleavage was redirected to new sites. When they linked the cleavage domain to a set of zinc fingers, they opened the door to designing reagents that would be directed to completely novel targets. Zinc fingers were identified in natural eukaryotic sequence-specific transcription factors and were known to bind DNA in very modular fashion—one finger per three base pairs—and fingers had already been identified for a number of different triplets. Thus, in addition to basic advances in DNA manipulation, three specific research threads led to the production of the first zinc-finger nucleases (ZFNs).

In a nutshell, early work with ZFNs showed that it takes two such constructs to cut DNA efficiently, since the cleavage domain must dimerize, and the dimer interface is quite weak. An optimal linker between the binding and cleavage domains was identified. Despite the fact that the nuclease domain came from a bacterium, ZFNs effectively cleaved targets that were assembled into chromatin.

The next major step was the demonstration of ZFN activity in whole cells and organisms. The first such experiments were done with *Drosophila melanogaster*, demonstrating both targeted mutagenesis by simple cleavage and reliance on inaccurate repair and homologous gene replacement when an appropriate donor DNA was supplied. Additional work showed efficacy in cultured human cells, in plants and in nematodes. By now, ZFNs and other nucleases have been used successfully to modify the genomes of more than 50 distinct organisms.

A drawback to the use of ZFNs was the difficulty of deriving reliable designs for new targets. Assembling new combinations of fingers from existing libraries was successful in some cases, but not in others. Methods for selecting novel combinations for individual new targets from mutagenized pools helped, but were not widely adopted for various reasons. Very reliable constructs were and are available commercially, but at prices inaccessible to some. Nonetheless, ZFNs have been used widely.

TALENs

The discovery of a simpler DNA-recognition code in transcription activator-like effectors (TALEs) in *Xanthomonas* bacteria led to a new platform for targeted cleavage. In these proteins, each module binds a single base pair, and individual modules reliably recognize each of the four types of base pair. Thus, new recognition domains could be assembled simply by reading the target DNA sequence and inserting the appropriate TALE subunit. Linkage to the FokI cleavage domain created TALE nucleases (TALENs) that, like ZFNs, needed to be provided in pairs to ensure cleavage. It is perhaps fortunate that, again like zinc fingers, the TALE modules naturally bind DNA in a chromatin context, in this case targets in plant-host chromosomes. TALENs were rapidly adopted in preference to ZFNs, particularly in research labs, for the simplicity of their design, for the higher rate of success of new designs, and the apparent sequence specificity of the ultimate reagents.

CRISPRs

The latest additions to the tool kit are the CRISPR/Cas RNA-guided nucleases that I like to simply call CRISPRs. In this case, recognition is mediated by a guide RNA, using Watson-Crick base pairing, making designs for new targets even simpler than for TALENs. In addition, a single protein, Cas9, is required, and it doesn't need to be modified when the target is changed. These features have led to very rapid adoption of CRISPR nucleases.

Common Characteristic

An important characteristic common to these three platforms is that DNA recognition and cleavage are mediated by functionally separable domains. This means that each can be manipulated independent of the other, which is, of course, critical for attacking new

targets by changing binding specificity. The nuclease domains have also been manipulated in various fashions, notably to produce single- rather than double-strand breaks at the target.

Ultimately, these targetable nucleases make breaks only at the desired DNA targets. Everything that happens afterward depends on cellular DNA-repair activities. This means that the outcomes of a nuclease-mediated targeting event may be different in different organisms or cell types, depending on the status of those repair activities. In addition, the ways in which the nucleases—and donor DNA, when homologous repair is sought—are delivered will depend on what is appropriate in any application.

Model Fruit Fly

We have investigated a number of these repair parameters using *Drosophila* as the model organism. We asked, *What gene products participate in nuclease-mediated targeting, and what are the consequences of disabling each of them?* Not surprisingly, we found that the Rad51 and Rad54 homologues were required for most (but not quite all) of homologous repairs. More importantly, in the absence of DNA ligase IV (Lig4), a dedicated component of nonhomologous end joining (NHEJ), the majority of repairs proceeded by homologous recombination (HR). In many experimental situations, HR is the desired pathway. It is not possible to propagate Lig4 mutants of quite a number of organisms, so alternative means of disabling the enzyme, or other NHEJ activities, are being sought.

Another important question regarding homologous repair is, *How much homology is needed to support efficient sequence replacement?*

Reference

Kim YG *et al.* (1996) Hybrid restriction enzymes: zinc finger fusions to FokI cleavage domain. Proceedings of the National Academy of Sciences of the USA 93(3) 1156–1160.

DANA CARROLL received a bachelor's degree in chemistry from Swarthmore College, and a PhD in biophysical chemistry from the University of California at Berkeley in the laboratory of Ignacio Tinoco, Jr. He was a postdoctoral fellow in Glasgow with John Paul, and at the Carnegie Institution Department of Embryology with Donald Brown. He has been on the faculty at the University of Utah School of Medicine since 1975, where he served as chair of the Department of Biochemistry from 1985 to 2009.

DR. CARROLL has a long-term interest in molecular mechanisms of DNA repair and recombination. He was a pioneer in the use of zinc-finger nucleases (ZFNs) for targeted genome modifications. For this work he received the 2012 Novitski Prize from the Genetics Society of America and the 2014 Sober Lectureship Award from the American Society for Biochemistry and Molecular Biology. His current research focuses on genome engineering using ZFNs, TALENs, and CRISPR/Cas nucleases.

Opportunities and Regulatory Challenges for Genome Engineering in Agriculture

Daniel F. Voytas
University of Minnesota
Minneapolis, Minnesota

Voytas@umn.edu

Plant agriculture faces numerous challenges in the coming decades. The burgeoning world population demands that more food be produced on less, and often increasingly marginal, land. Climate change and diminishing resources, such as water and fertilizer, will make it difficult to achieve the needed increase in productivity. Whereas multiple strategies must be deployed to achieve food security, it is clear that amongst these is the need to accelerate the rate of crop improvement. Recent advances in genome engineering promise to make this possible. From targeted mutagenesis to targeted gene insertion, genome engineering is transforming plant science, making it possible to create genetic diversity with precision, efficiency and control. For the basic plant biologist, genome engineering helps dissect gene function by linking genotype to phenotype. Information garnered about plant-gene function can then be harnessed to create genetic variation relevant to agriculture to achieve increased productivity.

Genome Engineering

Genome engineering, as typically practiced, uses sequence-specific nucleases that recognize unique sites in the plant genome and introduce targeted DNA double-strand breaks (DSBs) (Voytas, 2013). The repair of the DSB can be controlled to achieve the desired DNA-sequence modification at or near the break site (Figure 1). One repair pathway that cells use is non-homologous end-joining (NHEJ), wherein the broken chromosome is simply rejoined (Gorbunova and Levy, 1997; Salomon and Puchta, 1998). Oftentimes, a few to several nucleotides are gained or lost at the break site, creating a targeted mutation. If the mutation occurs in a coding region, it can alter a protein's amino acid sequence or cause a frameshift mutation that destroys or knocks out gene function. A second DNA-repair pathway is homologous recombination (HR) (Puchta *et al.*, 1993, 1996). Through HR, the broken chromosome uses a homologous template to copy information to the break site. The template is most often a sister chromatid or homologous chromosome;

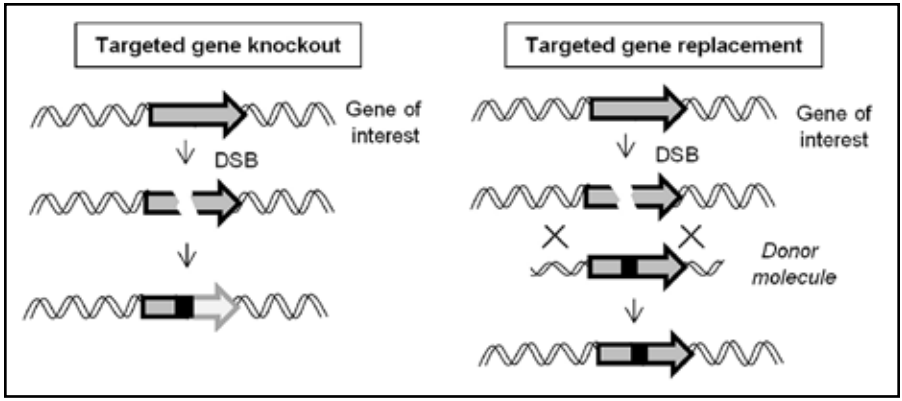

Figure 1. Plant-genome engineering using DNA double-strand breaks (DSBs). DNA-repair pathways can be exploited to introduce desired sequence changes to a plant's genome. Repair of DSBs by non-homologous end joining can result in deletions or insertions at the break-site. Therefore, targeting DNA breaks to a locus or gene of interest can achieve targeted mutagenesis (left panel). Alternatively, DSBs can stimulate homologous recombination with a user-supplied donor molecule. Donor molecules can be designed to contain small point mutations for the purpose of making small changes within genes (targeted gene replacement, illustrated in the right panel) or larger changes, including full genes or gene-regulatory elements (targeted gene insertion). (Courtesy of Nick Baltes.)

however, exogenous templates can be delivered to a cell, and, because they are user-specified, the templates can have specific DNA-sequence alterations that become incorporated at the break site. HR is, therefore, a powerful means to achieve precise alterations to the plant genetic code.

Whether created through NHEJ or HR, the key to achieving a targeted DNA-sequence modification is the DNA DSB. Much effort in the past 15 years has focused on creating reagents capable of recognizing specific DNA sequences in complex genomes to introduce targeted breaks at high efficiency. Four classes of sequence-specific nucleases have been widely deployed (Figure 2). One class is the meganucleases or homing endonucleases—enzymes that naturally recognize and cleave large DNA-sequence signatures (typically >30 bp). The DNA specificity of meganucleases can be altered such that they recognize and cleave novel DNA targets (Smith *et al.*, 2006; Pâques and Duchateau, 2007). Two classes of sequence-specific nucleases use engineered DNA-binding domains fused to the catalytic domain of the type II restriction endonuclease, FokI. These are the zinc finger nucleases (ZFNs) and the transcription activator-like effector nucleases (TALENs). For ZFNs, DNA targeting is achieved by custom arrays of zinc fingers, each of which typically recognizes three base pairs (Bibikova *et al.*, 2003; Carroll, 2011); for the TALENs, custom arrays of TAL effector repeats are assembled, with each repeat recognizing one base pair (Christian *et al.*, 2010; Bogdanove and Voytas, 2011). Both ZFNs and TALENs function as dimers: two DNA-binding domains are engineered to bring the FokI monomers into

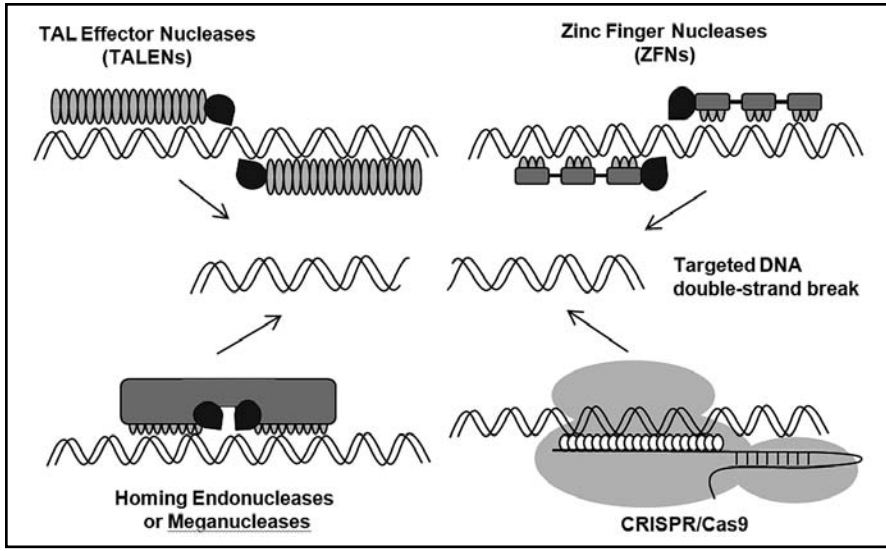

Figure 2. Illustration of the four classes of sequence-specific nucleases: Transcription activator-like (TAL) effector nucleases, zinc-finger nucleases, homing endonucleases or meganucleases and CRISPR/Cas9. All classes of proteins can be "reprogrammed" to recognize and cleave desired DNA sequences. (Courtesy of Nick Baltes.)

proximity on the DNA target. There they dimerize and create the DSB. More recently, CRISPR/Cas9 reagents have emerged as powerful and highly efficient tools for making targeted DSBs (Jinek *et al.*, 2012; Doudna and Charpentier, 2014). For CRISPR/Cas9, targeting is achieved when a guide RNA base pairs with its DNA target. The Cas9 nuclease then introduces the DSB. Because no protein engineering is required and targeting is achieved simply through base pairing, CRISPR/Cas9 has emerged as the reagent of choice for making targeted chromosome breaks.

Plant Varieties Created Through Targeted Mutagenesis

One of the simplest means of deploying sequence-specific nucleases is to create mutations through imprecise NHEJ (Voytas, 2013). For targeted mutagenesis, the nuclease is typically delivered to the cell as DNA, either transiently or by stably integrating the nuclease-encoding construct into the genome. If imprecise repair occurs after the break is created, a mutation results. Targeted mutagenesis is particularly valuable for studying gene function. Loss-of-function mutations and their consequential phenotypes are achieved by introducing frameshift mutations near the 5'-end of the gene. Traits of relevance to agriculture can be created through targeted mutagenesis, although the phenotypic variation afforded by loss of gene function is somewhat limited. That said, removing toxins, such as ricin from castor oil, or anti-nutritionals, such as trypsin inhibitors from soybean, are potential traits of value. Similarly, antigenic determinants that cause allergic reactions could be removed from nut or grain proteins.

A recently published example of a trait created through targeted mutagenesis is a soybean variety that produces oil with elevated levels of the monounsaturated fat, oleic acid (Haun *et al.*, 2014). Soybean oil typically has about 20% monounsaturated fats, and, in the past, polyunsaturated fats have been reduced through hydrogenation to improve the oil's storage and frying characteristics (Clemente and Cahoon, 2009). Hydrogenation, however, produces trans-fatty acids, which are unhealthy when consumed. Consequently, there has been a strong push to create soybean varieties that produce oil with elevated levels of monounsaturated fats.

In soybean seeds, the monounsaturated fat, oleic acid, is converted to the polyunsaturated fat, linoleic acid, through the action of fatty acid desaturases (Tang *et al.*, 2005). Soybean has two seed-specific fatty acid desaturase genes, designated *FAD2-1A* and *FAD2-1B* (Schlueter *et al.*, 2007). To mutate these genes, and test whether levels of oleic acid could be increased relative to linoleic acid, TALENs were created that recognize conserved DNA sequences in both *FAD2-1A* and *FAD2-1B* (Haun *et al.*, 2014). Constructs encoding these TALENs were stably transformed into soybean cells and expressed constitutively. As the transformed cells regenerated into soybean plants, the TALENs created mutations, including mutations in cells that gave rise to the germline. Among nineteen transgenic soybean lines that were regenerated, three transmitted to progeny mutations in one or more *FAD2* genes. Consequently, it was possible to recover plants from this population that were homozygous for mutations in *FAD2-1A*, *FAD2-1B* or both genes. In the case of the homozygous double mutant, the desired phenotype was achieved. Oil pressed from this plant's seeds had 80% of the monounsaturated fat, oleic acid, and less than 4% of the polyunsaturated fat, linoleic acid. In contrast, oil from wild-type plants had 20% oleic acid and 50% linoleic acid. This single loss-of-function mutation, therefore, created soybean lines that produce oil with a fatty acid composition that is healthier for human consumption.

In the above example, the construct encoding the TALEN was stably introduced into the soybean genome. The mutations that were created, however, were at *FAD2* genes located at other genomic sites. Consequently, it was possible to segregate away the TALEN transgene and obtain lines of soybean with mutations only in the *FAD2* gene targets (Haun *et al.*, 2014). In contrast to this example, which involves a stable, transgenic intermediate, sequence-specific nucleases can also be introduced into plant cells transiently (Townsend *et al.*, 2009). The nucleases encoded by DNA that enters the plant cell are expressed, and oftentimes the DNA never integrates into the plant genome. This transient expression of the nuclease creates targeted mutations, and transgenic plants are not intermediates in the mutagenesis protocol.

The real advantage of mutagenesis with sequence-specific nucleases is their precision. Traditional methods of mutagenesis that use chemicals, X- or gamma-rays, transposons or T-DNA provide virtually no control over where in the genome mutations are created. Consequently, large populations of mutagenized plants need to be generated and screened to identify those rare individuals with alterations in a particular gene of interest. Oftentimes, mutations that are recovered are not ideal, and perhaps, for example, result in only partial loss of gene function. Sequence-specific nucleases can be used to efficiently create

multiple mutant alleles for study, including complete gene knockouts. It is important to note that all classes of sequence-specific nucleases, when engineered properly, are highly precise (Pauwels *et al.*, 2014). They rarely cleave at unintended or off-target sites and, thus, typically only create mutations at the intended sites. This contrasts with traditional mutagens, which can cause considerable collateral damage to the genome.

Regulatory Aspects

Regulation is one factor that will determine how broadly and rapidly the products of genome engineering will be deployed in agriculture (Voytas and Gao, 2014). In the United States, plants that have genetic variation created using chemical mutagens or ionizing radiation are not regulated and can be planted directly in the field to test the phenotypic consequence of the induced genetic variation. In contrast, transgenic plants are subjected to exhaustive and costly regulatory scrutiny before they can be planted in the field (Lusser *et al.*, 2012). In many ways, this regulatory burden has restricted the use of transgenic approaches to create genetic diversity to a handful of high-margin row crops. As described above, targeted mutagenesis with sequence-specific nucleases is highly precise, and since the mutant plants often lack foreign DNA, this suggests that they might be treated more like traditional mutants in terms of regulation. In the United States, this appears to be the case. Recent opinion letters from the USDA indicate that plants with targeted mutations made by NHEJ and without transgenes fall outside their regulatory authority (Waltz, 2012; Jones, 2015). Opinion letters were rendered in two cases—for a low phytate line of corn made with ZFNs and a potato variety with improved storage and frying characteristics created by a TALEN-induced mutation. If this trend continues, then many new plant varieties could be made and commercialized without having to accumulate large, costly data packages for regulatory approval. This will likely extend the range of species for which biotechnology is used to create genetic variation of value, and horticultural and vegetable crop varieties will likely enter the marketplace with genomes altered using this technology.

Plant Varieties Created Through Homologous Recombination or Gene Targeting

The repair of DNA breaks by HR, referred to as gene targeting, allows a vast spectrum of DNA-sequence modifications to be introduced into a plant's genome (Voytas, 2013). These modifications can range from single-nucleotide substitutions that alter an amino acid in a coding sequence to the insertion of arrays of transgenes at defined chromosomal sites. The high level of control afforded by DNA repair through HR makes it possible to create plant varieties with complex traits, such as tolerance to biotic or abiotic stress or that more efficiently use inputs such as fertilizer and water. Gene targeting could be used to alter primary metabolism to create varieties that, for example, produce specialty carbohydrates or oils for industrial purposes or for fuel. Plants also produce a remarkable array of complex secondary metabolites, and genome engineering could create plant varieties that overproduce chemicals of pharmaceutical or industrial value. In many cases, achieving such complex traits will require the modification of multiple genes in a pathway.

To illustrate how gene targeting can be used to create a new plant variety, consider an approach to increase disease resistance, specifically resistance of potato to late blight. Late blight is caused by the fungal pathogen *Phytophthora infestans*, and is one of the world's most devastating crop diseases (Kamoun, 2001). If late blight were controlled effectively, potato yields could increase by as much as 50%. Existing methods for combating late blight involve multiple applications of fungicides to potato fields throughout the growing season, which is costly and can have a negative impact on the environment. Resistance can also be achieved genetically. In related species of potato, genes have been identified that confer late-blight resistance (Song *et al.*, 2003; Foster *et al.*, 2009). Traditionally, these genes would be introduced into cultivars of potato through breeding regimes that would take many years to complete. Alternatively, resistance genes could be introduced into the potato genome as transgenes to create resistant, transgenic varieties. This latter approach, however, may be undesirable from a regulatory point of view, in that the resultant resistant varieties carry foreign DNA.

With gene targeting, late-blight resistance in potato can be achieved in a much shorter timeframe than with traditional breeding and with only subtle alterations to the genome. For the potato resistance genes, orthologues that confer susceptibility exist, and they are highly similar to the resistant variant (Song *et al.*, 2003; Foster *et al.*, 2009). Only a handful of base changes distinguish the resistant and susceptible alleles; the DNA-sequence differences confer the ability of the encoded resistant protein to recognize or respond to the pathogen. To confer late-blight resistance through genome engineering, a sequence-specific nuclease, such as a TALEN, ZFN or CRISPR/Cas9 reagent, would be engineered to recognize and cleave the susceptible allele (Figure 3). A construct encoding the nuclease would be introduced into potato cells along with a repair template, which, through HR, would introduce into the susceptible allele the desired DNA-sequence variation from the resistance gene. Potato cells with the desired DNA-sequence modification would then be regenerated into plants, and they should be resistant to late blight. Resistance achieved through genome engineering could be accomplished in as little as a year's time, fast-tracking the production of plants with a trait of commercial value.

Further Regulatory Aspects

How will plant varieties created through gene targeting be regulated? It is likely that each new variety will be considered on a case-by-case basis. In the above example, the genetic variation that conferred resistance to late blight already existed in nature. Further, the resistant variety created through gene targeting is largely equivalent to a variety derived through traditional breeding—an unregulated process. Since DNA-sequence variation closely linked to the resistance gene would also be introduced through breeding, gene targeting is actually more precise. The potato genome modified through gene targeting has only the desired DNA-sequence alteration, and this could be easily confirmed using approaches such as whole-genome sequencing.

The need for case-by-case evaluation of plants derived from gene targeting is warranted because of the range of modifications that can be created. In the potato example, only a handful of DNA-sequence changes—identified from a wild, resistant relative—were

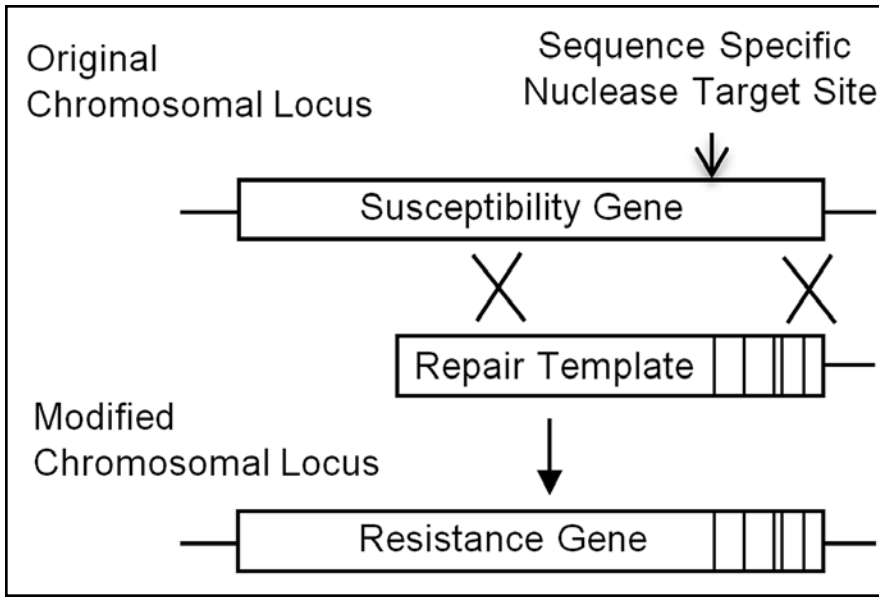

Figure 3. Achieving resistance to late blight through genome engineering. A sequence-specific nuclease is engineered to recognize and cleave the susceptibility gene. A repair template is provided that incorporates mutations (vertical lines) in the susceptibility gene, such that it now confers resistance to late blight.

needed to achieve the desired phenotype. However, variation could also be introduced that is not found in nature. For example, an enzyme's activity could be altered or optimized in the laboratory through *in vitro* evolution or directed mutagenesis to create a novel variant. The genetic changes that underlie the novel activity could then be introduced into the native gene in the plant genome. In considering how to regulate such a plant, a variety of factors will have to be considered, ranging from potential effects if the plant product is consumed (*e.g.* is the variant protein immunogenic?) or its impact on the environment (*e.g.* what are the consequences if the genetic variation moves into weedy relatives of the crop plant?). A clearer picture of how crop varieties created through gene targeting will emerge as new plant varieties are developed and brought to the regulatory authorities for consideration. The guidance provided will be invaluable for those parties using the technology, particularly with respect to estimating the costs needed to pass the regulatory steps prior to field release of a new variety.

Conclusion

Genome engineering has emerged as a powerful means to create genetic variation in plants and is rapidly being deployed for both basic and applied plant biology. The sequence-specific nucleases that enable targeted DNA-sequence modification are precise and accurate, and they alter the genome through well-understood mechanisms of DNA repair. The types of genetic variation that can be created through genome engineering

will contribute to agricultural productivity and help meet the world's burgeoning need for food and other agricultural products. Because genome engineering is a new approach to introduce genetic variation in plants, responsible regulation is required so that the technology can be best deployed for the public good.

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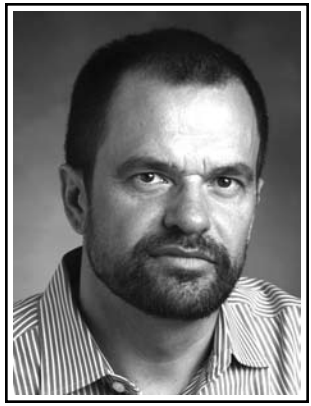

DANIEL VOYTAS is a professor in the Department of Genetics, Cell Biology and Development at the University of Minnesota (UMN) and director of the UMN's Center for Genome Engineering.

HE GRADUATED from Harvard College in 1984 and received his PhD in genetics from Harvard Medical School in 1990. He conducted postdoctoral research at Johns Hopkins University School of Medicine where he was a fellow of the Life Science Research Foundation. In 1992, he joined the faculty at Iowa State University where he was promoted to associate professor in 1997 and to professor in 2001. In 2008, he joined the faculty at UMN.

DR. VOYTAS advises agricultural biotechnology companies on the use of new methods of genome engineering for crop improvement and he serves as chief science officer for Cellectis Plant Sciences in New Brighton, MN. He is an elected fellow of the American Association for the Advancement of Science.

The Promises and Challenges of Precision Gene Editing in Animals of Agricultural Importance

Perry B. Hackett, Scott C. Fahrenkrug and Daniel F. Carlson
Recombinetics, Inc.
St. Paul, Minnesota
and
University of Minnesota
Minneapolis, Minnesota

hacke004@umn.edu

We live in changing times—it has always been that way. But, now the times are changing more rapidly, dramatically, and unpredictably. The world must accommodate an increasing population that demands greater nutrition per person, better health, and greater energy consumption per person. As the world's population rises to a predicted level of nearly 10 billion by 2050, most want sustainable growth without harm to the environment. The inconvenient truth is that it's not possible with current agricultural, economic, and environmental operating systems (Hoekstra and Wiedmann, 2014). The green revolution has essentially run its course (Conway and Toenniessen, 2000; Pingali, 2012; Stevenson *et al.*, 2013). Even with technological gains, hunger worldwide has been increasing over the past decade. Global climate change coupled with a demand for increased nutrition in the developing world exacerbates the stress on agricultural production (Eisler and Lee, 2014). Hence, developing new methods of increasing the production of agricultural products, crops and animals, with minimal impact on the environment is essential (Godfray *et al.*, 2010). Current approaches cannot meet demands. However, gene editing (Tan *et al.*, 2012, 2013) (Figure 1)—which allows geneticists to introduce (introgress) any natural trait into any breed without the use of recombinant DNA—has the potential of improving animal genetics for meeting increasing agricultural and biomedical needs with minimal environmental impact. However, there are policy issues associated with gene-editing in livestock and in biomedical research that must be addressed for their real-world applications (Pauwels *et al.*, 2014). We discuss several types of genome editing and current deficiencies in regulatory oversight that block enthusiasm for its adoption to agriculture.

The Problem

Genetically modified (GM) animals have been around for more than four decades (Gordon and Ruddle, 1981, 1982). These animals had expression cassettes driven by constitutive promoters that are active in most cell types and were delivered by vectors that could integrate semi-randomly in genomes (Figure 1, upper panel). As a result, there were fears of unacceptable (scary) results (Rollin, 1985) that were exacerbated by the engineering of a mouse that grew like a rat (Palmiter *et al.*, 1982, 1983). The first large animals of potential commercial importance were fish (Zhu *et al.*, 1986; Hackett and Alvarez, 2000; Devlin *et al.*, 2009). Since then, several lines of transgenic livestock have been engineered for producing valuable biomedical medicines and for agriculture (Tan *et al.*, 2012). Animals engineered to become bioreactors for manufacture of enzymes and antibodies that are not for general sale or consumption have been cleared by regulatory authorities. But, in the United States, not a single animal engineered for food production has been approved by the US Food and Drug Administration (FDA), which regulates GM animals.

These demonstrations of transgenic technologies were greeted with more concern than enthusiasm by the general public and especially by several non-governmental organizations. The concerns focused on four areas:

- Health effects due to the *un-naturalness* of products the modified genome might encode;
- Environmental effects due to uncontrolled release of transgenes (*i.e.* GM animals) and reduced diversity of natural genomes;
- Social concerns that huge corporations would have undue influence over diets; and
- Moral concerns that were summed up by the phrase "playing God."

These concerns were applied to animals as well as crops.

The first crops were genetically engineered in 1985, *e.g.* tobacco with a firefly luciferase gene (Lamppa *et al.*, 1985). Astonishingly, as shown in Figure 2, over the past 15 years, GM crops have been adopted increasingly year after year with respect to acreage, plant varieties and countries. In contrast, even though farm animals can be controlled to a far greater degree than crops, not a single animal has made it through full regulatory review in any country.

This is in part due to the different attitude taken by the FDA, which regulates GM animals and the US Department of Agriculture (USDA) and Environmental Protection Agency (EPA), which regulate GM crops. Foreign countries have in large measure deferred to FDA to formulate regulations and procedures for evaluation of GM animals. The precision of gene editing along with our greater knowledge of molecular genetics and cell biology gained over recent decades supports the belief that GM animals may become a reality.

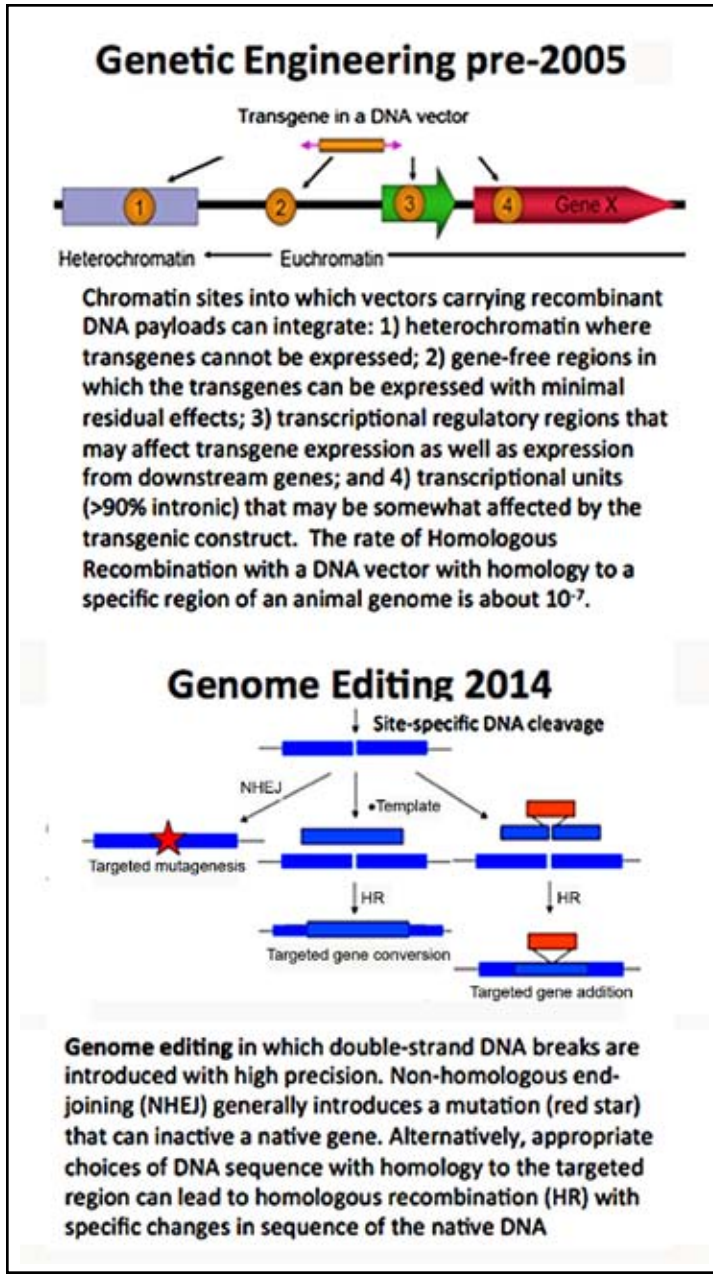

Figure 1. Genetic engineering using recombinant DNA vectors compared to genome editing using site-specific DNAases: The gain in precision between the two methods is 10^7.

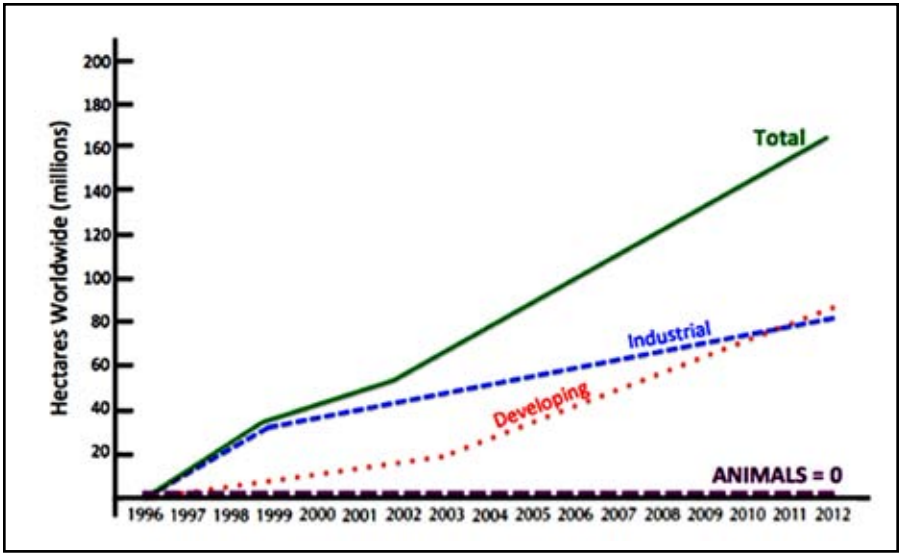

Figure 2. Crops and animals modified by molecular genetics procedures (GM) approved for agricultural purposes. No government-approved GM animal has been developed for agricultural use.

The Promise of Gene Editing in Animals

Gene-edited animals can be fruitfully employed in three distinct areas, as shown in Figure 3. Each of these areas, illustrated in the figure, would be a game-changing event globally with respect to personal health. In 2013 in the USA, more than 121,000 individuals were on the waiting list for transplantable organs; only 29,000 organs were transplanted. However, with precision inactivation (knockout, KO) of specific genes required for organ development *in utero*, pigs could be used as bioreactors for production of donor-specific organs/tissues by a process called *blastocyst complementation* or *exogenic organ production*. This is theoretically applied by injection of induced pluripotent stem cells (iPSCs) into a KO-pig blastocyst from which the donor cells fill the vacant developmental niche, resulting in a human organ to match the donor. This would be analogous to an autograft, which will avoid immune responses when transplanted into a patient. Likewise, pigs are far closer to humans in physiology than other commonly used model animals.

Gene editing allows production of animals with specific conditions that mimic human disorders, which will allow more-accurate pre-clinical evaluation of novel drugs and advanced medical devices before human clinical trials. The most dramatic promise of gene-edited animals is in agriculture worldwide. In the Anthropocene era (Vince, 2011), characterized by global changes in climate with attending alterations in spreads of animal-disease vectors, the exchange of new genes (traits) from different breeds in

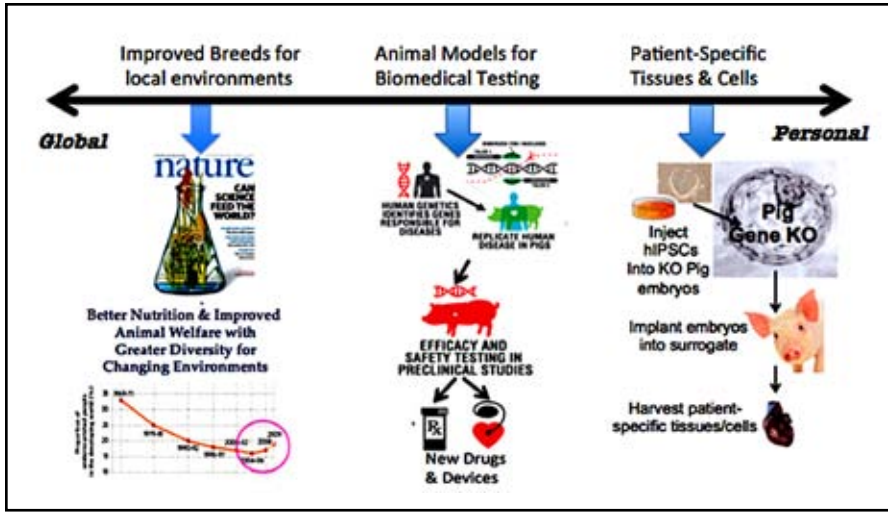

Figure 3. Promise of gene-edited animals. The major areas of application are, from left to right, 1) improved animal genomes adapted to local environmental conditions to produce food more efficiently, 2) improved animal models of human disease that provide more reliable pre-clinical information regarding safety of new drugs and medical devices, and 3) animals designed to harbor patent-specific organs, tissues and cells for transplantation that will not induce adverse immune responses.

various regions and microenvironments of the world will be vital for adaptation of animals and their abilities to provide improved nutrition while simultaneously improving their welfare. Additionally, gene editing will allow novel tweaking of genomes in order to introduce new approaches to the control of animal diseases as well as to improve animal health and efficiency.

The three major technologies for introducing site-specific double-stranded DNA breaks into genomes—zinc finger nucleases, ZFNs, transcription activator-like element nucleases, TALENs, and RNA-guided endonucleases, RGENs, of which the CRISPR-Cas9 system is best known (Gaj *et al.*, 2013; Kim and Kim, 2014)—are effective in livestock genomes (Carlson *et al.*, 2012; Tan *et al.*, 2013). The steps for creating livestock with specific genome-edits are illustrated in Figure 4. The process begins with introducing double-strand DNA breaks in the genomes of somatic cells, typically fetal fibroblasts, expanding the cells into colonies that then are screened for the desired outcomes. This process is between 10% and 70% efficient, depending on the gene, the size of the edit, the genetic locus and cell type. Counter-intuitively, introducing single-nucleotide changes is generally less efficient than introducing longer alterations (Tan *et al.*, 2013). Table I shows the approximate efficiencies of introducing defined gene edits into the genomes of pigs, cattle, goats and sheep.

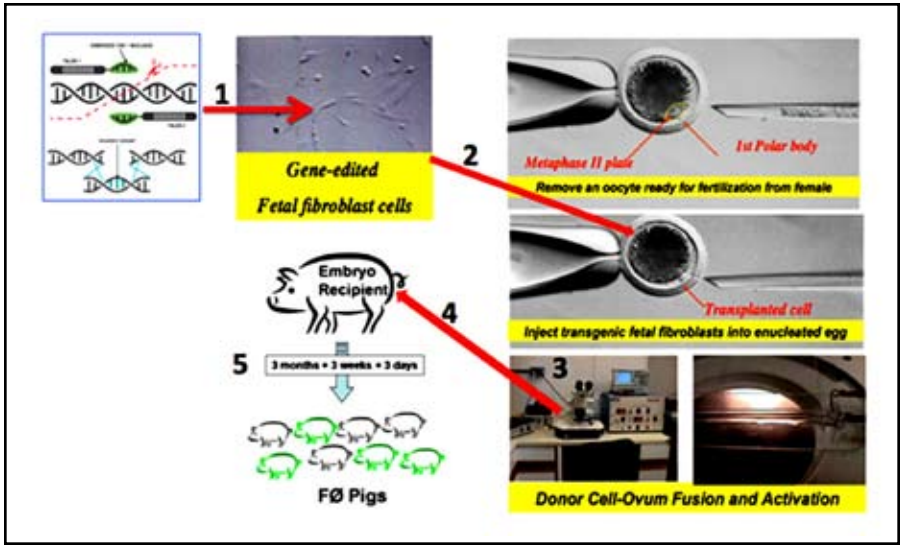

Figure 4. Route of obtaining gene-edited livestock (pigs). 1) Introduced gene-edits into fetal fibroblasts (1 month; 10–70% efficiency). 2) Transfer validated gene-edited cells into enucleated oocytes. 3) Activate embryos by electrical pulse to fuse the cell with the oocyte. 4) Transfer activated embryos into surrogate female to produce 5) F0 pigs with the desired gene-edit (4–9 months; 1–5% efficiency).

Table I. Summary of homology-directed recombination for livestock fibroblasts.

- >98% success (60+ genes/loci successfully targeted; 1 failure
- Heterozygosity (monoallelic conversion) up to 70%
- Homozygosity (biallelic conversion) up to 40%
- Single-nucleotide polymorphism(SNP) edits with 1 bp alteration about 10% efficiency
- SNP edits much more efficient with silent mutations

The *POLLED* gene in cattle illustrates the power of gene editing in large animals. Dairy cattle, like many other mammals, naturally have horns. Early in the domestication process of dairy cattle thousands of years ago, horns were valuable for survival, whereas today horns have no intrinsic value because the animals are confined to secure enclosures. However, horns do pose a significant risk both to the animals and to humans because of inadvertent nicking and, consequently, are removed efficiently and cost-effectively, but not without suffering (Graf and Senn, 1999). Two mutations that prevent development of horns in certain breeds of cattle have been mapped on the bovine genome (Medugorac *et al.*, 2012; Seichter *et al.*, 2012) that may encode an lncRNA rather than a protein (Allais-Bonnet

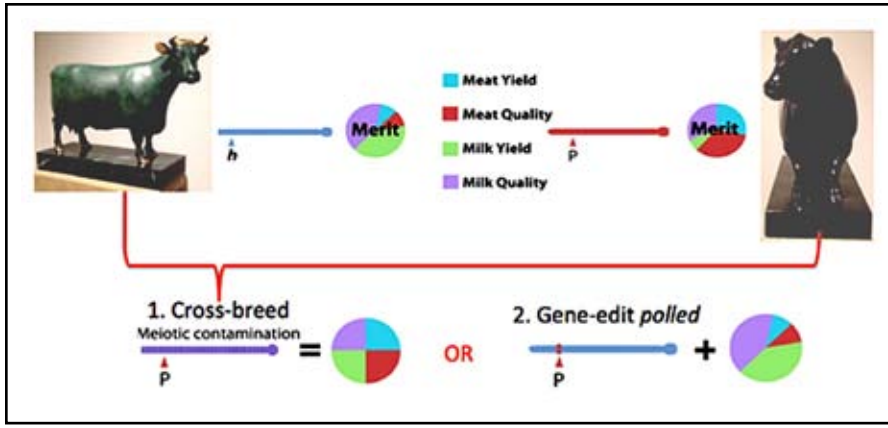

Figure 5. Introgression of the *POLLED* allele into dairy cattle. Top: Merits for traits of commercial value are shown by the circles for dairy (left) and beef (right) breeds. The *POLLED* locus is shown by the P (or h for the Hereford breed) on chromosome 1 (BTA1) for the two breeds. Bottom: Cross-breeding to introgress P into a dairy background will mix all the valued traits for both breeds. Alternatively, gene editing of the h locus to introduce the P allele maintains the dairy merits.

et al., 2013). The standard method for introducing *POLLED* in dairy breeds would be by crossing with a hornless breed (Figure 5), but that produces a hornless animal without the best combination of traits for the dairy industry. Recovering the dairy merits would take nearly a quarter century of backcrossing and selection.

Gene editing saves about eight generations of backcrossing and the entire attendant screening for alleles desirable to industry. Figure 6 shows molecular introgression of *POLLED* into the Hereford breed. The efficiency of introducing this particular site-specific mutation with this particular pair of TALENs was 1% to 5%, substantially below the average rate shown in Table I. However, because the genomic edits can be introduced into the chromosomes of fibroblasts, even this relatively low rate is sufficiently high for easy selection of appropriate genomes for transfer into embryos.

Gene editing is not limited to single changes. Because of the high efficiency of the procedure, multiple selected mutations can be simultaneously introduced into genomes. Thus, gene editing offers parallel, precise changes in genomes of animals rather than sequential changes. This can save decades of time. Moreover, as illustrated by the recent elucidation of the genetic basis of *POLLED*, as greater numbers of genomes of various breeds of livestock are sequenced, our abilities to identify further traits conferring disease-resistance, drought-tolerance, temperature-tolerances (high and low), *etc.*, will be enhanced. All findings of this sort will provide a bank of alleles for molecular introgression. The process is fast, efficient, and essentially unlimited in the combinations of traits that can be moved as needed. In the coming decades, in which significant variations in global as well as local environmental conditions are predicted, gene editing offers the ability to substantially expand the diversity of animals that will be better adapted for climatic

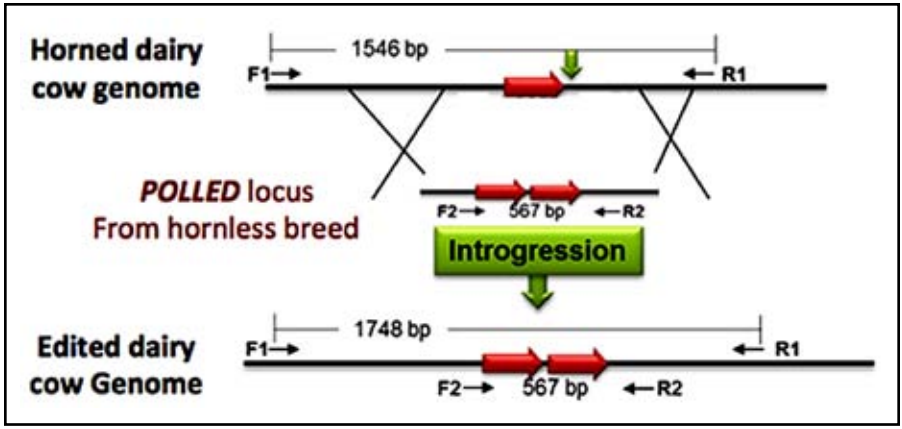

Figure 6. Molecular introgression of the *POLLED* allele into dairy cattle by gene editing. A double-stranded oligonucleotide serves as a template for homology-directed recombination (HDR) following introduction of a double-stranded DNA break (DSB) in the genome following cleavage by a specific pair of TALENs. Because gene-editing is precise, genomes can be screened by PCR for exactly the desired changes. F1, F2, R1 and R2 are specific DNA oligonucleotide primers for PCR analysis.

changes. This will benefit the animals as well as their caretakers and the populations that depend on agricultural production. The promise of gene editing in livestock is enormous (Howe *et al.*, 2008).

Challenges for Gene Editing in Animals

The challenge for application of gene editing in animals is evident from Figure 2: not a single commercial animal for agricultural purposes has been approved in the United States. Regulatory reluctance to issue approval for the first proposed commercial animal, faster-growing salmon, has been stymied by regulatory delays (Figure 7) (Van Eenennaam and Muir, 2011; Maxmen, 2012b; Ledford, 2013; Editorial, 2014). There are suggestions that molecular introgression using the procedures described above will meet the same regulatory purgatory (Maxmen, 2012a). One way of appreciating the consequences of regulatory reluctance to approve any genetically modified livestock are the hundreds of citations on genetically modified animals 10–20 years ago, reviewed by Tan *et al.* (2012), compared with the paucity of citations today. If there is no method of gaining approval, there is no incentive for development.

The letters (many form) about the transgenic salmon on FDA's website[1] raised the same concerns listed above (p. 40) for transgenic organisms in general:

- *Health effects*—possibly allergic responses to a transgenic product, but more commonly general uncertainty in what eating a transgenic food might mean over many years.

[1] http://www.regulations.gov/#!docketBrowser;rpp=25;po=0;dct=PS;D=FDA-2011-N-0899.

Figure 7. Year-after-year delays in approval of transgenic salmon by the FDA.

- *Environmental effects*—diversity may be reduced and/or transgenic animals may take over ecosystems.
- *Social effects*—transgenic products come from large corporations catering to large farms that dominate over smaller "family" farms; hence, greedy corporations benefit at the expense of consumers.
- *Moral* concerns—tampering or tinkering with nature, often referred to as "playing God."

Regulatory agencies may take emotional reactions of citizens into consideration in decisions, but the primary driver should always be scientific understanding. In that light, what have we learned from thirty years (and more than $100 billion) of molecular genetics and agricultural research, much of which can be understood by the large investment into the molecular, cellular and developmental biology of humans, which represent a pretty good model system for livestock?

Health Effects

Regarding health effects, if a genetic alteration is not detrimental to the animal, it is highly unlikely it would be to humans. The many cultures and societies of humans eat almost every type of life form. All food is foreign to someone, but not necessarily unhealthy. Generally it is processing of food, not the original product that can lead to adverse health effects. Regulatory pandering to public fears of food safety (DeFrancesco, 2013) must change.

Environmental Effects

Environmental effects for gene-edited animals will be no different from any other introduction of a related animal; the changes are too minor. For instance, although there are peer-reviewed studies that suggest ecological disaster from escape of even a few genetically altered fish (Muir and Howard, 2002; Devlin *et al.*, 2006) hundreds of thousands of farmed fish escape from Norwegian fisheries with little discernable effect (Glover *et al.*, 2012; Skilbrei *et al.*, 2014). There is theory and there is the real world, which often goes ignored (Hackett, 2002; Fedoroff, 2013). More importantly, as noted earlier, gene editing offers the *opportunity of introducing far greater diversity* into ecosystems because it is so economically efficient that it can be used by mega-farms and even the smallest family farm in a unique micro-ecosystem where agricultural efficiency can bring the greatest benefits.

Social Effects

The issue of huge corporations dominating the availability of genetically modified products is in large part a direct consequence of the cumbersome regulatory processes that take years for approval, if they come at all. That is, governmental policy demands expenditures of millions of dollars for regulatory approval that only large companies can afford.

Moral Concerns

Moral concerns are beyond the scope of most scientific discourses. But, an often-overlooked point on this issue is that genetic modifications with modern techniques are mere engineering, not creation.

Regulatory Issues

Regulatory problems are far greater than just addressing the common concerns above. The regulatory system is designed to fail (Figure 8). It begins with the notion that the procedure for introducing genetic alteration is a greater issue than the outcome. That is completely based on fears in the 1980s about the unknowns of genetic engineering. That attitude has remained in place for thirty years, even though our understanding and our techniques have advanced beyond what was imaginable back then. A second problem is that scientists thrive on conducting experiments to address unresolved scientific questions. However, lab testing has severe cost and infrastructural constraints that restrict the numbers of animals and minimize the variations and conditions that can be considered. In contrast, the natural world is characterized by large numbers of organisms, innumerable variables, and confounding interactions that are poorly understood. Hence, singling out and testing only a few of the myriad variables inevitably leads to irrelevant results that, because they are so controlled and do provide reproducible results, are often published in premier journals. The result is that the question remains unanswered for which, ironically, the scientists are rewarded: more publications and more work. Regulators are also rewarded; they recognize that issues brought up by the scientific community remain unresolved and, thereby, no decision needs to be made. The results are evident. No genetically modified animals derived from modern genetic techniques have passed regulatory approval.

Broken Partnership

Gene editing represents the ultimate level of genetic engineering wherein precise changes can be made in genomes to achieve exact goals. The old ways of looking at genetic engineering are outdated. Regulators and their advisors must update and apply new ways of evaluating the coming tidal wave of gene-edited animals. The partnership between the regulatory agencies and the funding agencies is broken. The new tools, understanding and approaches to improve agricultural efficiency to meet needs of the 21st century have been developed, but they are not available to those who paid for the basic research. That has to change.

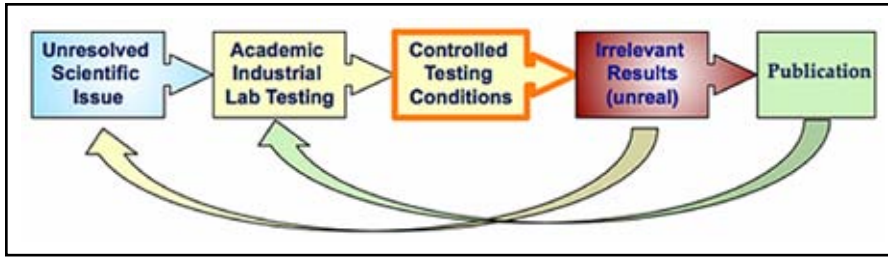

Figure 8. Regulation of genetic modification of animals is designed to fail.

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PERRY HACKETT has 50 years of experience in molecular genetics and genetic engineering. He has degrees in physics (Stanford) and biophysics (University of Colorado). Since 1980, he has been a professor of genetics, cell biology and development at the University of Minnesota, where he is a founder of the Center for Genome Engineering. His focuses are on molecular genetics and genome research. Recently he has been developing methods for gene editing in large animals including livestock. He has used bacteria, fish, mice and dogs as model systems for investigating various transgenic technologies in vertebrate animals. The *Sleeping Beauty* Transposon System, invented in his lab as a way of delivering genes to vertebrates, is in human clinical trials for treatment of lymphoma and other cancers.

DR. HACKETT is a cofounder of Discovery Genomics, to develop the *Sleeping Beauty* Transposon System for human gene therapy, and Recombinetics, to genetically engineer livestock for biomedical and agricultural purposes. Both companies are based on technologies developed in his and his students' labs. Currently, Dr. Hackett is the chief science officer of Recombinetics. He serves on three editorial boards and three scientific advisory boards in the areas of transgenesis, genome engineering and gene editing.

TALENs and CRISPR/Cas9 for Rice-Genome Editing

Bing Yang
Iowa State University
Ames, Iowa

byang@iastate.edu

Rice, *Oryza sativa* L., is an important staple crop that feeds more than half of the world's population. Cultivated for more than 5,000 years, a huge number of natural and cultivated genotypes exist, representing the richest genetic resource among crop species. Rice also serves as a model species for cereal crops due to its syntenic relationship with other grass species (Gale and Devos, 1998). It was the first crop to have its genome sequenced (Goff *et al.*, 2002; Yu *et al.*, 2002), and the genome sequence of cultivar Nipponbare has been well annotated (Sakai *et al.*, 2013). Rice has been a subject of genetic manipulation by a variety of approaches, including "traditional" mutagenesis (*i.e.* chemical- and radiation-induced mutation) and forward and reverse genetics.

Research in our laboratory has been focusing on host/microbe interactions by using bacterial blight of rice as a model. The pathogen, *Xanthomonas oryzae* pv. *oryzae*, causes an important disease in Asia and Western Africa, resulting in severe losses in rice-grain production (Nino-Liu *et al.*, 2006).

The objectives of our research include:

- identification of virulence factors that facilitate the pathogen's ability to colonize the host and develop symptoms,
- identification of host targets for the pathogen's virulence factors that cause disease susceptibility,
- elucidation of the molecular mechanism of disease susceptibility and resistance mediated by the interactions between the pathogen's virulence factors and the host's target genes or gene products, and
- engineering resistance based on the best information obtained from study of the disease, an approach that is widely applicable to other related diseases in crops.

We are particularly interested in a group of bacterial proteins from *Xanthomonas*, transcription-activator-like (TAL) effectors, on which some strains of *Xanthomonas* depend for pathogenesis. More than 100 distinct TAL-effector genes have been cloned or identified

from sequenced bacterial genomes; they are highly conserved, with about 90% identity at the nucleotide or amino acid level (Boch and Bonas, 2010). A typical TAL effector consists of the N-terminal domain, middle repetitive region and C-terminal domain. The N-terminus contains a secretion signal used by the bacterial type-III secretion system to translocate the TAL effectors from bacterial cells into host plant cells. The C-terminus contains three functional nuclear localization motifs for transporting TAL effectors into the nuclei of host cells. The C-terminus also contains a functional trans-activating domain, a characteristic feature of eukaryotic transcription activators. The most striking feature of TAL effectors is the central repeats that are mainly 34 amino acids in length. The repeats are nearly identical except for the two amino acids at positions 12 and 13, the so-called variable di-amino acids. The di-amino acids actually determine the specificity of DNA binding for each repeat. More than 20 types of repeats exist in native TAL effectors, but four types are predominant and each corresponds to one of four nucleotides with NI to A, NG to T, NN to G, and HD to C (using the single-letter code for amino acids[1]) (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). The discovery of the TAL-effector recognition code has two immediate implications:

- the code could be used to predict and validate the DNA sequences of host-target genes involved in bacterial diseases of crops, and
- the code could be used to guide the custom-engineering of DNA-binding proteins or domains with novel specificity.

Fusion Proteins of TAL Effectors and the FokI Nuclease Domain are Active Nucleases

As one of the obvious applications with programmable TAL-effector DNA-binding domains, TAL-effector nucleases (TALENs) have become a promising genetic tool for basic and applied research. We started working on development of TALEN technology by using TAL effectors AvrXa7 and PthXo1 and their respective target sequences. First, the two native TAL effectors were fused with the nuclease domain of the restriction enzyme FokI, generating the fusion proteins (putative TALENs). Second, the known DNA sequences respectively targeted by PthXo1 and AvrXa7 in the promoters of rice genes *Os8N3* and *Os11N3* were fused into a non-functional reporter gene *LacZ*; the two halves of *LacZ* contained the duplicated 120-bp regions separated by the PthXo1- and AvrXa7-targeted sequences in *Os8N3* and *Os11N3*. Third, constructs expressing the fusion proteins of AvrXa7-FokI and PthXo1-FokI were co-expressed with the *LacZ*-containing reporter construct in yeast. If functional as site-specific nucleases, PthXo1-FokI and AvrXa7-FokI would cause DNA double-strand breaks (DSBs) at the spacer regions between the two halves of the *LacZ* gene, and the repair to DSBs would lead to reconstitution of a functional *LacZ* gene and β-galactosidase activity, the gene product of *LacZ*. Our proof-of-concept experiments, indeed, demonstrated the feasibility of producing active TALENs by fusing the TAL effectors as DNA-binding domains and the FokI nuclease domain. The work was published in *Nucleic Acids Research* (Li *et al.*, 2011a).

[1]N=asparagine; I=isoleucine; G=glycine; H=histidine; and D=aspartic acid.

We further demonstrated the ability to engineer novel TAL-effector DNA-binding domains by using four types of TAL-effector repeats that contain NI, NG, NN and HD at positions 12 and 13 of the repeats that preferentially recognize the nucleotides A, T, G and C, respectively. The four types of TAL repeats were assembled by a modular assembly method. Briefly, each of the repeats as independent modules contains a unique 4-bp overhang with single-base polymorphism after digestion with BsmBI at its 5'- and 3'-ends. For construction of an 8-repeat TALEN array recognizing a specific 8-nucleotide sequence, a corresponding repeat is selected from each set of the repeats for the specific nucleotide of that position. By putting 8 separate repeats together through DNA ligation, an array of 8 repeats can be assembled. Similarly, one or two additional 8-repeat arrays can be assembled; further ligation of two or three 8-repeat arrays results in 16 or 24 repeats of novel TAL-effector binding domains and TALENs, once fused with FokI nuclease domain, recognizing the user-chosen 16 or 24 bp of the target sequence.

To demonstrate the capacity of engineered designer TALENs (dTALENs) to modify endogenous gene loci in eukaryotic cells, we chose genes *URA3*, *LYS2* and *ADE2* as the targets in yeast. Five pairs of TALENs were designed and engineered by using our TALEN modular-assembly method with two pairs targeting two sites of *URA3*, two pairs targeting two sites of *LYS2* and one pair targeting one site of *ADE2*. When transferred with each pair of TALEN genes, the yeast cells were selected for mutations that were derived from the repair to the TALEN-induced DSBs on growth media containing chemicals such as 5-fluoroorotic acid (5-FOA, for *ura3*-mutated cells), or α-aminoadipate (α-AA for cells with mutated *lys2*), or containing limiting adenine concentrations that result in the formation of pink colonies formed by *ade2*-mutant cells. The results indicated that each pair of TALENs indeed induced site-specific mutations that were either insertions or deletions in the intended target genes with corresponding phenotypes of yeast cells. The results were published also in *Nucleic Acids Research* (Li *et al.*, 2011b).

TALEN Applications in Rice to Engineer Disease Resistance to Bacterial Blight

Our ultimate goal in developing TALEN technology is to apply it for our basic scientific research and for breeding disease-resistant rice varieties. As a case in point, we focus on bacterial blight of rice. Years of research effort by our group and by scientists in other laboratories around the world allow us to propose a working model for the outcome of rice-blight disease controlled by the interactions between the TAL effectors from the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo) and the host target disease-susceptibility (S) genes.

First, the pathogen produces and translocates its virulence proteins, including TAL effectors, into host cells through a type-III secretion system; once internalized, TAL effectors are localized into the nuclei of the host cells and bind to the promoter elements [effector-binding elements (EBEs)] of S genes; TAL effectors transcriptionally activate the S-gene expression, which leads to more susceptibility of host plants to bacterial infection. It has been discovered that some Xoo isolates depend on the TAL effector PthXo1 to activate *SWEET11*, one of the sugar-transporter genes in rice. Some isolates

use PthXo3, AvrXa7, TalC or Tal5 to induce *SWEET14* for disease susceptibility, whereas some pathogenic isolates utilize *SWEET13* for disease (Yang *et al.*, 2006; Antony *et al.*, 2010; Streubel *et al.*, 2013).

It would be ideal to make a small change at the TAL-effector binding site in the S gene so that the TAL effector would not recognize the target site of the S gene and the modified plants would become resistant to the bacterial infection. Collectively mutating all three S-gene promoters would enable the plants to become durable and broadly resistant, *i.e.* to all of the Xoo strains that depend on the induction of either one or all of the S genes. The precise modification at the intended site of the S gene would be achieved by using TALENs designed to specifically cause DSBs in the S genes. The TALEN-mutagenized S gene would provide disease resistance similarly to a naturally occurring S-gene mutant such as *xa13,* a resistant rice gene that has a mutation at the PthXo1 binding site in the promoter of *SWEET11* (Chu *et al.*, 2006; Yang *et al.*, 2006).

The precise gene mutagenesis using TALENs in rice also involves multiple steps of transgenics (Li *et al.*, 2014). The steps include 1) immature rice embryos are induced to generate embryogenic callus cells in tissue-culture medium; 2) the callus cells (calli) are transformed with a TALEN-gene-containing construct that also has an antibiotic-resistance gene for selection of transgenic cells or plants containing the TALEN construct; 3) the transformed calli are selected on hygromycin, an antibiotic that kills the non-transformed wild-type embryogenic cells; 4) transgenic plantlets are generated from calli that may contain desired mutations at the intended site caused by TALENs; and 5) the transgenic plants are genotyped for site-specific mutations.

To first demonstrate the feasibility of employing TALENs to effect site-specific mutations at the TALEN-binding site of the S gene and thus produce rice plants resistant to bacterial blight, we engineered two pairs of TALENs that targeted the AvrXa7- and PthXo1-binding sites in the promoter of *SWEET14* (or *Os11N3*). We expected that the TALEN-induced mutations that interfered with the inducibility of *SWEET14* by AvrXa7 and PthXo1, would not affect the developmental function of *SWEET14* in rice. The T1 generation of the transgenic rice plants, in the progeny of selfed primary transgenic plants, were genotyped by PCR-amplifying the relevant region and sequencing the amplicons. Indeed, site-specific mutations of small insertions/deletions were found at the promoter of *SWEET14* at high efficiency. Also, the rice plants homozygous for the 4-bp or 9-bp deletion were resistant to bacterial blight when inoculated with Xoo isolates that depended on AvrXa7. Importantly, the construct that contains the transgenic TALEN genes and the hygromycin-resistant gene could be segregated out in some progeny through genetic crossing, resulting in plants that contained only the desired mutations and valuable agronomic traits, but not the transgenes. The results were published in *Nature Biotechnology* (Li *et al.*, 2012).

The transgene-free rice plants allow us to carry out the second round of gene modification by using constructs that contain new TALENs and the hygromycin-resistant gene, in this case by targeting another SWEET S gene, *SWEET11*. One pair of TALENs was engineered based on the PthXo1-recognizing DNA sequence in the promoter of *SWEET11*. Similarly to the production of *SWEET14* mutations, rice plants with muta-

tions occurring at the intended site of *SWEET11* were obtained. Wild-type rice was also used to generate mutations in *SWEET11* with the engineered TALENs. Those plants contained mutations in both *SWEET14* and *SWEET11* promoters as expected, and were subjected to bacterial inoculation with a collection of 95 Xoo isolates. The plants with only *SWEET11* mutations were resistant to 16 of 95 Xoo isolates; plants with only *SWEET14* mutations were resistant to 71 of the 95 isolates, whereas plants with both *SWEET11* and *SWEET14* mutations were resistant to 87 of the 95 Xoo isolates. The results clearly demonstrate the broad spectrum of resistance in rice plants with mutagenesis of two S genes (unpublished data).

We have been using TALEN technology also to generate genetic materials of rice to gain basic understanding of the roles of rice SWEET genes in plant growth, development and production in addition to disease susceptibility. There are 15 SWEET genes that are highly homologous and expressed in different types of tissues. Our goal is to generate knockout plants of individual genes or combination of multiple genes. So far, we have generated knockout plants of 8 SWEET genes. Characterization of the mutant plants and production of the rest of the SWEET genes are in progress.

CRISPR/Cas9 System for Targeted Gene-Editing in Rice

The type-II CRISPR/Cas RNA-guided nucleases are the most recent addition to the tool kit of sequence-specific nucleases. Intense interest has been focused on the CRISPR/Cas9 system from *Streptococcus pyogenes* following initial reports of its successful use for gene editing (Jinek *et al.*, 2012). In this system, Cas9 nuclease coupled with tracrRNA (trans-activating crRNA) can be guided by a ~20 nt guide (or seed) sequence in crRNA (CRISPR RNA) to hybridize with a specific complementary DNA sequence (*i.e.*, the target site) that is followed by a 5'-NGG or 5'-NAG PAM (protospacer adjacent motif) sequence to induce a precise cleavage of the target sequence 3–4 base pairs upstream of the PAM site (Jinek *et al.*, 2012). Alternatively, parts of crRNA and tracrRNA sequence can be fused in a synthetic gene to produce a single-guide RNA (sgRNA) that is equally as effective as the separate crRNA and tracrRNA complex in targeting a specific DNA sequence for Cas9-directed cleavage (Cong *et al.*, 2013; Mali *et al.*, 2013). Unlike ZFNs (zinc-finger nucleases) and TALENs, the CRISPR/Cas9 system, referred to here as Cas9/sgRNA, is DNA-methylation insensitive. It is also more affordable, remarkably easier to use, and well-suited for multiplex gene targeting and high-throughput genome-wide gene editing at a similar or even higher efficiency than ZFNs and TALENs.

We have developed a Gateway-based Cas9/sgRNA system for rice-gene editing. Specifically and briefly, a common Cas9-expressing destination vector and an intermediate vector for cloning up to two oligo-derived sgRNA genes were constructed. The major cloning work involves sequential insertion of two oligo-derived small dsDNAs in the intermediate vector. The resulting sgRNA construct can be easily combined with the master Cas9 binary vector using Gateway clonase into a single construct wherein, in rice cells, Cas9 is expressed under the maize ubiquitin 1 promoter while the sgRNA genes are expressed under the rice U6 promoters. Restriction enzymes BtgZI and BsaI will be used to create 4-bp overhangs downstream of the U6 (or U3) promoter in the

intermediate vector, and the complementary oligos with appropriate 4-bp overhangs, after being annealed to each other, will be phosphorylated, annealed and cloned into the respective pENTR-sgRNA.

As an example, we made a construct expressing Cas9 and the sgRNA-targeting the rice SWEET13-coding region. The construct was transferred into rice embryogenic callus cells and transgenic plants were obtained through tissue culture and transformation similarly to the TALEN work in rice. Nine independent transgenic lines were obtained; PCR-amplification of the relevant region and sequencing of the PCR amplicons revealed a high efficiency of mutagenesis at the targeted site of *SWEET13*. Each line contained the mutations that occurred independently on the two chromosomes (so-called di-allelic mutations), resulting in a mutagenesis frequency of 100%.

We also demonstrated proof-of-efficiency of Cas9/sgRNAs in producing large chromosomal deletions (115–245 kb) involving three clusters of genes in rice protoplasts and verification of deletions of two clusters in regenerated T0 generation plants. Part of our Cas9/sgRNA work has been published in *Nucleic Acids Research* (Zhou *et al.*, 2014).

Conclusions

- We have demonstrated that fusion proteins of the native TALE and the FokI nuclease domain enabled site-specific DSBs (Li *et al.*, 2011a);
- We developed a modular assembly method to engineer designer TAL effectors with novel DNA-binding domains, and the custom-made TALENs were capable of inducing gene editing in yeast (Li *et al.*, 2011b, 2014);
- TALENs were successfully applied to edit the promoters of two disease-susceptibility SWEET genes to render the otherwise susceptible rice resistant to a broad range of bacterial-blight pathogen field isolates (Li *et al.*, 2012);
- CRISPR/Cas9 has been established for genome editing in rice, leading to extremely high efficiency in small/local DNA changes and efficient large chromosomal-segment deletions (Jiang *et al.*, 2013; Zhou *et al.* 2014).

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Zhou H *et al.* (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Research doi: 10.1093/nar/gku806.

BING YANG'S research interests focus on: the molecular mechanisms of plant/microbe interactions and crop disease-resistance engineering by using the bacterial blight of rice as a model; and development and application of TALEN and CRISPR technologies for targeted genome editing in plants. Over the past 15 years, he has identified and characterized several important naturally occurring TAL effectors in the rice pathogen *Xanthomonas oryzae* for their disease-promoting ability, and, most recently, he has helped harness the disease-causing TAL effectors for targeted gene editing. His group generated the first disease-resistant crop species by using the TALEN technology.

DR. YANG has a PhD in plant pathology from Kansas State University. He is an associate professor in the Department of Genetics, Development and Cell Biology at Iowa State University.

Keynote Presentations

Q&A

Moderator: Abel Ponce de León
University of Minnesota
Minneapolis-St. Paul, Minnesota

Daniel Voytas: A question for Dana. We now have at our fingertips all these DNA-breaking reagents, and you talked about ways to direct the outcomes of repair—homologous recombination, non-homologous end-joining—so, in the future do you think that we will be able to control the DNA-repair machinery, both in terms of achieving higher efficiencies and very predictably the outcomes of the repair.

Dana Carroll: Yes… I'm working on it. Quite a number of people are working on trying to improve the efficiency of homologous recombination, which, for a lot of us, is lagging. There is a lot of different approaches, and I don't know if it will come through adding components that will enhance homologous recombination, or whether it will come through knocking down and joining; maybe there's an approach that we haven't anticipated.

Robert Millman (MPM Capital): Dr. Voytas, in your expression system, did you use a selectable marker?

Voytas: In my potato example, no selectable marker was used. We knew the framework of the technology and expected efficiencies, so we treated the cells and began screening to regenerate plants.

Karen Kindle (Boyce Thompson Institute): Do the invertase mutations have any developmental, phenotypic or flavor effects?

Voytas: That's the next question. We have just generated the products and we hope, with the USDA's approval, to go to the field to fully evaluate the trait.

Patrick Di Bello (University of Arkansas): Has the FDA given you any indication of how they will regulate the potatoes?

Voytas: No. The EPA must also consider environmental impact.

Vibha Srivastava (University of Arkansas): Regarding the efficiency of gene targeting, you observe 5 percent in potato and 7 percent in tobacco, which are easily transformed, but what do you expect in species that are more difficult to transform?

Voytas: For many species, getting the nuclease and the donor molecule into the cell remains a challenge. The potatoes were easy because almost every cell was transformed—in excess of 80 percent of our cells took up the reagents, so many of the plants that we regenerated had the target modifications. This will continue to be a challenge. You might expect 10 percent of the cells that take up the reagents will undergo the modification you are interested in. You have to identify the cells that took up the reagent, and, among those identify those that have undergone the gene replacement.

Yinong Yan (Pennsylvania State University): I agree with Dr. Hackett that we should focus on the product, not the process. Even though *Agrobacterium* is a plant pest, regulation of *Agrobacterium* as a vector should be abolished.

Voytas: Opinions rendered so far say that if the product does not have the pathogenic sequences, it should not be regulated.

Adam Bogdanove (Cornell University): What do you think is more important: educating consumers about the technology or putting forth products that have a clear consumer benefit?

Hackett: Regarding educating the general public, I don't think you can do it. It's hard enough—when I teach undergraduates in genomics—to get them to pay attention to what I am talking about, even with the threat of tests. What I am finding—because I make maximum use of clickers in the classroom and interrogating the students constantly on where they're at—is that over the past 5 years, and it has just been five years, two obvious changes have been occurring, each of which is a sea-change equivalent to the acceptance of gay marriage in America. Number one, of incoming freshmen, only 2 percent believe that they *don't* eat GMO food; ninety-eight percent think either they are, or they might

be, eating GMO foods, and it doesn't concern them in the slightest. Secondly, most of them feel that regulatory policies fail to take advantage of recent developments, but they also feel that regulatory agencies can be trusted to save them. These numbers and trends result from questions to the students before they receive any instruction, in the first hour of class.

Voytas: To educate the public presupposes that they understand the subtleties of this highly sophisticated technology. As an analogy, when I have dinner with my mother and say, "Hey Mom, I've whipped up a potato that is resistant to chlorsulfuron." she is likely to respond, "That's fine, but God already made a perfectly nice potato that I prefer to eat." But, if I say, "Today I whipped up a potato that has less neurotoxin when fried," she might be more predisposed to it. She doesn't have to understand the subtleties of the science and technology, but she can grasp when something is more healthy. This is the level of approach appropriate for most of the population.

Hackett: In Brazil, you are allowed to eat transgenic this, that, and the other, and labels on the foods have a small yellow triangle that shows a capital "T." The transgenic products sit on the shelf side-by-side with non-transgenic counterparts, and the only difference between them is cost. It's akin to decisions on whether to eat organic or natural produce, for which my students don't really have a feeling for the difference. I trust the Brazilian government that the items on the shelf with the "T" are safe, for the most part—like we trust "non-organic" to be safe, although we may buy organic.

William Haun (Cellectis Plant Sciences): Perry, one of your slides indicated that one founding reason why the system is broken is that the process is regulated, not the product. In Canada, they regulate the plant product rather than the process; regulation is triggered if the trait is novel. Does that system apply to animals also, and, if so, is it at least a step in the right direction?

Hackett: At the *Second International Workshop for Regulation of Animal Biotechnology* in Brazil in August, 2014, a Canadian regulator said that the US policies on transgenic crops are "an utter disaster," and I suggested that, in fact, they are an outstanding example of success. So, we couldn't have been further apart in our views. He didn't like the clumsiness of the statutes, whereas I was looking at the outcome that for 20 years more products in more countries with more acreages are being devoted to transgenic crops, especially so now that the greatest percentage increases are occurring in developing countries where small-scale farmers have the most to gain. In the United States, it is encouraging to see that gene-editing of plants is being suggested as not requiring regulatory oversight. However, that concept remains under consideration *vis-à-vis* transgenic animals. The US agency that regulates animals has not released a single transgenic animal in a quarter-century; they spend so much time trying to expand their control without thinking about revising statutes to bring them more into line with twentieth-century technology.

Greg Martin (Boyce Thompson Institute): Availability of these new methods raises the question of how many interesting target genes in plants and animal do we know enough about to be in a position to start editing them?

Hackett: This technology is equivalent to high-end computing. When IBM first came out with its large computers, the chairman stated that there was need for five of them. Now, everyone of us has a laptop, each of which greatly surpasses those "large" computers in capability. I think that the possibilities of design changes in animals and plants are beyond the imagination of anyone in this room.

Voytas: I second that. Thinking back to my PhD work—I spent five years sequencing 30 kB, whereas it can be done in a millisecond with current technology. And sequencing the human genome was beyond imagination. We are talking about one or two genes being modified, whereas—in line with Perry's comments—in a few years we may be able to make many dozens of nucleotide changes simultaneously.

Karen Kindle (Boyce Thompson Institute): How do intellectual property issues affect you as academics who may wish to see your products in the public domain? Any suggestions for researchers, particularly those in small companies?

Voytas: Those in the genome-engineering community have been very good about making reagents accessible to help address basic biological questions. If a product arises from a targeted modification, that's when intellectual property has to be taken into consideration. A handful of companies have pieces of applicable IP that may have to be accessed for commercialization. Good news, in terms of getting the technology more broadly accepted, is that we have multiple competing platforms, which, basically, drives down the price. If it is too expensive to use a TALEN to modify a tomato, then a CRISPR approach may be a valid alternative.

Abel Ponce de León: Where genomic introgression has been achieved, the intellectual property is basically on the animal or plant *per se*, not necessarily on the knowledge. Have any of your companies addressed this already, and where are they in the process?

Hackett: We have filed patents on several of the animals, but I would like to go to the bigger aspect. It's unbelievably expensive to get taxpayer investments in basic research back to the taxpayers. I started two companies, and it cost me probably a total of $180,000. I'm way in the hole right now, but, hopefully, something may come out. To get any of this stuff out, whether it's *Sleeping Beauty* transposons with chimeric antigen receptors to treat cancer or to get some of these animals out to developing-country farmers who need them, takes an incredible about of money. Investors are not going to give anybody any money to get this technology out to the people who paid for its development in the first place, unless there is IP to protect the rest of the development into a product. Actually the University of Minnesota holds up the dispersion of the reagents that we have. They have

something called a materials transfer agreement, which I hate. I used to share what I had until they put a stop to it because they want everything to run through their hands.

Brent Woodward (Cooperative Resources International): Are you willing to tell us more about the regulatory minefield faced by Recombinetics?

Hackett: First of all, we are completely open. A request was put out by INAD[1] on whether or not Recombinetics and other companies were planning on using gene editing with the idea that the FDA would be able to have a certain amount of discretion to not demand field trials and the like, that would be way too expensive for a small company to afford. Such a letter was sent, and retracted a few days later after a meeting with regulators in the context of another meeting at which it was realized that this meant that Recombinetics was recognizing the legality of the FDA's position, that gene editing was, actually, under their purview. It is our opinion that, in fact, because this is no different from any natural mutation—it doesn't leave any footprint, so to speak, it's not transgenic DNA in the slightest any more—that it really doesn't have anything to do with better regulations. And so, the lawyers actually advised us to retract the letter.

Steve Pueppke (Michigan State University): Is any other country regulating more effectively and doing a better job?

Hackett: Zuoyen Zhu, who was the first scientist to genetically engineer a fish for food, in Wuhan, China—growth-enhanced carp—spent a Sabbatical in my lab and gave a talk in 1987, in which he said that the fish would be released to the Chinese public in 1995, approximately, when adequate stocks would be available. A scientist in our group, Anne Kapuscinski, asked when trials would be completed to determine safety; he replied, 1995. In fact, none of these fish have been released, although China needs fish. But they have deferred to the United States, as have other regulators in other countries. That's the problem—people are waiting for the United States to do it the right way, thinking that we have the most expertise.

Audience Member: Reference has been made to engineering with TALENs and CRISPERs. Will they become part of bioengineering modules that will be used in synthetic biology or do you see them as distinct approaches?

Voytas: If you think of synthetic biology in terms of constructing new organisms to produce products of value, then certainly these TALENs and CRISPERs are enabling tools.

Alan Collmer (Cornell University): Like many other land-grant universities, Cornell has sincerely expressed aims such as "knowledge for the public purpose." I am wondering if

[1]Investigational New Animal Drug for the FDA's Center for Veterinary Medicine (CVM).

the land-grant university system could be a particularly useful voice for guiding us towards more rational regulations and also IP policies that are designed to benefit the public.

Voytas: If you take as a precedent the opinions that have been rendered by the USDA on these technologies, I can see going to horticulture departments and saying, "Do you want to change the color of your petunias?" or "Do you want to knock out a few genes to get rid of anti-nutritionals," then "Here is the technology, here's how to do it, go ahead." If the regulatory barrier is broken down, there is no cost to deploying the technology at land-grant institutions for practical purposes. Clarity is still tentative; only a few opinion letters have been published, but if they reflect the eventual trend, with clearer guidelines the technology will be ready to be deployed.

Ponce de León: Let me add something to that. The majority of the members in the North American Agricultural Biotechnology Council are land-grant universities, and through the Council we are trying to bring to the fore conversations involving various stakeholders with interest in making progress with these technologies, including discussing of pros and cons. We have to acknowledge as scientists that the regulations have political components and regulators have to accommodate both scientific and politic considerations. Through our discussions, we hope that by the end of the conference we will develop novel ideas to propose alternative solutions to manage the conflict and allow us to move forward.

Ralph Hardy (North American Agricultural Biotechnology Council): And returning to the regulatory area, the mistake that was made in the United States in terms of regulations, back in the 1980s, was to elect to use existing legislation, This was probably good in the short term, but it was disastrous in the long term. We've heard about the Canadian situation, where they regulate the product rather than the process, and Helen Shearer will address this later[2]. NABC's 2013 conference focused on the fruit and vegetable area where there are very few genetically modified crops. One problem is small market share, which deters interest on the part of large companies and the other problem is regulatory. Recently, NABC issued a brief white paper suggesting ways of facilitating the commercialization of genetically engineered fruits and vegetables[3]. Also, the National Research Council has begun a new study on oversight and regulation of genically engineered crops. My experience is that our NABC reports are helpful, but those from the NRC carry more weight on the Washington scene than most other sources of information. We have a unique opportunity to provide that NRC committee with our guidance on how to improve the regulatory system, which we should keep in mind during the *Tie-Up Session* discussion at the end of the conference.

[2]Pages 193–199.
[3]http://nabc.cals.cornell.edu/Publications/WhitePapers/SpecialtyCrops.pdf.

CRISPR/Cas9: Tools and Applications for Eukaryotic Genome Editing

Fei Ann Ran
Broad Institute
Cambridge, Massachusetts

ran@fas.harvard.edu

I will provide some background on the CRISPR/Cas9 technology, some of the rationale for how we came to develop and use this tool, and I will address immediate questions concerning the specificity of the technology. I will also discuss some of the more interesting applications.

Figure 1 reflects how the cost of DNA sequencing has decreased dramatically over the past two decades due to technological progress. As a result, there has been an explosion of data, not only in the sequences of different species, but in sequence differences between individuals within species, between cell types and between diseased and healthy cells. It suffices to say that this is an exciting time to be working in the field of genome engineering.

Genome Engineering

Typically, genome engineering is achieved by leveraging the cell's own repair machinery. This can come from the error-prone NHEJ pathway that leads to insertion/deletion (indel) mutations, which can be used to knock out genes, or, alternatively, we can supply a repair template to overwrite the site of a double-stranded break (DSB) for more-precise genome engineering via the HDR pathway (Figure 2).

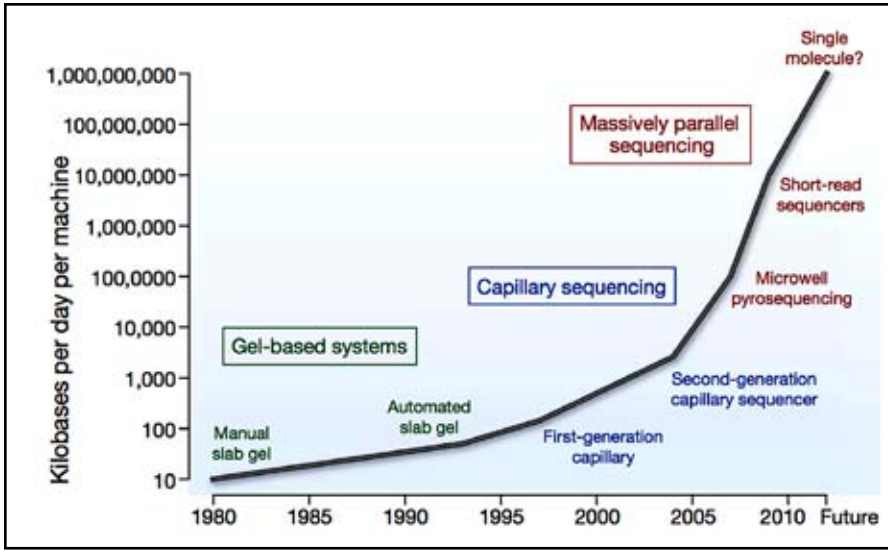

Figure 1. Advances in DNA-sequencing technologies.
(Stratton MR *et al.*, 2009)

When we started working on CRISPR/Cas technology[1], several well developed tools were already being used—and still are being used—to achieve impressive results in biotechnology, medicine, agriculture, and other fields. At the outset, we were interested in developing an alternative technology to make cloning easier at lower cost with greater scalability.

The CRISPR locus, including the hallmark repetitive patterns of crRNA, was discovered in the genome of *Escherichia coli* over 25 years ago, and, at the time, no one knew what they were. In the 2000s, it was discovered that this was a defense system against viral infection. Figure 3 illustrates a phage injecting its genome into a bacterium; a portion of the viral genome is inserted by the bacterium into its own genome. These inserts were initially called spacers, which are vitally important for CRISPR-system function. One of the requirements for what sequence can be incorporated and inserted into these CRISPR-loci in bacteria is what's called a protospacer adjacent motif (PAM). This is important because a given species may have one or multiple types of CRISPR systems, and each CRISPR system may have a unique PAM.

The CRISPR system that we started working with—now one of the most widely adopted—comes from *Streptococcus pyogenes* (Figure 4) and the PAM for that species is

[1]Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins are found in many bacteria and most archaea. CRISPR-Cas systems use sequences derived from plasmids and phages to activate Cas endonucleases to neutralize those plasmids and phages via RNA-guided sequence-specific DNA cleavage, thus blocking their transmission and creating simple acquired immunity.

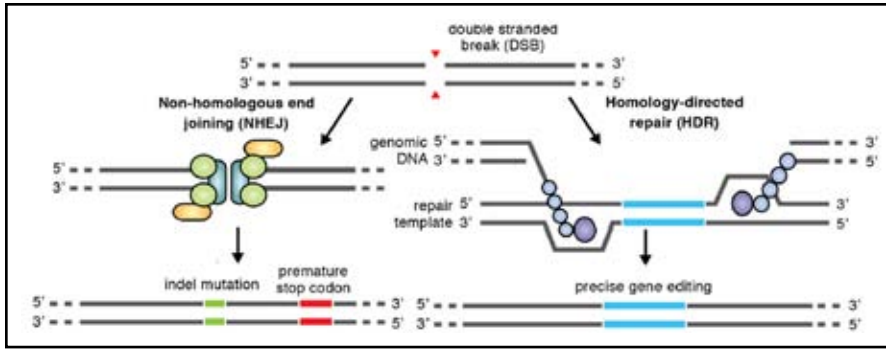

Figure 2. DNA double-stranded breaks facilitate alteration of the genome. (Ran *et al.*, 2013)

an NGG-trinucleotide motif, which means that the *S. pyogenes* CRISPR system incorporates only sequences adjacent to NGG. After integration, the bacterium can carry out the "execution" part of its defense. During this stage, there are several key players. One is the CRISPR array that becomes transcribed as a long precursor CRISPR RNA (pre-crRNA). This is a string of direct repeats flanked by spacers and this array can go on for 30 to 60 different spacers, as a single long transcript. In the presence of the trans-activating CRISPR RNA (tracrRNA) and the Cas9 nuclease, the pre-crRNA:tracrRNA duplex gets processed to its mature form, consisting of single units of processed spacers and direct repeats hybridized to the processed tracrRNA. Now the mature crRNA:tracrRNA duplex can guide the Cas9 to target any sequence that is complementary to the spacer. The protospacer adjacent motif (PAM) is again crucial for DNA cleavage by Cas9. Cas9 will cleave only targets that are immediately adjacent to a PAM.

When we started working on this, the tracrRNA hadn't been discovered yet. This discovery came from Emmanuelle Charpentier's lab, and helped kicked off genome engineering using CRISPR/Cas9. At that time, two other developments also emerged (Figure 5): (1)

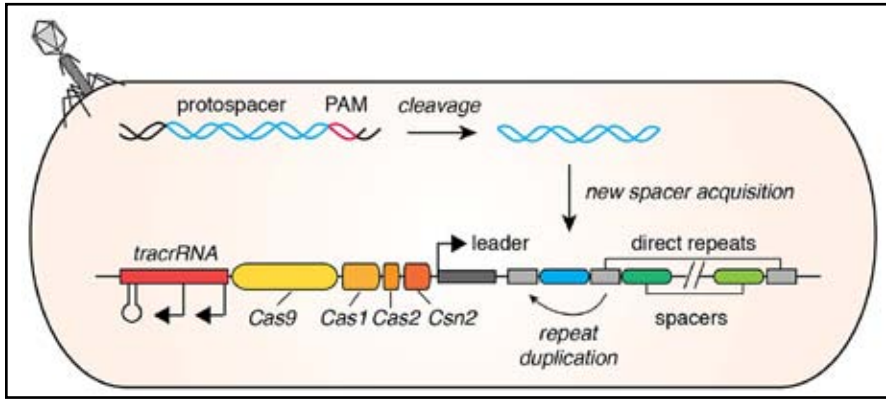

Figure 3. Clustered regularly interspaced palindromic repeats (CRISPRs).

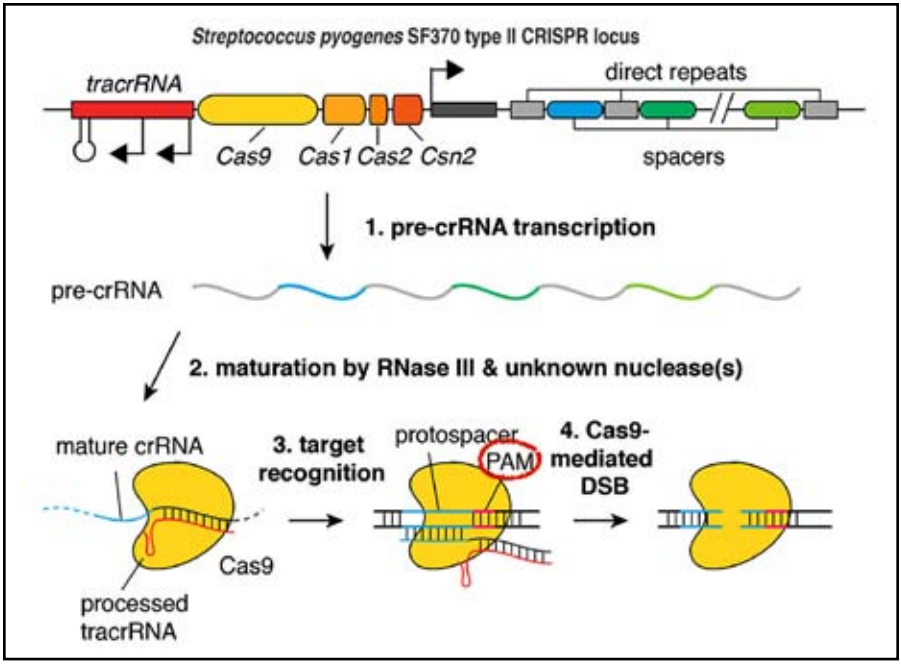

Figure 4. *Streptococcus pyogenes* CRISPR system.
(Cong *et al.*, 2013)

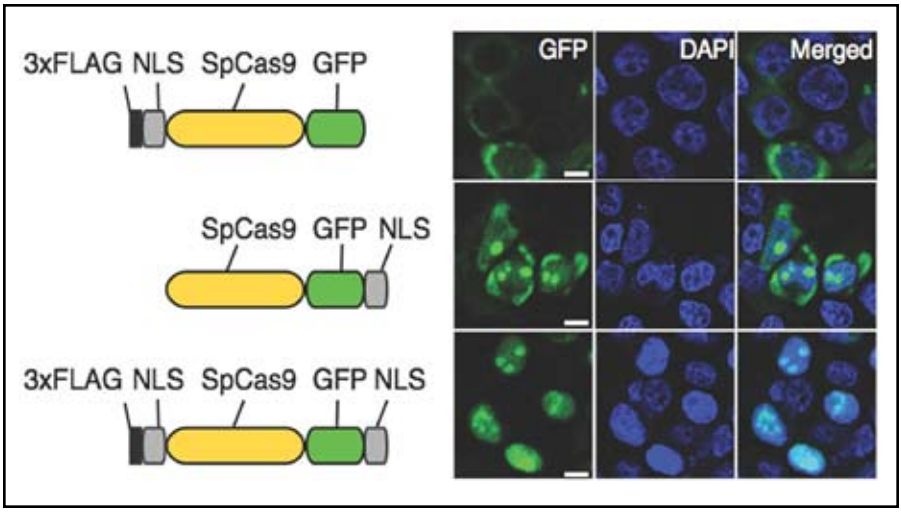

Figure 5. Adapting *S. pyogenes* Cas9 (SpCas9) for eukaryotic expression[2].
(Cong *et al.*, 2013)

[2]DAPI=4',6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to A–T-rich regions in DNA.

you can fuse the spacer and the repeat and the tracrRNA into a single chimeric RNA; and (2) you can use a single chimeric RNA and Cas9 to program the cleavage of DNA targets in an *in vitro* cell-free lysis reaction.

We built upon these exciting discoveries, but at the same time, nobody knew if this was going to work in mammalian cells. We modified two systems to get this working robustly in eukaryotic systems. One issue was that, obviously, bacteria don't have nuclei, whereas mammalian and other eukaryotic cells do, and so we tagged NLS (nuclear localization signal) sequences to Cas9 and also codon-optimized it for better eukaryotic expression. By doing this, we were successful in moving the Cas9 enzyme into mammalian nuclei. These experiments were done in human embryonic kidney (HEK) cells (Figure 5).

We started these experiments with the same type of chimeric RNA as described earlier. But we weren't having luck targeting every locus. So, we went back to optimize the RNA components and extended the tracrRNA portion of the chimeric RNA to its original full length that is expressed by the bacteria. We call this single-guide RNA (sgRNA) (Figure 6). The sgRNA has an invariant scaffold region and a spacer region or the guide proper region that base pairs with the target and once it brings Cas9 to the locus of interest, Cas9 makes a double-stranded break about 3–4 base pairs upstream of PAM.

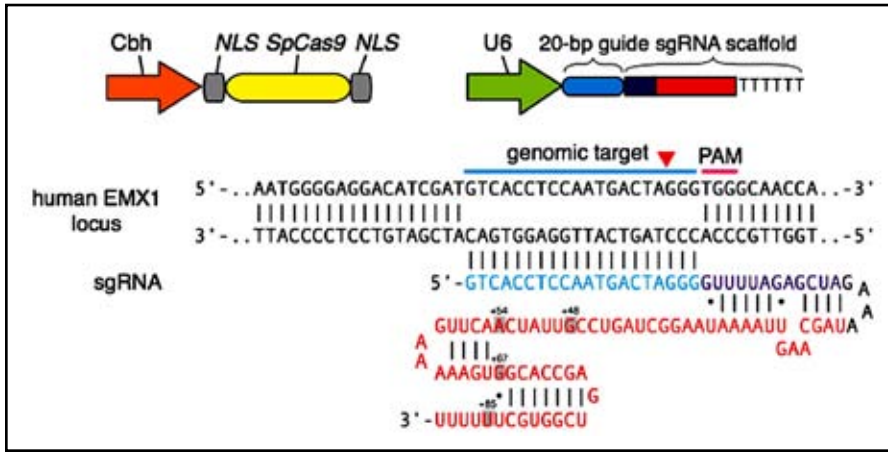

Figure 6. New single-guide RNA (sgRNA) design improves cleavage efficiency. (Hsu *et al.*, 2013)

With these two modifications, we were able to increase the efficiency of Cas9-mediated genome engineering in mammalian cells. Figure 7 shows an enzymatic assay, SURVEYOR, which we use to measure the efficiency of genome editing and—without going into detail—the numbers on the bottom are the percentages of transfected cell populations that have acquired indel mutations. This is an example of the more error-prone NHEJ way, which can be leveraged to do simple gene knockouts by creating mutations in the coding region of the gene. In the absence of any type of selection, with a transient transfection we see very high modification rates in mammalian cells.

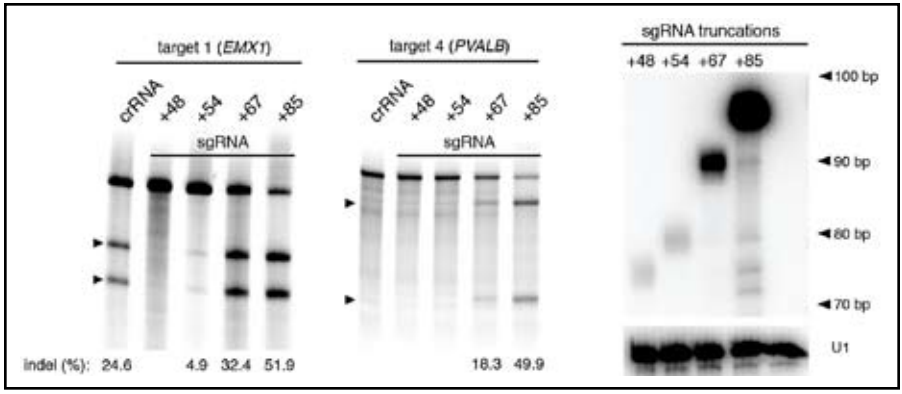

Figure 7. Extended single guide RNA (sgRNA) improves cleavage efficiency.
(Hsu *et al.*, 2013)

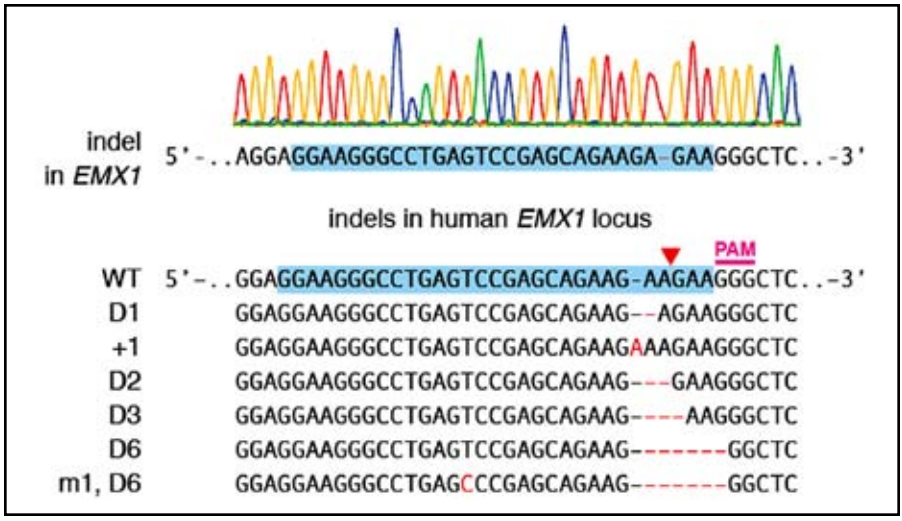

Figure 8. Cas9-mediated indel mutations.
(Cong *et al.*, 2013)

Figure 8 shows what the indel mutations look like when sequenced. Again, most are centered about 3–4 bases upstream of PAM. Cas9 was a very easy-to-engineer technology, because all that was necessary to target a locus was to provide an RNA template to Cas9. We thought we could multiplex the system, in other words knock out multiple genes in the same cell. Initially, we tried knocking out just two genes in the cell, which involved co-delivering two guides in Cas9. Again in a transient transfection of mammalian cells, both genes underwent fairly significant levels of indel modification. Since then other people have iterated those to a much higher order of multiplexing.

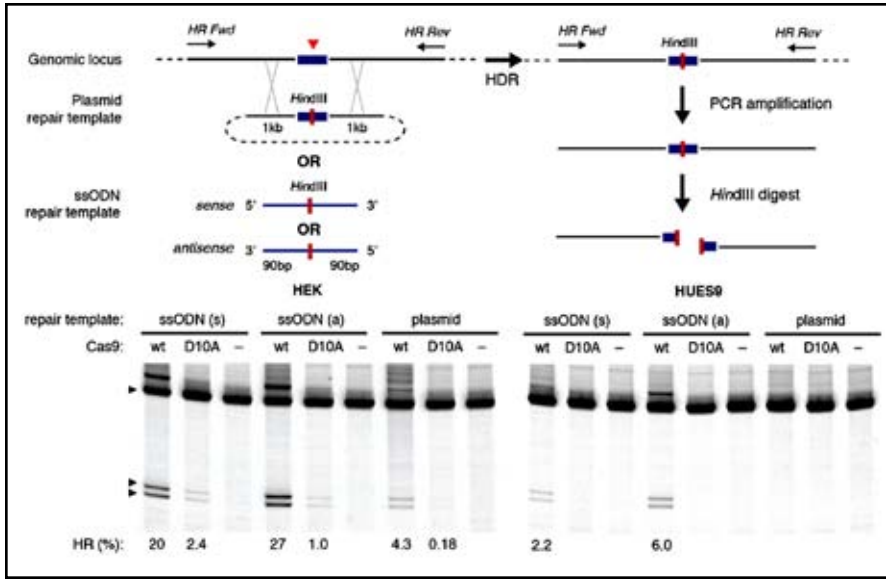

Figure 9. Using CRISPR-Cas9 to mediate precise gene editing.
(Ran *et al.*, 2013)

Similar to zinc fingers and TALENs, Cas9 is also a mediator of HDR through creating double-stranded breaks. Figure 9 provides an example of using Cas9 for introducing a pair of restriction sites into the genome through HDR. One can introduce restriction sites, epitope tags, or SNPs into a locus of interest by building a traditional homologous repair template in the form of a plasmid with 1–3-base flanking arms, or one can use a single-stranded DNA oligo to repair the template to introduce these types of small changes. In the absence of any selection, we see again fairly high levels of HDR being mediated by Cas9, and one can titrate these numbers with additional screening or selection.

By way of a quick summary, I hope I have made a convincing case that Cas9 is an easy-to-use system for both introducing indels as well as mediating HDR. Recently, the crystal structure of Cas9, alone or in complex with the guide RNA and target DNA, was solved by three groups. Figure 10 shows that the enzyme has a bi-lobed structure. At the top is a domain mostly responsible for recognizing guide RNA and target DNA, and at the bottom of the Cas9 enzyme are the nuclease domains. These domains create a positively charged groove where DNA and RNA sit.

We created a pipeline for rapid generation of cell-line models in a span of about a month from the *in vitro* design of sgRNAs—which we have a website tool to help—to reagent construction and functional validation and expansion of cell lines (Figure 11). We published this in *Nature Protocols* in 2013 and have deposited Cas9, and GFP, puro, and nickase versions, at Addgene.

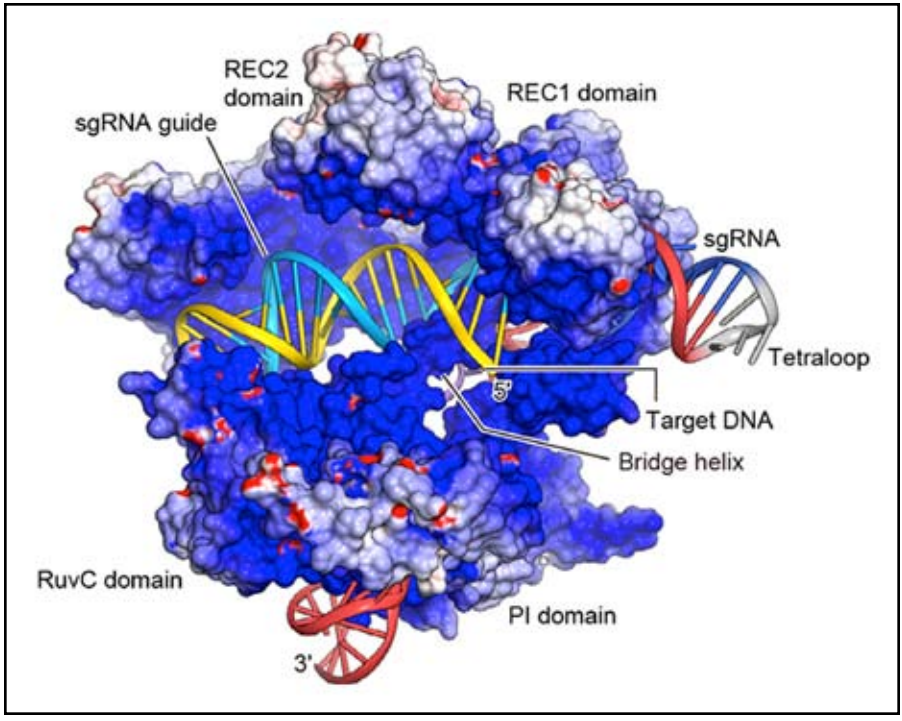

Figure 10. A new system for efficient mammalian genome cleavage.
(Anders *et al.*, 2014; Jinek *et al.*, 2014; Nishimasu *et al.* 2014)

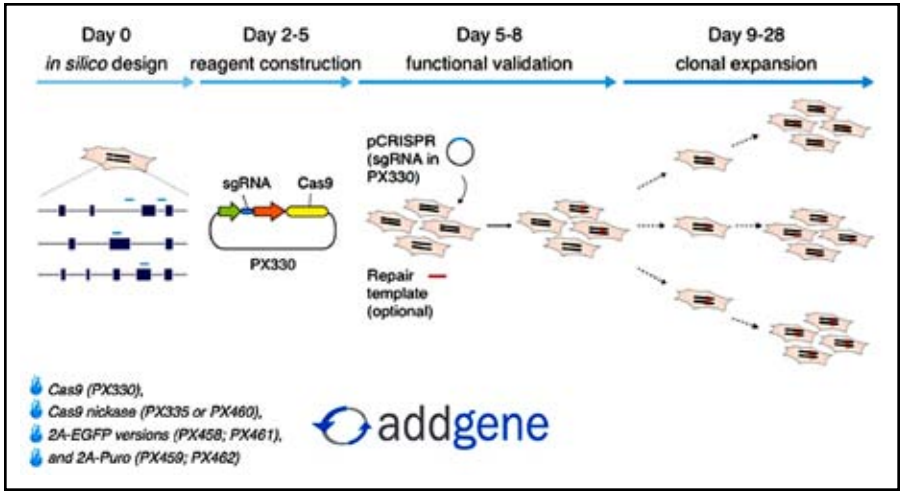

Figure 11. Pipeline for rapid generation of cell-line models.
(Ran *et al.*, 2013)

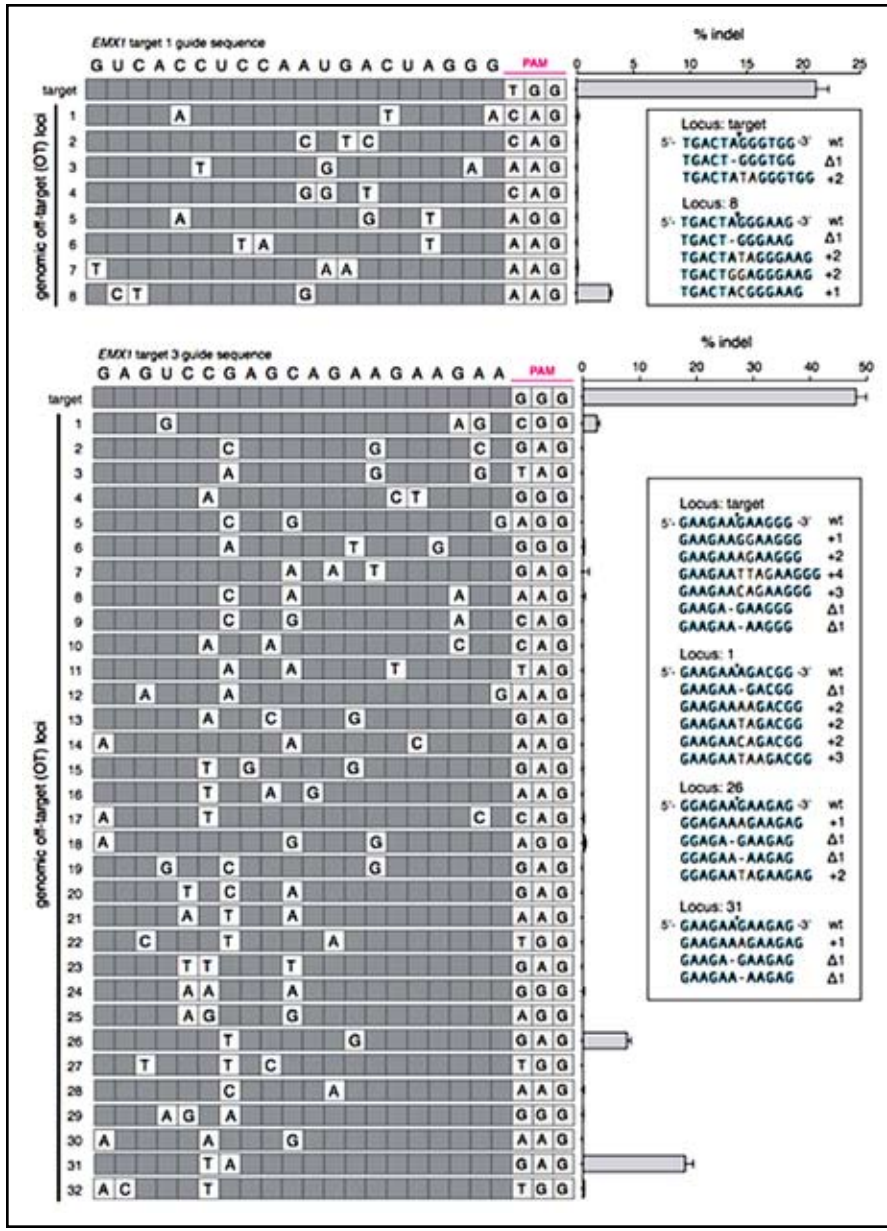

Figure 12. Cas9 can have off-target effects.
(Hsu *et al.*, 2013)

Knowing now that Cas9 works well, one of the immediate concerns in everyone's mind was the specificity of the system. Figure 12 shows that Cas9 does have off-target activities and these tend to occur when there are mismatches between the guide RNA and the target DNA on the PAM-distal side of Cas9. Several groups have come up with independent ways of improving the specificity. One idea is that you can actually truncate the guide sequence, which is usually 20 nucleotides long. Shortening this to a 17-, 18- or 19-nucleotide sequence is sufficient to improve the specificity of Cas9 by a significant amount. Another idea is to use an enzymatically dead version of Cas9 and tow around a Fok1 nuclease and rely on the obligate heterodimeric properties of Fok1 to increase that specificity.

The tack that our group took—which was introduced by Dana Carroll[3]—was using Cas9 as a double nickase. If you situate two units of Cas9 nickase on opposite strands of DNA, then a nick plus another nick equals a double-stranded break. This works efficiently and it works across a wide number of distances. Double nicking can happen as far as 100 nucleotides away from each other.

Returning to specificity, Figure 13 shows that Cas9 nickase can increase specificity of the system by several orders of magnitude. One of the other advantages of using double nicking is that it creates staggered cuts, which is reminiscent of cloning using restriction enzymes. It turns out that we can do something similar in cells, and if you have a repair template or insertion template that has corresponding arms that can be inserted directly into the staggered cuts, then we can essentially do ligation-based cut and paste of the template directly into cells. So, this is another alternative strategy to HDR or indel for specific genome editing.

To sum that part up, for specificity considerations what one would like to do ideally is select unique parts of the genome to target and avoid sites with large numbers of off-target matches in what is considered a seed region or the PAM-proximal region of sgRNA. Also, one can use techniques such as paired sgRNAs for double nicking or shorter truncated sgRNA guides, or both together, to improve the specificity of the system. And to improve the activity of the system, the guide should always begin with a G and avoid poly-T tracts to prevent premature transcriptional termination. Taking these together, one can design very efficient and specific guides.

Finally, I will talk briefly about applying Cas9 towards *in vivo* cell editing. One of the main challenges to using Cas9 in adult somatic tissues is delivery. Currently, one of the most clinically promising vehicles is adeno-associated virus (AAV), which is used for several human clinical trials and AAV1 was approved as a gene therapy vehicle in Europe. For AAV delivery, the *S. pyogenes* Cas9 (SpCas9) that everybody has been using so far is a little too big to fit with its sgRNA and all the regulatory elements in a single vector. So our lab has developed an additional Cas9 from *Staphylococcus aureus* (SaCas9), which is small enough to be squeezed into a single AAV vector along with its sgRNA. SaCas9 has a different protospacer adjacent motif (PAM), NNGRRT, which is relatively permissive, as it's required to be present next to the target for Cas9 binding and cleavage. We were

[3]Pages 25–27.

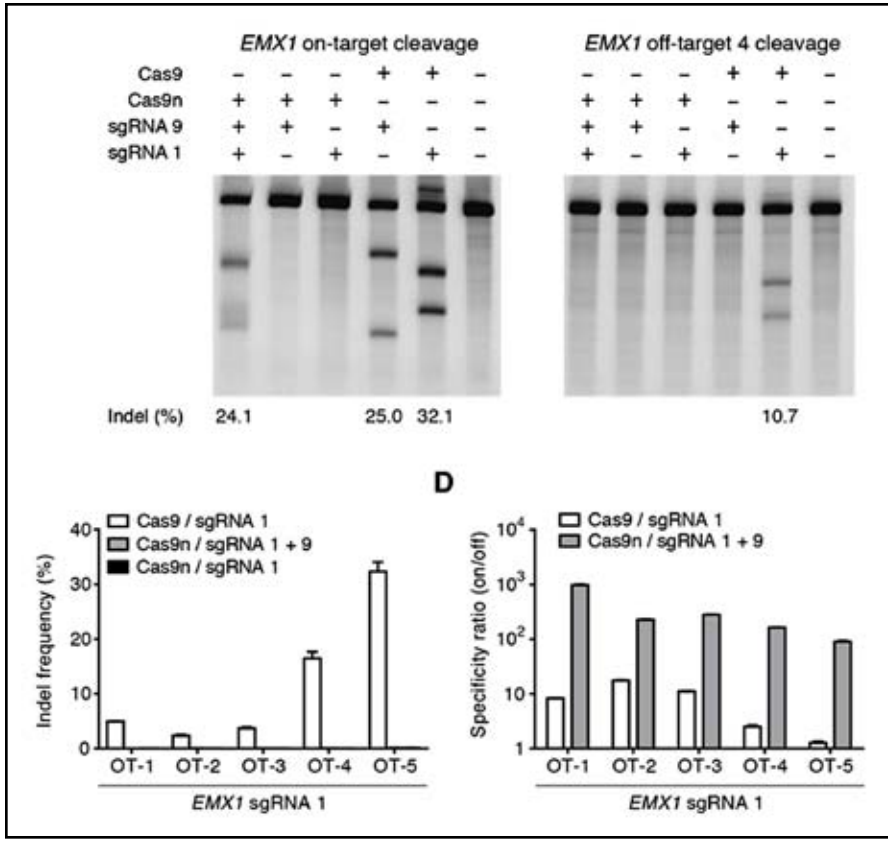

Figure 13. Double nicking improves specificity.
(Ran *et al.*, 2013a)

able to package SaCas9 and its sgRNA into AAV with the AAV8 capsid and inject the particles into animals via the tail vein. Depending on the serotype of the AAV, other tissue types of interest can be targeted.

Figure 14 shows some of our preliminary data. We targeted the ApoB gene in the mouse liver; ApoB knockout leads to an oil-droplet-accumulation phenotype that we can observe. Next, we tried a promising target for treatment of hypercholesterolemia, *Pcsk9*, which regulates the cycling of LDL-receptors. One week after injecting AAV bearing SaCas9 and sgRNA against *Pcsk9* into the animals, we saw a 40% gene modification in the liver and a depletion of serum Pcsk9 levels in the treated animals. This is a work in progress, but we are excited about the possibility of expanding the use of Cas9 towards DNA-editing in somatic tissues of adult animals.

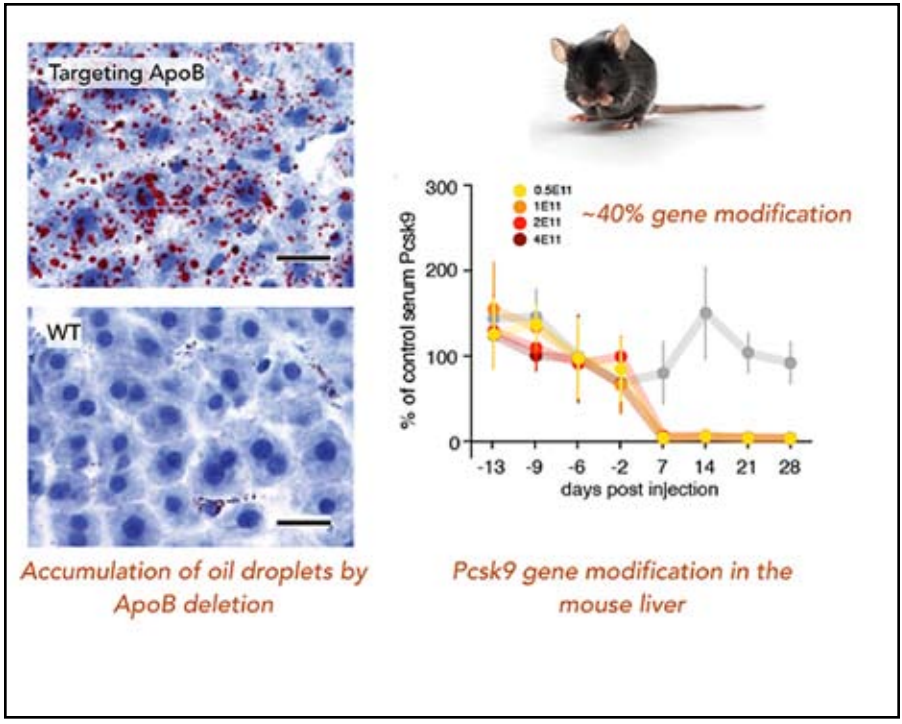

Figure 14. Sa Cas9 can be delivered by AAV to target genes *in vivo*.

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ANN RAN is a postdoctoral fellow in Feng Zhang's laboratory at the Broad Institute of MIT and Harvard and a junior fellow at the Harvard Society of Fellows. She graduated *cum laude* with a BS from Yale in 2006, and received her PhD in biochemistry from Harvard in 2014. In the Zhang lab, she co-developed Cas9 for mammalian genome engineering and established methods for rapid generation of cell lines using Cas9, with reagents now widely distributed by Addgene.

DR. RAN has presented her work on improving Cas9 specificity using the double-nicking method and elucidating Cas9 and guide-RNA structure-function relationships at a number of conferences. She received the Meselson Prize at Harvard for the "most beautiful experiment" in 2013 and was a finalist for the Regeneron Creative Innovations Prize.

The Roles of TAL Effectors in Nature in Relation to their Unique Properties as DNA-Targeting Tools

Adam Bogdanove
Cornell University
Ithaca, New York

ajb7@cornell.edu

I will present perspectives on the unique properties of TAL effectors as DNA-targeting tools, and what we can gain from understanding how they work in nature and in plant disease. I'll begin by comparing and contrasting the CRISPR/Cas9 and TAL-effector nuclease (TALEN) systems to highlight some of the unique features of the latter (Figure 1). TAL-effector nucleases work as dimer proteins. Each individual monomer of the TAL-effector domain is about 102 kDa. Targets are encoded by repeats in each protein. In contrast with CRISPRs, typically you need one dimer per target, so it's not readily multiplexed, certainly not as readily as the CRISPR/Cas9. The targeting specificity is typically 15 to 20 bases times 2. Therefore, on the surface, in these base configurations, TAL effectors inherently have great specificity, but, as we heard from Dan Voytas[1] and Ann Ran[2], a number of tricks have been applied within the CRISPR/Cas9 architecture to improve their specificity. So, they are complementary, effective systems.

[1]Pages 29–37.
[2]Pages 69–81.

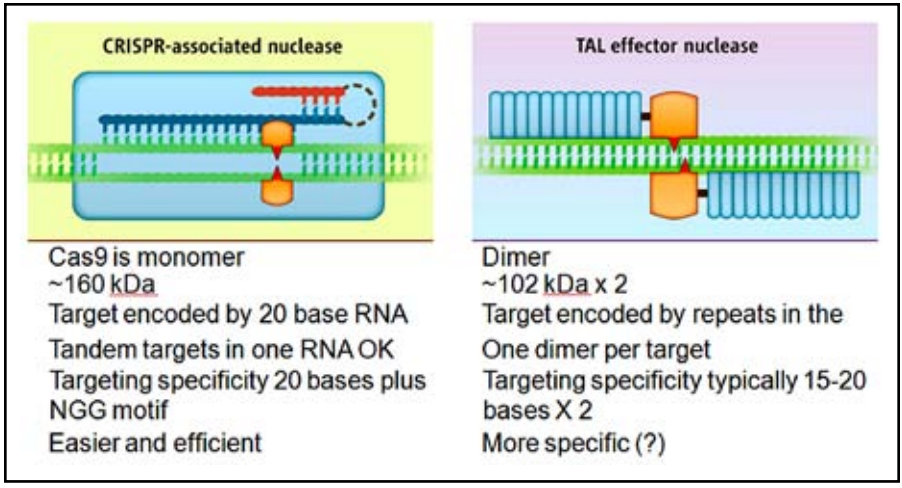

Figure 1. Comparison of DNA-targeting systems.
(Images adopted from van der Oost, 2013)

TALEN Utility

I was interested to see what would happen in the genome-editing field as CRISPRs gained momentum with possibly less emphasis on TAL effectors. However, a proliferation of TALEN kits have been deposited with Addgene[3]—which Dan Voytas[4] and I are familiar with because we deposited there—and over 1,400 requests have been filled for our kit. Also, we and others have web-based tools for TALEN design, which receive thousands of hits per week. Clearly, TALENs remain a popular and, therefore, apparently useful reagent. Figure 2, showing numbers of publications that cite TALENs, reveals a striking rise over the past five years. It remains to be seen whether this trend will continue, but TALENs have been used in all of the organisms of agronomic and agricultural importance in Figure 2. I don't know why anyone would mess with such a thing of beauty and perfection as the catfish, but that has been done. These data exclude progress toward gene-therapy approaches by using TALENs in human-cell cultures.

Certainly, TALENs are a viable and still-popular reagent, but it's interesting to gain more perspective on their unique properties from their native context. We think of them as straight-forward, Lego-block-like modules that can easily be assembled with a one-repeat to one-nucleotide targeting specificity. However, if we step back and think about where they come from, and the selective pressures that are on them, we can gain some interesting insights.

[3]A global, non-profit plasmid repository dedicated to making it easier for scientists to share.
[4]Pages 29–37.

Figure 2. Publications that cite TALENs.

Xanthomonas spp.

The DNA-targeting domain of TALENs comes from the transcription activator-like effectors of *Xanthomonas*, a genus of plant pathogenic bacteria that comprises 20 species within which there are several variants. Collectively, they cause diseases in over 350 plant species. Some of the more economically important diseases are listed in Figure 3. It is noteworthy that not all strains of the pathogenic species deploy TAL effectors and, in those that do, the number of TAL effectors deployed by a pathogenic strain may be anywhere from 1 to 25. There is quite a bit of variability in how consistently bacteria in different contexts actually use TAL effectors as virulence factors to manipulate host-gene expression.

Figure 4 illustrates what TAL effectors do when they function in an important way. They are delivered into the plant cell by the type-III secretion system (T3SS) of the bacterium and, by virtue of some nuclear-localization signals (NLS) in the C-terminal part of the protein, they are imported into the plant-cell nucleus. A translocation signal (T3S) on the N-terminal end gets them out and into the plant cell. A striking feature is an acidic activation domain (AD) on the C-terminal end. That's why they were called transcription activator-like, because they looked to be nuclear localized and they had this sequence at the end that looked like an activation domain typical of a transcription factor. Only in 2007 were they conclusively demonstrated to be translocated transcription factors and to activate gene expression in a very specific way in the host. The targets are recognized by virtue of the repeat region, which directs them to specific locations in the host genome, and then they recruit the transcriptional machinery in some way—yet to be characterized—to drive expression of the downstream gene. That downstream gene in

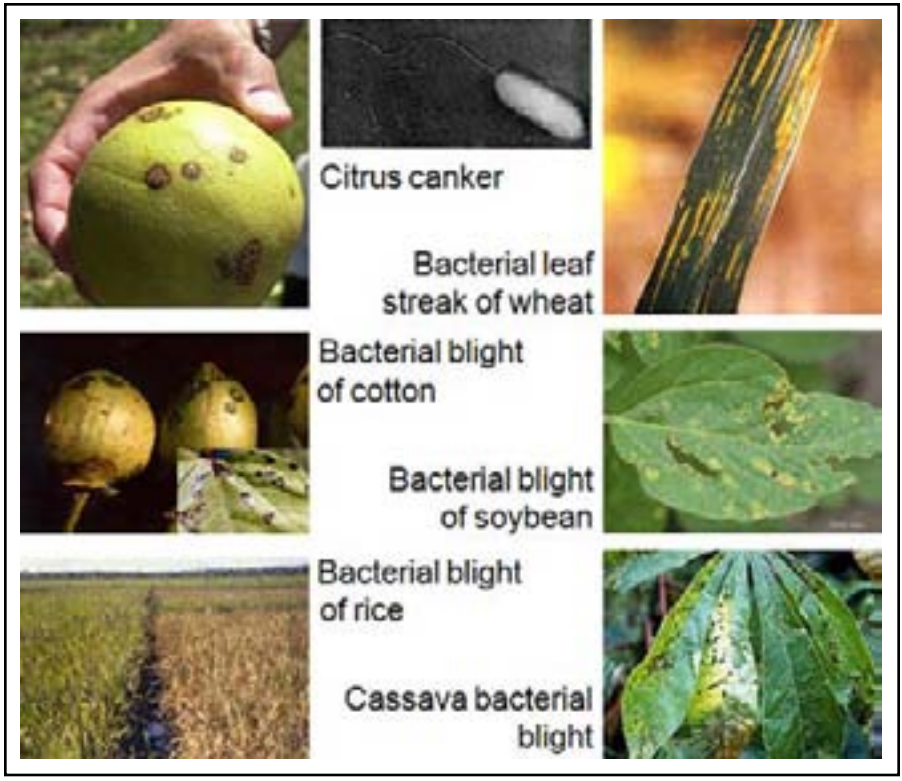

Figure 3. *Xanthomonas* species cause >350 plant diseases.

some way contributes to proliferation of the bacteria for symptom development, and we call it a disease-susceptibility gene, "*S*" in Figure 4. Over evolutionary time, plants have responded in several ways with different types of mechanisms to resist pathogens that are deploying TAL effectors. One that is conceptually quite simple to understand is to acquire polymorphism at the TAL-effector binding site upstream of a major susceptibility gene to prevent activation by the corresponding TAL effector and confer genetically recessive or passive resistance, essentially just taking out the susceptibility (Figure 5).

Another resistance mechanism—discovered in Susan McCouch's lab at Cornell—is polymorphism in a general transcription-factor subunit that provides resistance very broadly to pathogens using TAL effectors (Figure 5), and that's our only genetic evidence for direct interaction of TAL effectors with the transcriptional machinery. The most elegant evolutionary solution to this problem of pathogens with TAL effectors is to juxtapose a TAL-effector binding site upstream of a gene whose activation doesn't confer susceptibility, but triggers a resistant response, in the literature variously called an activation trap or an executor resistance ("*R*") gene—"*Executor*" because it is executing a resistance pathway (Figure 5).

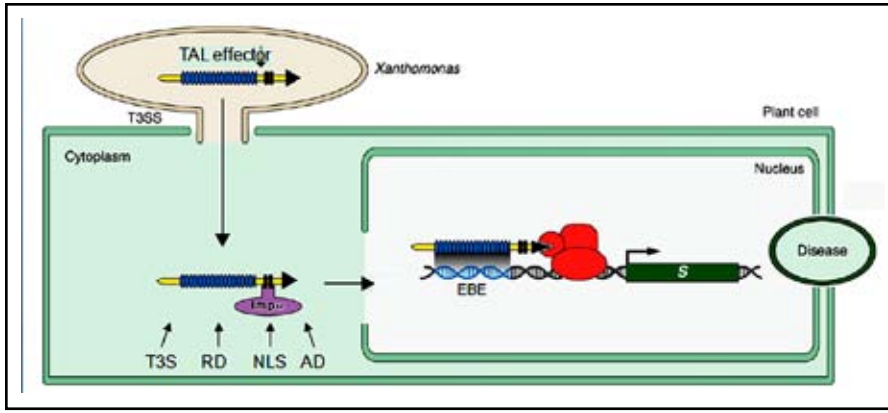

Figure 4. Transcription activator-like effectors are secreted transcription factors that activate host genes to increase susceptibility.
(Bogdanove *et al.*, 2010)

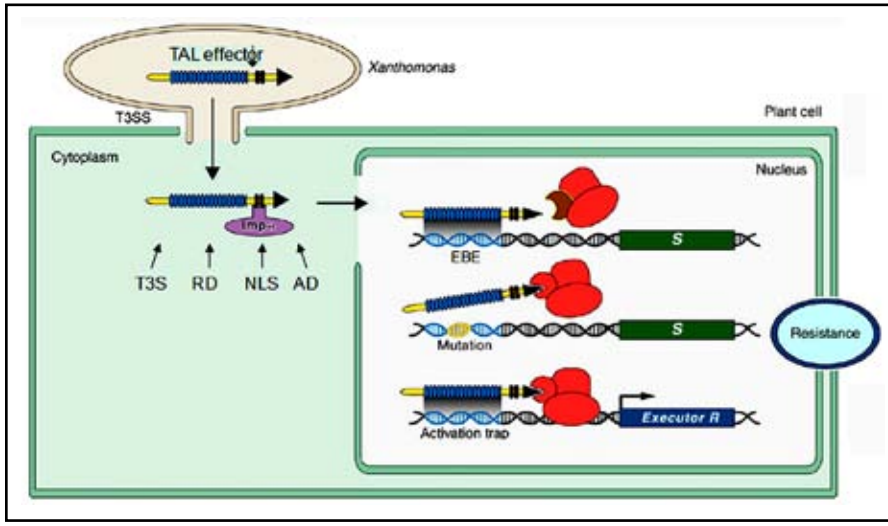

Figure 5. Plants have evolved resistance mechanisms.'

In this context, opposing selective forces act on TAL effectors: (1) for targeting flexibility to accommodate susceptibility-gene polymorphism from one plant variety to the next; and (2) at the same time for stringent specificity to avoid falling into one of these activation traps. If you have evolved a target for an "*S*" gene and you enter a genotype in which there is an "*R*" gene with the same binding site there, or a very similar one, you want to be able to distinguish those. So, in essence, we have two opposing sets of selective forces on TAL effectors, and our working hypothesis is that nature's solution is a modular DNA-recognition mechanism that allows rapid evolution of new specificities by

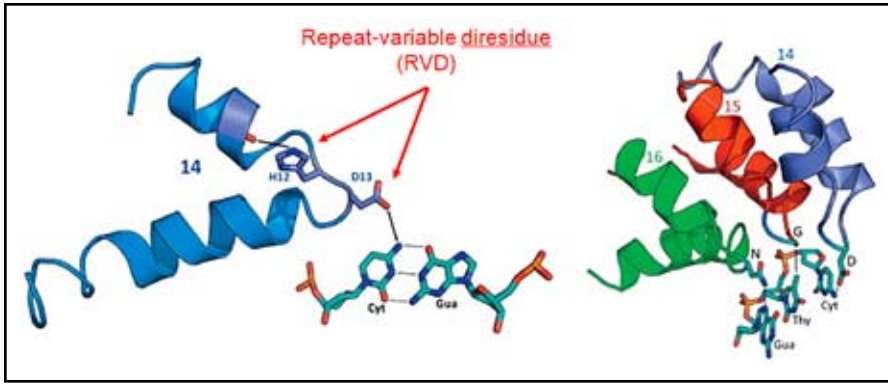

Figure 6. Modules are 33–35 amino-acid repeats. Each forms a 2-helix bundle and recognizes a third base. (Mak *et al.*, 2012)

recombination of those modules, but also—as discussed below—allows a tunable targeting specificity, not only specificity in itself in a qualitative fashion, but the stringency of that specificity can be modulated in a quantitative fashion.

TAL-Effector Modules

The TAL-effector modules are 33 to 35 amino acid repeats and any given TAL effector may have between 5 and 30 of these repeats in the central repeat region (Figure 6). Each of these repeats forms a two-helix bundle and each repeat recognizes a single nucleotide, so, continuously, the number of repeats and the composition of those repeats define the number of nucleotides and the composition of that nucleotide stream in the target. The repeat variable diresidue (RVD)—two amino acids—resides on an inter-helical loop and the 13th residue side chain reaches out and makes base-specific contact. Residue 12 reaches back and, through interactions with the first helix, shapes the conformation of the loop to affect DNA-binding specificity.

If you stack these repeats up in a lateral-like fashion to track the DNA, each repeat interacts with one base and the repeats all together assemble into a superhelix that wraps the DNA (Figure 7) for interactions in the major group with each of those RVDs and their corresponding nucleotides.

RVD-Nucleotide Interaction

Figure 8 illustrates the four major repeat types with each of the most common RVDs that are used in genome editing. A straightforward correspondence exists between the RVD sequences and the nucleotides that they prefer, but even though people typically use NI for A and HD for C and NN for G or A and NG for T, the heights of the letters in Figure 8 tell us how often each RVD is found associated with that nucleotide in nature. The stringency is not entirely strict, so that NI is sometimes found in association with C and can also be happy in an array opposite a C. So, there is some subtlety already in the major RVD-nucleotide interactions. However, beyond that, there is a range of RVDs that actually occur in nature that weren't characterized until recently. A group at Peking

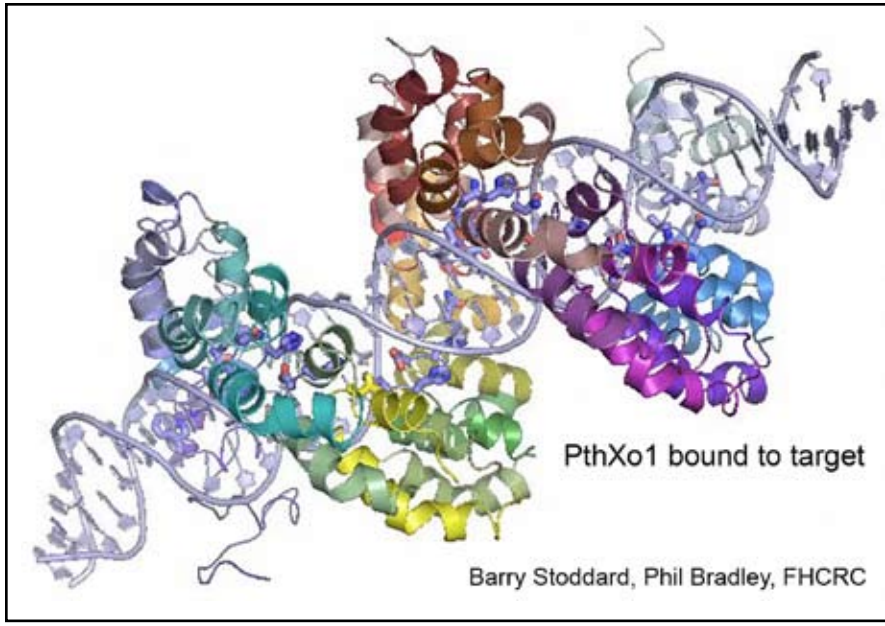

Figure 7. Repeats assemble into a superhelix that wraps the DNA.
(Mak *et al.*, 2012)

University tested all possible RVDs—all amino acid residues in the 12th position and all the amino acid residues in the 13th position—assayed by using a TAL effector as a transcriptional activator, in a human cell line where they varied three repeats as a homomeric triplet (Figure 9). They tested each of these different RVDs as a homomeric triplet in the middle of the repeat array on substrates with corresponding triplets of either A or T or C or G. Each of the quadrants in the array shows activity for A or T or C or G according to the colors, green, red, blue or yellow, respectively, and the gradient schemes (0 to ≥20).

For example, at HD the specificity is recapitulated with very strong activity on C and virtually no activity on the other bases (Figure 9). At NN, the one for dual specificity, it's recapitulated very nicely in their assay as well, you see G and A. And NK—which was discovered early as a substitute for NN that gives specificity for G—is very specific for G, but the activity is low relative the NN variant. And then there are weird ones, like RV, which has not actually been observed in nature, but might be useful as a wildcard RVD; it shows good activity for any of the four bases. So, this pool of unexploited RVD variation exists in TAL effectors found in nature.

Frameshift Accommodation

An elegant study was done by colleagues in Germany and France who were puzzled by the presence of the atypical repeats in TAL effectors. A typical repeat is 34 amino acids, with the RVD at 12 and 13 (Figure 10). But occasionally you see these ones with an insertion at the C terminal part of the repeat, or a deletion of the very C terminal end of the repeat or an insertion in the N-terminal part of the repeat. They wondered if these

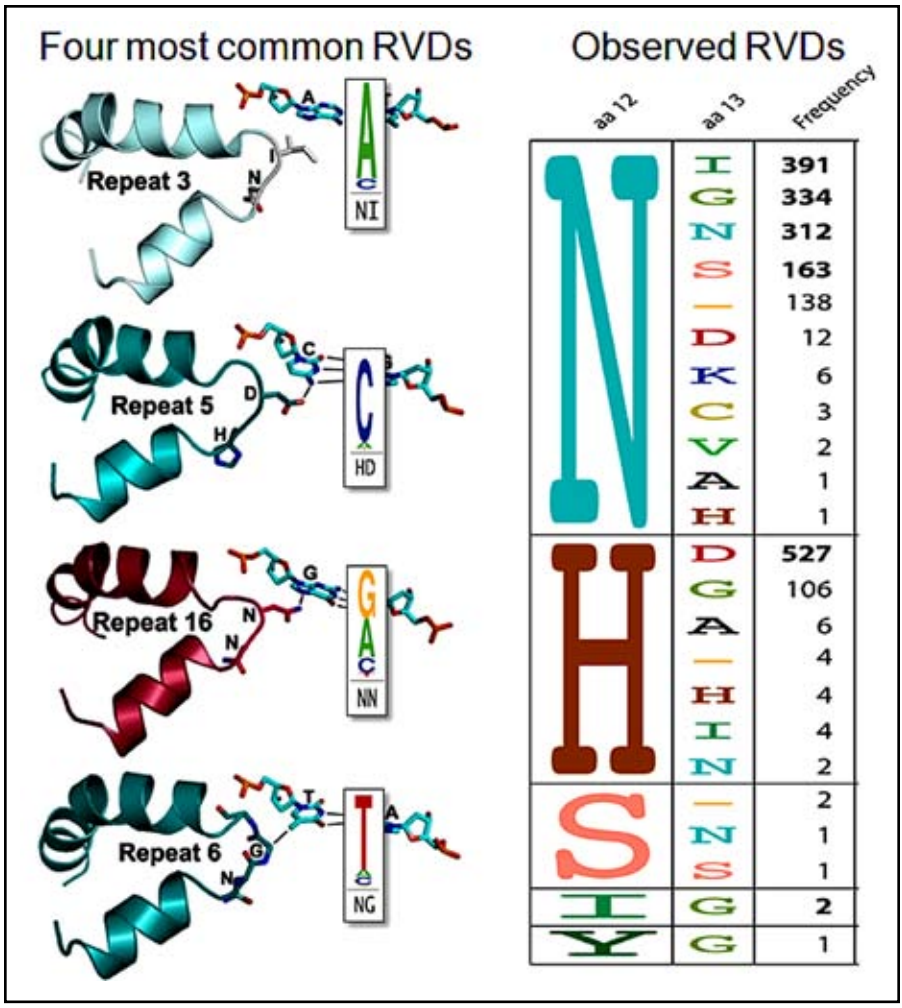

Figure 8. Major repeat types and common RVDs.
(Moscou and Bogdanove, 2009; Boch and Bonas, 2010)

are doing anything different. It turns out that they provide a frameshift accommodation. If you take an array of standard 34 amino acid repeats, and you put it on a template, then if you pop out a purine, for example, you move the template and TALEN-effector register out of frame (Figure 10), so binding dissolves after this point and you lose activity entirely. They found that the presence of the aberrant repeats allows a disengagement of the repeat from the interaction and accommodation of that frameshift. So all those downstream shift back up and then match the template (Figure 10). So far, this is completely unexploited in DNA-targeting applications, but it's a very interesting feature of TALEN effectors occurring in nature.

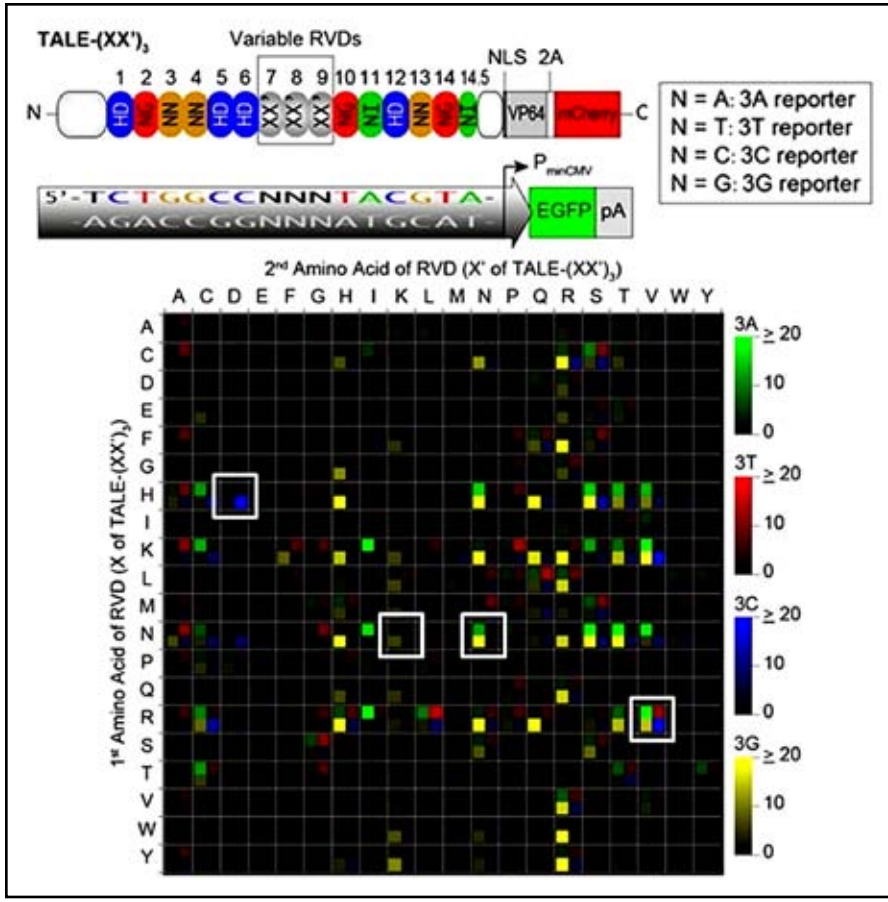

Figure 9. All possible RVDs.
(Yang *et al.*, 2013)

Array Length

Another curiosity that we've explored recently is the effect of variation in the length of the array, the number of the repeats in the array, on specificity and activity. If you look simply at a collection of TAL effectors in nature, there is quite a variation in the number of repeats, as mentioned before. Some are probably pseudogenes, essentially just 2 RVDs long, and some are as long as 34 RVDs but the peak—the most common in nature—is somewhere between 16 and 20 RVDs (Figure 11). This is also the most common length used in most DNA-targeting applications. To nail down whether this finding is functionally significant, Fabio Rinaldi, a postdoc in my lab, assisted by undergraduate Ava Fan, took on the question of the relationship of length to affinity and specificity. Figure 12 shows the experimental set up. They took, as an anchor, the first 10 RVDs of the

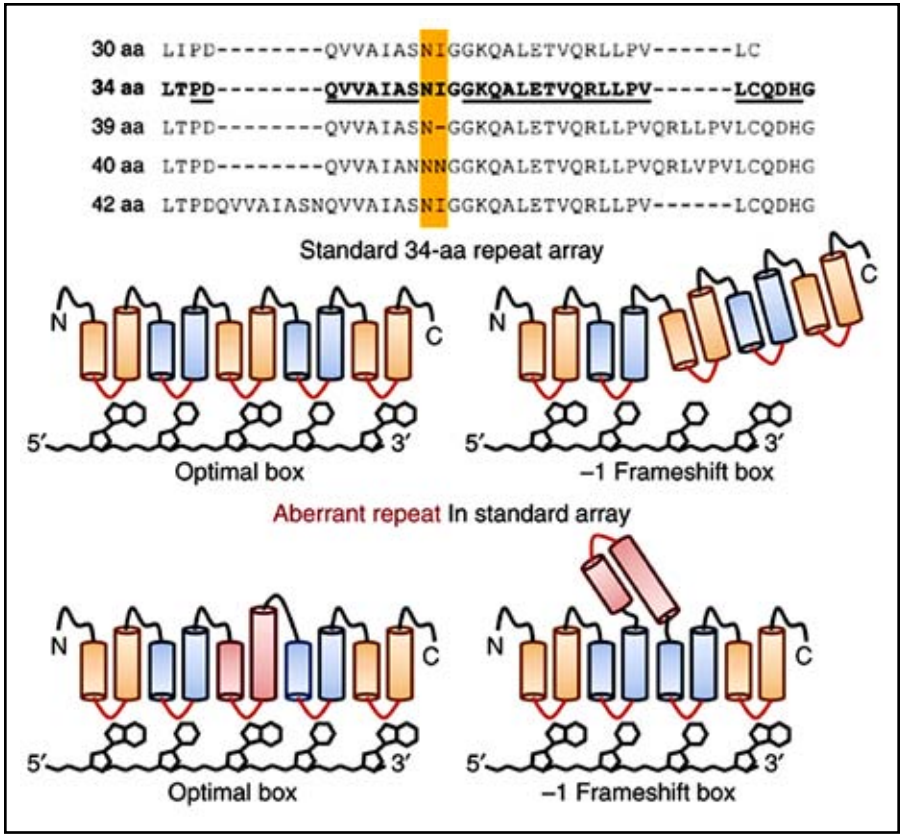

Figure 10. Atypical repeats accommodate single-bp deletions.
(Richter *et al.*, 2013)

well studied TAL effector PthXo1 and then they used repeating quadruplets of the four major RVDs. So, they ended up with a 10-mer, a 14-mer, an 18-mer, a 22-mer and a 26-mer, which they tested on substrates that matched perfectly and had corresponding repeating subjects of A, C, G, and T. So each was anchored by the same register and the effects of lengthening the array on specificity and affinity could be assessed. As a control, they tested interactions of these proteins also on a scrambled-DNA target representing nonspecific DNA interaction.

A lower dissociation constant means stronger binding and higher affinity. It turns out that when you measure the 10-, 14-, 18-, 22- and 26-mer arrays on the target DNA, a drop in the Kd occurs, with a diminishing-return scenario after about 18 RVDs (Figure 13); the gain in overall affinity for lengthening the array is diminished. In contrast, the non-specific interaction on the scrambled DNA showed that affinity drops in a more linear fashion. The ratio of the affinity on the non-specific DNA to the affinity on the specific DNA produced a gaussian-like distribution or bell curve (Figure 14). And, interestingly

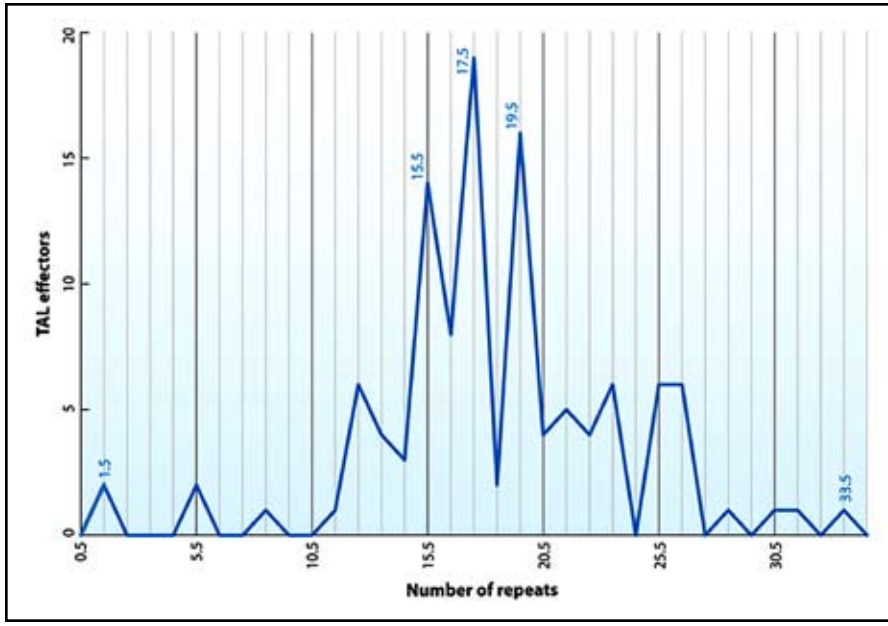

Figure 11. Frequency of array length in nature.
(Boch and Bonus, 2010)

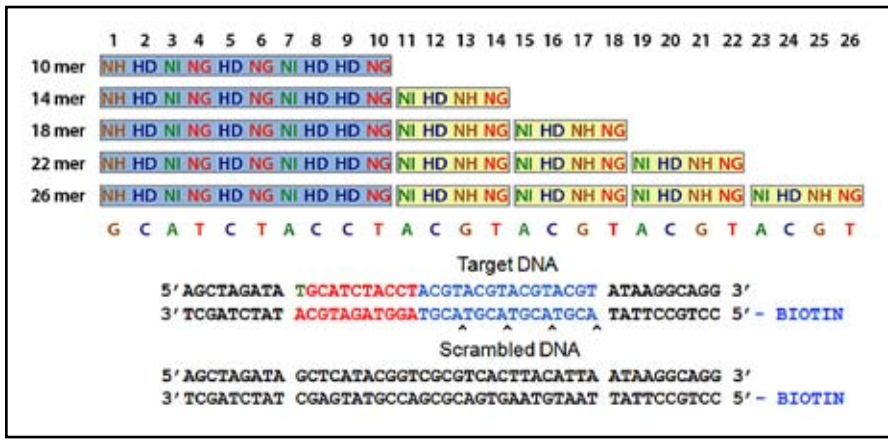

Figure 12. Relationship of length (# repeats) to affinity and specificity.

then, this ratio represents the overall specificity. And we see there is a clear optimum somewhere between 16 and 20. In nature, it seems that the most commonly observed length of TAL effectors is the one that gives the best balance between targeting affinity and minimal off-targeting.

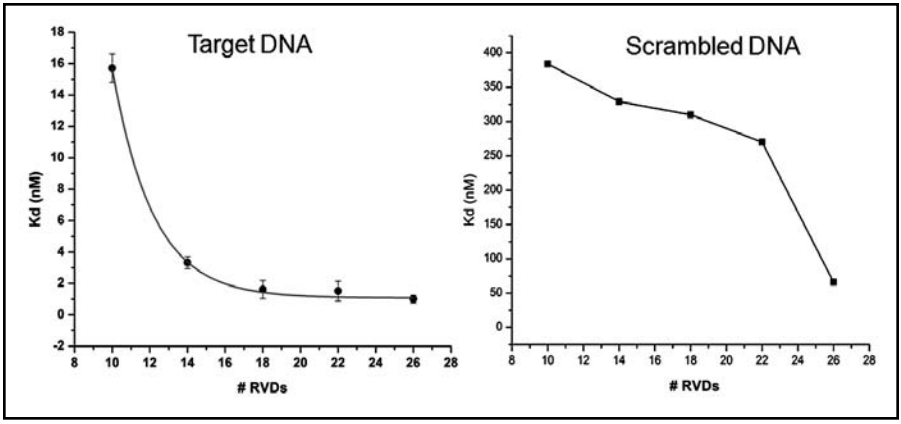

Figure 13. Size versus affinity.

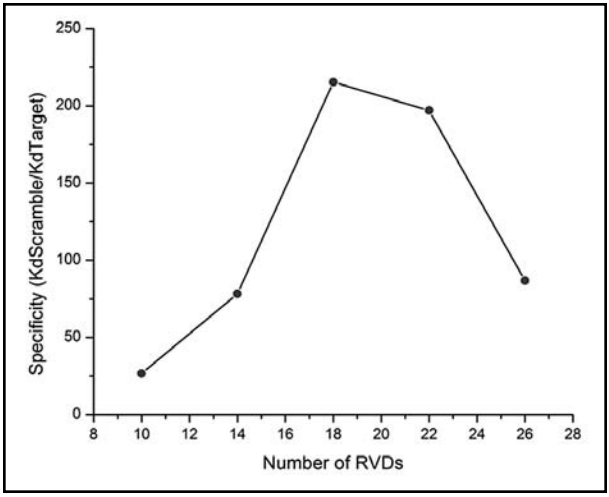

Figure 14. Size versus specificity (Kd_{scr}/Kd_{tar}).

To summarize those aspects, it's fair to say that with varying RVD composition and varying numbers of repeats and depending on the presence of these aberrant repeats that I mentioned, affinity and specificity of a TAL effector array can vary. And when I'm talking about specificity, it's important to keep in mind not just qualitative specificity but also quantitative.

From Nature

I want to wrap up with an observation from nature that Bing Yang[5] already touched on. We haven't exploited this technology to the extent that we can in DNA-targeting applications,

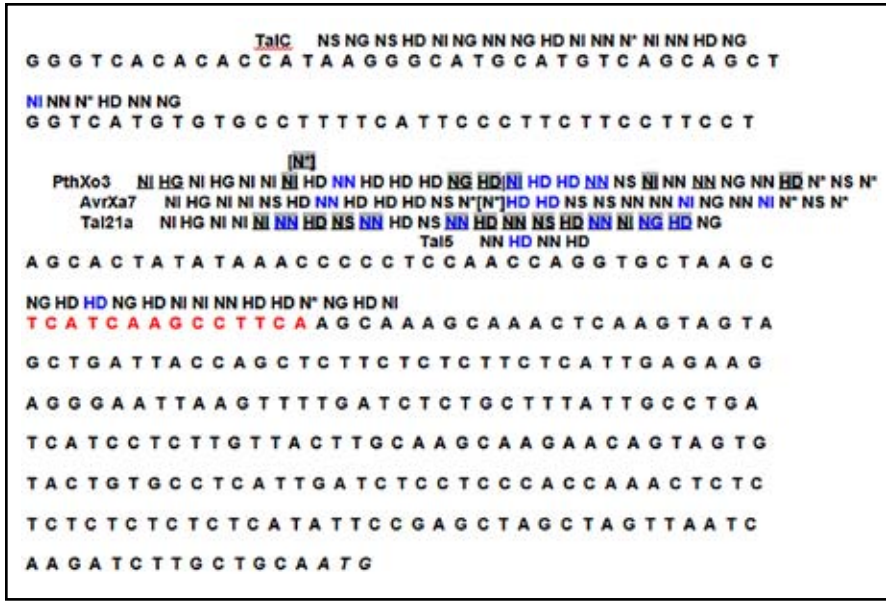

Figure 15. Distinct TAL effectors from different strains target the rice bacterial blight *S* gene *SWEET14*.
(contributions from many research groups)

but to what extent does evolution exploit these adaptations? In the context of bacterial blight of rice disease that Bing introduced, if we look at one of the susceptibility genes, *SWEET14*, it turns out that there are at least five distinct TAL effectors from different strains that go after this gene promoter. And the variation across these is very telling. We have some that are fairly long, some that are fairly short, some that target the same general sequence and others that target distinct sequences. And in the group to focus on, I've underlined differences in proteins PthXo3 and the Tal21A, from protein AvrXa7 (Figure 15). The blue colors show where there are mismatches, alignments of RVDs opposite their non-preferred nucleotides. Something special about AvrXa7 is that it is caught by an activation trap in another genotype. It triggers resistance in rice that carries the gene Xa7. Tal21A and PthXo3 don't fall into that trap; they target the same spot in the susceptibility gene, but they avoid the activation trap. The differences here are not so great between AvrXa7 and Tal21A; it's essentially a deletion of four repeats at the end and then several modifications, which maintain activity on this susceptibility-gene substrate, but clearly interfere with binding to the activation trap. But with PthXo3, additions and a couple of differences also help this protein to avoid the activation trap. The little bracketed RVD is one of those aberrant types, so it can accommodate the sequence by looping out this particular repeat and the presence of that repeat combined with those other differences

[5]Pages 53–59.

clearly is exploited to prevent getting trapped by Xa7. It's fair to say just from this simple example that adaptations are exploiting these unique properties of TALEN effectors.

My take-home message is that we are continuing to better understand the subtle properties of TAL effectors in part by studying them in their native context, but we are very far from having fully exploited them in DNA-targeting applications.

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ADAM BOGDANOVE is a professor of plant pathology and plant-microbe biology at Cornell University. His research centers on diseases of rice caused by the bacterium *Xanthomonas oryzae*, with a special focus on TAL effectors, *i.e.* transcription factors injected by the bacterium into the plant to activate host genes during infection. He discovered the modular mechanism by which TAL effectors recognize their DNA targets and has pioneered the use of TAL effectors as customizable DNA-targeting domains for a variety of applications including genome editing and synthetic biology.

DR. BOGDANOVE earned his BS degree in biology at Yale University in 1987, and his PhD in plant pathology at Cornell in 1997. Following postdoctoral work at Purdue University and later at the Boyce Thompson Institute, he joined the Department of Plant Pathology at Iowa State University in 2000 and moved to Cornell in 2012.

Non-Transgenic Trait Development in Crop Plants Using Oligo-Directed Mutagenesis: Cibus' Rapid Trait Development System

Greg Gocal
Cibus
San Diego, California

GGocal@Cibus.com

A huge technological pivot is going on in crop breeding. If the end of the 20th century was the era of transgenic breeding or GMO technologies, the 21st century is silently turning to non-transgenic breeding or non-GMO technologies. Due to significant regulatory issues, cost of development and technology constraints, transgenic breeding technologies have been limited to a few crops grown on large acreages, *i.e.* about 10% of the available cultivated acres globally[1]. The promise of non-transgenic breeding technologies is their extraordinary breadth. Crops bred using these technologies can be planted on all farming acreage worldwide. And these technologies can address better, more novel non-transgenic traits both in large- and in small-acreage crops. Small crops are accessible because the development cost is so much less than for transgenic crops.

This shift to non-transgenic technologies has been led by advances in precision gene-editing technologies that include molecular scissors such as CRISPRs (clustered regularly interspaced short palindromic repeats) and TALENs (transcription activator-like effector nucleases), bolstered by the explosion in genome-sequence information. Our understanding puts us at the threshold of a new era in non-transgenic crop breeding. With powerful

[1]FAO statistical databases, United Nations.

advances in crop-breeding technologies at the core of this silent revolution in crop breeding, these new technologies will enable the development of new traits more rapidly and more precisely. Because of their promise, these powerful technologies are quickly becoming the most important tool to ensure global food security through sustainable agriculture in the foreseeable future.

Cibus is a non-transgenic-trait-development company at the apex of this revolution. With a proprietary technology built over more than a decade, Cibus has a large and broad patent portfolio in this space (over 170 issued or pending). Cibus has developed precision gene-editing platforms for a variety of crops that are accurate and predictable with reliable timelines. As opposed to younger companies just starting to work with these technologies, Cibus has a broad product line both in commercialization and in development. This revolution in agriculture, food and microbes promises both to address the growing needs for a safe and sustainable food future and to replace the need for the current transgenic technologies that, today, dominate the industry. Cibus is a leader in this revolution.

Overview

Technology and an understanding of genetics has been at the core of accelerating plant breeding (Figure 1). Articulated by Gregor Mendel, single traits were tracked as visible phenotypes from one generation to the next, with outcomes limited by the genetic diversity available within the parental lines. To increase this diversity, various classical mutagenesis techniques were employed, including the treatment of whole plants, seeds or tissue pieces with various DNA-damaging chemicals (*e.g.*, ethyl methanesulfonate [EMS]) or radiation (*e.g.*, gamma radiation). This approach helped to address one of the limitations of traditional breeding, the wait time for new mutations, thus accelerating the breeding process. That said, the mutations obtained are random, so influencing specific outcomes was not possible with this method. Currently more than 2,500 such products in 180 crops are sold in commerce without labeling or other restrictions applied (http://www.fao.org). Many characteristics of current crops, such as orange- and yellow-colored sweet peppers, are the result of this process, known as chemical mutagenesis or mutation breeding.

With advancements in the field of molecular biology, marker-assisted selection (MAS) enabled the development of complex traits leveraging both existing variation among all available lines of a crop and that induced using classical mutagenesis approaches. The marker is a small piece of DNA that always coexists in a plant with the desired trait or a proportion of that desired trait. By quickly and easily identifying plants containing the marker associated with the desired trait, the breeding process is faster and more efficient using MAS. MAS is, however, limited by the diversity that exists in the crop already. If the trait does not exist in the crop, there is no way to breed for it using MAS.

The development of transgenic techniques, inserting DNA foreign to the plant, beginning in the late 1970s, enabled movement of entire genes to generate single traits. Initial transgenic traits used genes from bacteria or other non-plant sources; however, transgenic traits can also be developed by inserting genes from wide crosses that could not occur in nature. Although extremely powerful, the location at which the transgene lands is ran-

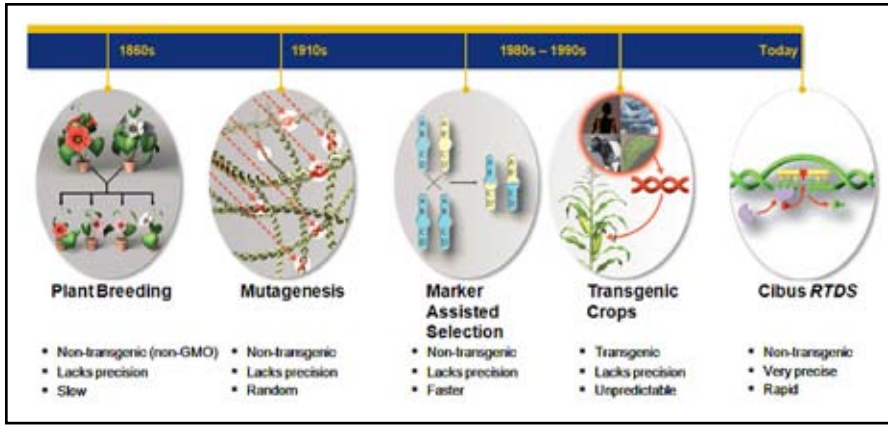

Figure 1. History of plant breeding from Mendel to today.

dom, occurring in well expressed and less well expressed regions of the genome alike. To ensure the appropriate level of transgene expression requires that many transgenic lines be assessed for the trait's performance. Random transgene insertion may inactivate an existing gene, one that is critical for some aspect of the crop's development, therefore agronomic performance of the crop must also be assessed. Even when this does not occur, because a completely new sequence (the transgene) is present on one arm of a chromosome pair, it may reduce recombination frequency at that point, making it difficult to reduce linkage drag associated to the new locus.

Gene-editing techniques, including Cibus' Rapid Trait Development System (***RTDS***™), enable the development of non-transgenic traits with laser precision. These traits can be developed more rapidly, with extremely reduced cost and global consumer acceptance.

RTDS in Plants

Gene editing has been a holy grail in biology with technology to obtain precisely targeted mutations being explored for almost 40 years. Initially, work to develop precise single-nucleotide polymorphisms (SNPs) focused on simpler and less-complex plasmid DNA targets (Hutchison *et al.*, 1978). A decade later, Fred Sherman used chemically synthesized oligonucleotides to achieve targeted mutations in yeast (*Saccharomyces cerevisiae*) (Moerschell *et al.*, 1988).

As early as 1998, oligonucleotides were used to achieve targeted mutations in the genome of the gut bacterium *Escherichia coli* (Zhang *et al.*, 1998). Then, with the advent of the lambda-Red system, precise targeting in *E. coli* became routine in 2001 and, by 2011, improvements in that technology enabled more than 10 SNPs to be targeted simultaneously in a single genome (Isaacs *et al.*, 2011). These were combined by breeding to obtain more than 300 targeted mutations within a single genome (Isaacs *et al.*, 2011).

In 1996, oligonucleotides were first used in mammalian cells, initially to correct an episome (Yoon *et al.* 1996), and then within the nuclear genome to correct the point

mutation in the human β-globin gene that causes sickle-cell anemia (Cole-Strauss *et al.*, 1996). At that time, since the oligonucleotide chemistry used was an RNA/DNA hybrid molecule, the first generation of the ***RTDS*** technology was termed chimeraplasty.

A few years later in the first application in plants, chimeraplasty was used to target the acetolactate synthase (ALS) gene in a tobacco cell line known as Nt-1 (Beetham *et al.*, 1999). The enzyme encoded by this gene target is responsible for the biosynthesis of branched-chain amino acids including leucine, isoleucine and valine. By targeting this gene the researchers were able to modify it, allowing the cells to become herbicide resistant. The herbicide in this example belonged to a class known as sulfonylureas. Included in this report was evidence that ***RTDS*** could also modify a transgene (a marker gene known as GFP—green fluorescent protein—introduced transgenically into either tobacco cells or whole plants). The researchers were able to reactivate an inactivated form of this gene.

This work was further supported by a complementary study by researchers at Pioneer Hi-Bred, Inc., who modified a similar gene in maize (Zhu *et al.*, 1999, 2000). They modified the tobacco ALS homolog in maize known as the acetohydroxyacid synthase (AHAS) gene. The converted cells were also herbicide resistant. Additionally, they modified a GFP transgene in maize. Modified cells were then cultured, and plants were regenerated and allowed to mature. The subsequent progeny of these plants confirmed that the gene mutations were heritable and stable.

Kochevenko and Willmitzer (2003) also confirmed the utility of ***RTDS*** to modify plant genes by repeating and extending the work of Beetham *et al.* (1999) with the ALS gene in tobacco with a more extensive study that included the regeneration of whole plants from cells that had undergone ***RTDS*** conversion. Sequence analysis and enzyme assays showed that the DNA had been converted at the target site and the ALS enzyme was resistant to the herbicide. Applying chlorsulfuron to the regenerated tobacco plants confirmed that the herbicide-tolerant phenotype exhibited Mendelian inheritance. This paper confirmed that the gene-repair oligonucleotide (GRON) -targeted mutations are distinct and precise. Further, Okuzaki and Toriyama (2004) also targeting the AHAS gene, successfully converted two independent sites in rice, selecting with chlorsulfuron and bispyribac.

Trait-Development Process—Herbicide Tolerance in Canola

Cibus has used conversion of a visible marker gene to GFP in the model system *Arabidopsis*, canola and other crops, yielding improvements in conversion efficiency of up to three orders of magnitude.

One method of practicing ***RTDS*** involves regenerating protoplasts to whole plants. By applying the process described in Figure 2, mutations were obtained in canola AHAS homologs, one of which was key to developing Cibus' herbicide-tolerant ***SU Canola***™ product, which is currently in the launch phase in the United States.

Despite over a decade of experience in continuously improving the canola cell-culture system, the latest technology advancements have made this process twice as fast and 16 times more efficient. This provides opportunities to greatly accelerate project timelines and has made non-selectable changes a reality.

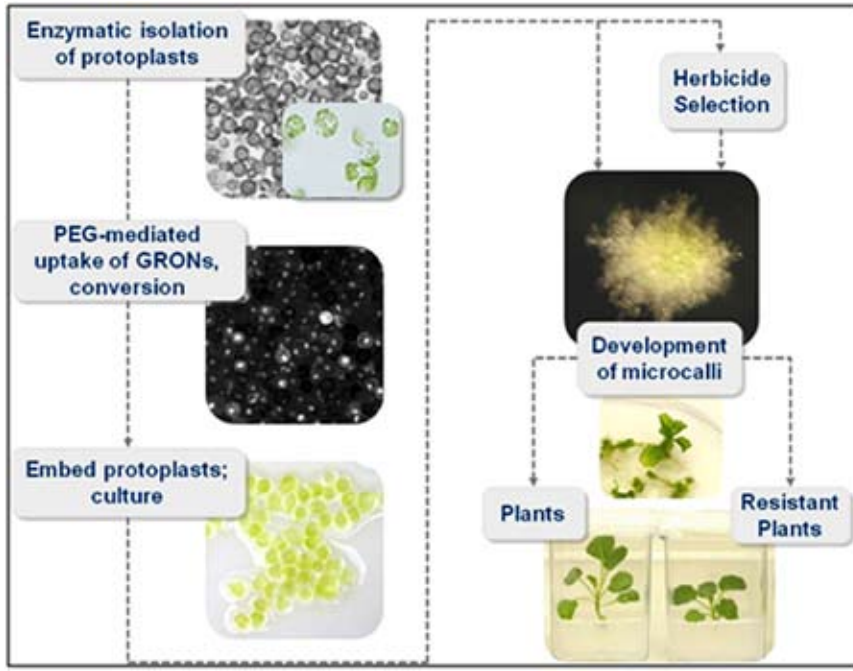

Figure 2. ***RTDS*** process to develop herbicide tolerance in canola.

RTDS *Conversion Process*—GRONs are Chemically Synthesized Directed Mutagens

GRON structure and chemistry are designed purposefully. The GRON is designed with a mismatch in one or a few bases compared to the target gene's sequence. GRONs are chemically synthesized structures consisting of both DNA and modified nucleotides or other end-protective chemistries. The GRON is blocked from undergoing recombination due to its chemical structure, with the GRON acting as a mutagen and ***RTDS*** being a targeted mutagenesis system and not a transgenic or GM process. Company scientists, through their understanding of the target gene's sequence, design the GRON to effect (a) specific sequence change(s), the replacement, insertion or deletion of nucleotides. GRONs are produced with an automated chemical synthesizer and purified like any other chemical agent; they contain no biologically derived material. The GRON is formulated without the need for a delivery vector, which ensures that no foreign or extraneous DNA is inserted into the plant.

Over the years, scientists have continued to actively work on ***RTDS***, focusing on understanding the mechanism to help optimize GRON design. Incrementally, alterations in design have been developed and tested. In our standard bacterial assay, GRONs are orders of magnitude more efficient, than our original designs. The efficiency in the

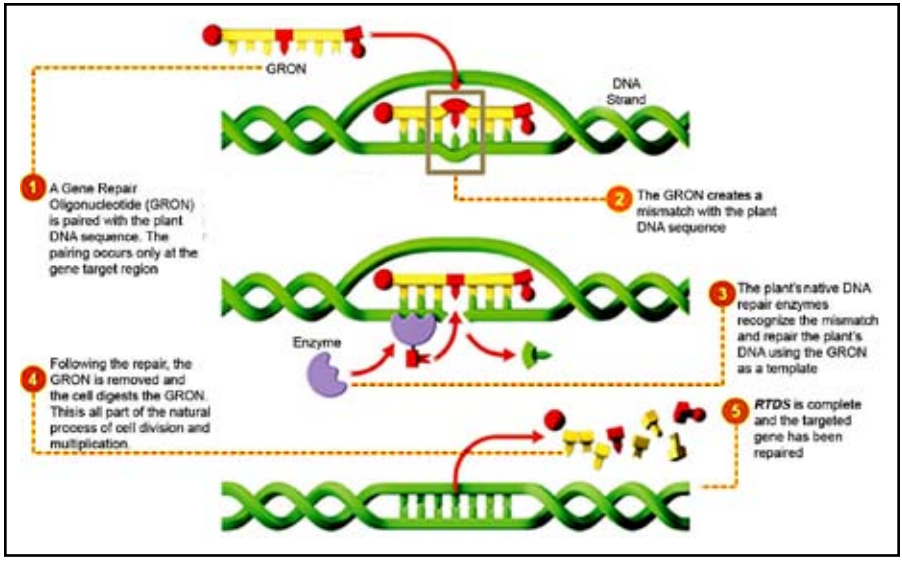

Figure 3. ***RTDS*** conversion process.

bacterial assay has also translated to higher efficiencies in eukaryotic cells, *i.e.*, plant, mammalian and yeast.

Mechanism of GRON Action

RTDS is a mutagenesis technology that uses the natural or inherent mismatch-repair system to effect a change. In eukaryotic cells, the GRON crosses the cell membrane, traverses the cytoplasm to the nucleus, locates and binds selectively and specifically to its target sequence and effects (a) specific sequence change(s) in its target gene. Nucleases and other degrading enzymes in the cells then break down the GRON (Figure 3).

A working model of the ***RTDS*** mechanism has emerged from studies in bacteria and mammalian cells. These studies suggest that the GRON will modify the nucleotide sequence in the genome via a process of genome/GRON "pairing" followed by GRON-directed mismatch repair (Figure 3). In *E. coli*, studies by Cibus scientists have clearly shown that the process is *mutS*- and *recA*-dependent. These two proteins are an integral part of the cell's own endogenous repair system. Rice *et al.* (2000) and Gamper *et al.* (2000) have shown that cell-free extracts from a variety of plants—including banana, corn and tobacco—were able to precisely modify a target-DNA sequence and that mutation efficiency varied with the chemistry of the supplied GRON. Among others, the effects of chemistry of single-strand GRONs have also been studied by Andrieu-Soler *et al.* (2005), Radecke *et al.* (2006), and de Piédoue *et al.* (2007), showing that some designs are toxic, leading to delayed progression through the cell cycle and, in some cases, selective apoptosis.

This mismatch signals the cell's "mismatch" repair system to change the gene's sequence such that the mismatched nucleotide is removed enzymatically, and the new sequence of the gene is resynthesized with the GRON acting as the template over the targeted portion of the gene. The working model that has emerged from studies in bacteria, yeast and mammalian cells indicates that the GRON will modify the nucleotide sequence in the genome via a process of genome/GRON "pairing" and then GRON-directed DNA repair.

Once inside the cell, the GRON is transported to the nucleus and hybridizes to the targeted gene sequence. Specific hybridization is a critical step for determining the efficiency of the gene-correction process. The GRON would then be used as the template for a DNA polymerase to correct the removed nucleotide or nucleotides, thereby producing a continuous sequence using the cell's own source of nucleotides. The ability of the GRON to specifically hybridize with great affinity to its target, and its resistance to degradation, allows the cellular gene-repair mechanism time to locate and replace, insert or delete the targeted DNA nucleotide(s) on both strands of the genomic DNA. When the DNA strands are corrected to the GRON's DNA sequence, the GRON is degraded and the gene then functions under its natural control mechanisms.

Regulatory Considerations

Using ***RTDS***, new traits can be added to a plant with only very minor changes to the genes and their resulting proteins. Results presented above show single-nucleotide substitutions in the AHAS gene lead to tolerance to three classes of AHAS inhibitors. The resulting plants are substantially equivalent to non-converted plants, with no risk of allergenicity or toxicity. Further, the GRON-induced mutation is made within the native pattern of expression for the target gene. There is no possibility that flanking genes may have expression patterns altered in unexpected ways.

In 2004, the USDA confirmed that ***RTDS*** is a modern form of mutagenesis and should not be regulated by state or federal agencies. In the United States, Cibus' commercial ***SU Canola*** product is now in the launch phase. In Canada, in late 2013, Cibus and its partner BASF received PNT (plant with novel trait) approvals for herbicide-tolerant canola. Work is underway to develop and register hybrids for the Canadian market as well as to achieve herbicide registration.

In Europe, ***RTDS*** is known as oligo-directed mutagenesis (ODM). Towards assessing ODM, in 2011, an Expert Working Group on Novel Plant Breeding Techniques, appointed by the European Commission, concluded that ***RTDS*** should be treated as mutagenesis and excluded from regulation under 2001/18/EC. This position has been endorsed by recent reports from a number of Member State advisory groups, including ZKBS in Germany and ACRE in the United Kingdom.

Opportunities—Broad Applicability Across Multiple Organisms

This technology can be used in a multitude of applications including repair of gene defects and mutations that modify genes and, therefore, their protein functions. Extensive research, at Cibus and by academic scientists, has demonstrated the successful application of the GRON-directed gene repair or gene mutation in human, animal, yeast, plant, and

bacterial cells, both in culture (*in vitro*) and in live animals, plants, yeast and bacteria (*in vivo*). Therefore, a large number of genes in a wide spectrum of living organisms appear to be accessible to ***RTDS*** through a commercially viable process.

Conclusions

Cibus' technologies are the core of our Rapid Trait Development System (***RTDS***). They have been developed over the past decade, focused on the molecular and cellular aspects of obtaining targeted spelling changes in target genes. Molecular aspects have focused on characterizing gene targets, mutation discovery to achieve desired traits in these targets as well as developing and improving our single-nucleotide polymorphism (SNP) screening technology. Cell-biology aspects have focused on developing crop platforms that enable SNPs to be obtained efficiently in single cells and regenerating those cells into normal fertile plants—plants that are the same as the parent with one or a few precisely targeted SNPs. Speed to market for future products has been cut materially because of advances in Cibus' technologies and with our systematized approach. These advances mean:

- Precision in trait modification
- Faster product-development timelines
- A proven and reproducible methodology for trait development
- Clear regulatory path

Due to technology constraints, development costs and regulatory hurdles, the current transgenic technologies have fallen short and cannot meet the needs of the market. In order to meet what has been reported as a doubling in global food production by 2050, we need a reliable method. This is the promise of precision gene editing for non-transgenic crop breeding. This is the promise of Cibus.

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WITH AN extensive background in molecular biology, **Greg Gocal** has been central in developing the ***RTDS*** technology in plant and microbial systems. Currently, he leads the technology group at Cibus, and is part of the senior executive team tasked with exploring the many commercial opportunities to deliver on ***RTDS'*** potential applications. In the past, he has focused primarily on developing the molecular aspects of the technology, including target characterization, mutation discovery, molecular screening and assay development. Also, he has played a key role in the development of Cibus' first commercial product, ***SU Canola*** (sulfonylurea tolerant), and in helping achieve regulatory approval for this trait in Canada. Over the past decade, his research leadership has led to multiple commercial partnerships for the company. In addition, his experience has expanded to include the development of key intellectual property through to products moving to market.

DR. GOCAL has authored numerous peer-reviewed publications, several book chapters and is a co-inventor of many applications within Cibus' patent estate. He earned a BSc in biochemistry/botany and an MSc in plant physiology from the University of Calgary, and received his PhD in plant molecular biology from the Australian National University.

Plenary Session 1–Technologies

Q&A

Moderator: Greg Martin
Boyce Thompson Institute
Ithaca, New York

Dana Carroll (University of Utah): Adam, when you showed the natural variation in numbers of the TAL effectors, there were peaks at fifteen, seventeen and nineteen and a half, and valleys at sixteen and eighteen. Is that significant?

Adam Bogdanove: It's odd, but I don't know what the significance is. There are hints that it might be significant in that TAL effectors at the 5' end of the binding site often coincide with a TATA box or TATA-box like element. It could be that there is some periodicity in positioning of the activation domain for interaction—totally off the top of my head—with the transcription machinery. That's one thought.

Perry Hackett (University of Minnesota): For Dr. Ran—cells are loaded with degraded messenger RNAs, and I am wondering if they can, in collaboration with the guide RNAs that you have, lead to totally random mutagenesis inside the cell, wherein the degraded RNA might hybridize with the guide RNA in order to completely redirect it towards a non-homologous site.

Ann Ran: If I understand you correctly, your question is whether any mRNA in the cell can degrade—

Hackett: First it gets cut up by endonucleases. You have all these fragments and, before it gets completely degraded, you might have them acting as pseudo-guides.

Ran: That is a possibility. In principle, Cas9 may hijack other RNAs in the cell that have structural similarity to an sgRNA. So far, there is no good way for us to determine that directly, but we and others are trying to develop unbiased ways of detecting double-stranded breaks that might shed light on that possibility. So, we don't know yet.

Donald Weeks (University of Nebraska): First a comment on Perry's question—secondary structure of the rest of the single-guide RNA has to interact with Cas9 productively, and not any old messenger will do that. And for Ann—my question is directed towards your double nuclease cuts. I noticed that it appeared that you were getting cuts very close to each other with those two separate Cas9 single-guide RNAs. That dictates, doesn't it, that one has to hop on, make a cut, and the other has to come along and make a separate cut? Were those efficiently made? Do you see those very often?

Ran: The guides are each twenty nucleotides long, and they each recognize a twenty base-pair DNA target. So there are two Cas9 nickases that are situated close together, based on the position of the guides. The position of the guides can overlap to a certain point, but the overlap tolerance is fairly small; it can be up to about four bases and beyond that you cannot achieve double-stranded breaks. That may imply a steric hindrance limit at which Cas9 double-nicking no longer occurs, but as you pull the Cas9s farther apart, double-nicking can occur again up to a certain point; if it's longer, efficiency decreases.

Tom Turpen (Citrus Research and Development Foundation): Greg, what's the basis of the site specificity of your oligo mutagenesis?

Greg Gocal: It's homology pairing, so your oligo is homologous to the DNA sequence that you are targeting, and, generally, mismatches don't help you, although, with increased efficiency, you can push extra mismatches in terms of the DNA editing.

Turpen: So, no nuclease break is involved?

Gocal: There doesn't need to be, no.

Dan Voytas (University of Minnesota): Greg, as I understand it, the modified oligos are a mutagen—a target mutagen, but a mutagen. You showed improvements in the technology and I am wondering—now you are changing one base at a time, but I can imagine in the future changing hundreds of bases simultaneously. As the technology improves, is it your opinion that they would also have that status?

Gocal: If you're engineering a pathway that didn't exist within a crop, you may fall under an FDA or EPA regulation, despite viewing the technology as mutagenesis. In Canada, you are always going to be regulated on the basis of the trait. Speaking for myself and not Cibus, I think that this is what we will end up getting in Europe. They will regulate the trait and not the process by which the trait was developed. Time will tell. From a regulatory standpoint in Europe, we are cautious in approaching each of the individual countries and, so far, we have made progress.

Weeks: Greg, the speakers who have talked about the TALEN and CRISPR technologies have talked about off-site targeting. How mutagenic are your oligos, *vis-à-vis* other sequences in the genome? Do you have any idea?

Gocal: We have evidence from designing oligos even for targeting our model system where we put additional mismatches in or have mismatches that are displaced from the target which is generally in the center of the oligo—what we see is that as the mismatches are fifteen to twenty bases apart, you tend to disfavor those in terms of the conversion frequencies. So, at least at its basic form, additional mismatches generally push you away from the target, so then if you have an oligo of sixteen base pairs it would be relatively unique in small genomes. As you get into bigger genomes, you really have to look at what the similar sequences are. You might have several hundred members in a gene family, so the answer can be complex, related to the homology of the oligo to the target sequence you are looking at.

Joachim Schiemann (Julius Kühn Institute): Greg, did I understand you correctly that you are saying that your ODM-generated plants are not transgenic, therefore you do not follow the route for approval for genetically modified plants by approaching EFSA? You are saying okay, it's not transgenic, so you are only asking different member states. Is that right?

Gocal: A group within Europe assessed oligonucleotide mutagenesis and concluded that ODM is no different from mutagenesis. Based on that opinion, which was shared publicly, we have gone to individual member states to see if they agree with that opinion or not, in terms of being able to plant or not in those countries.

Schiemann: But the question is whether an assessment by a group of experts is legally binding. So, you think it is legally binding.

Gocal: We believe it is legally binding. The opinion of the public is a different issue.

Maria Federova (DuPont Pioneer): Canola is a global commodity. Are you looking to clarify the regulatory status with global importers of canola—not just the United States, Canada and the EU?

Gocal: We have approached the Japanese to look at that. We have approached Australia. In the decision by some countries, one of the motivators in making the decision is that our canola is now part of the global economy because we have sold it in the United States for the past several years all the time with increasing acreage. Between the regulation decision in Canada and our discussions with various groups in Europe, we feel that we are on the right track.

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EXZACT™ Precision Technology: Scientific and Regulatory Advancements in Plant-Genome Editing with ZFNs

Gary Rudgers and Lakshmi Sastry-Dent
Dow AgroSciences
Indianapolis, Indiana

gwrudgers@gmail.com

Approaches to improving agricultural crops have relied on either mutational or traditional breeding and selection methods or through the random introduction of novel genes into crops by transformation (*e.g.* using *Agrobacterium*). These approaches are inherently time-consuming, inefficient and expensive, especially for developing complex, multi-trait products that are now standard for the industry. To better serve the market, new technologies have been developed that focus on targeted genome editing. One such technology is Dow AgroSciences' EXZACT™ Precision Technology that facilitates precise changes in plant genomes including DNA deletions, edits (point mutations) and targeted gene additions (trans-, cis-, intra-). Using EXZACT™ Precision Technology, developers and breeders can now introduce genetic traits more efficiently and precisely than ever before.

EXZACT™ Precision Technology is based on zinc-finger nucleases (ZFNs) that are composed of a DNA-binding domain (zinc-finger proteins) and a nuclease domain derived from the restriction endonuclease *Fok1.* DNA binding is mediated by ZF modules, where each ZF recognizes a specific three-base-pair DNA sequence. Typically four to six individual ZFs are linked together for specific recognition of a unique DNA sequence (12–18 nucleotides) (Figure 1). The *Fok1* nuclease that generates the specific double-stranded DNA cleavage at the targeted DNA sequence requires dimerization of the two *Fok1* domains for cleavage activity. Therefore, functional ZFNs are dimeric proteins consisting of two unique zinc-finger proteins (Figure 1) that bind to two unique DNA

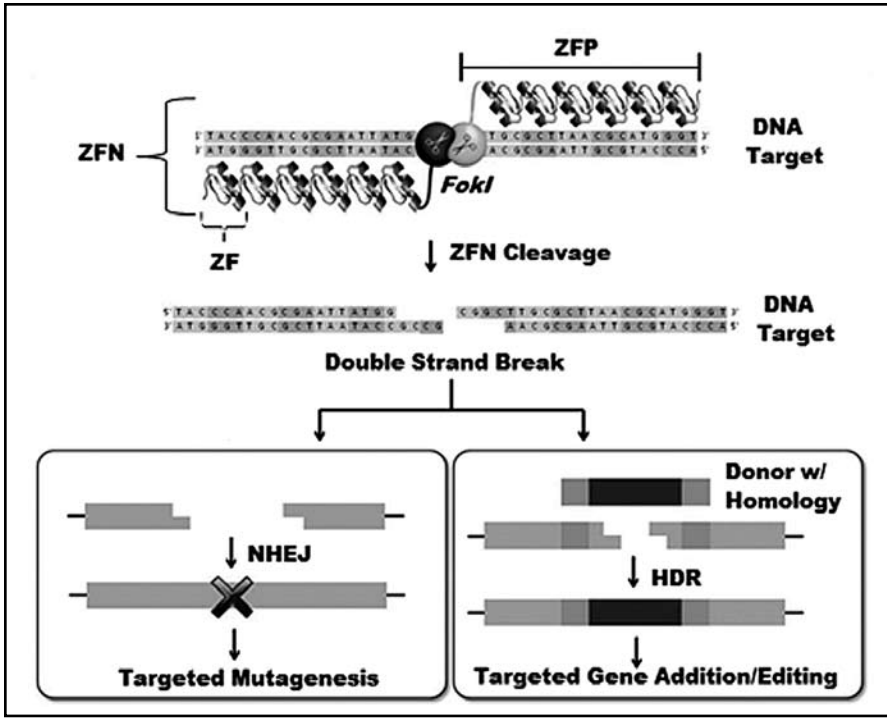

Figure 1. Model of ZFN DNA cleavage and repair in the presence (HDR pathway) or absence (NHEJ pathway) of a DNA-repair template. Depending on the DNA-repair pathway and repair donor-DNA template, genomic deletions, edits or additions can be generated, screened and selected.

sequences (24–36 nucleotides) in the correct orientation, at the same time, to generate a targeted double-strand DNA break.

The EXZACT™ Precision Technology platform exploits the functionality of ZFNs and plant cellular DNA-repair mechanisms to facilitate targeted deletions (*ZFN-1*), editing (*ZFN-2*) and addition (*ZFN-3*) of DNA (or genes) in model and specialty-crop species. When ZFNs are delivered into plant cells, they bind to a specific, predetermined genomic sequence and introduce a DNA double-strand break that activates the innate cellular DNA-repair pathways. Repair by non-homologous end-joining (NHEJ) may result in the creation of small insertions or deletions (INDELS) at the break site, leading to targeted mutagenesis and effective disruption of the genomic sequence (Figure 1). If the initial target sequence is a gene, NHEJ-induced targeted mutagenesis leads to loss-of-function or altered-function alleles. The double-stranded DNA breaks can also be repaired by homology-directed repair (HDR) in the presence of a donor DNA molecule with homology to the break site, resulting in targeted donor addition. With the HDR, presence of a gene of interest (GOI) in the donor leads to targeted gene addition, whereas point mutations in the donor give rise to a genome-editing mutation (Figure 1).

Should one of the text references be Figure 2 instead of 1?

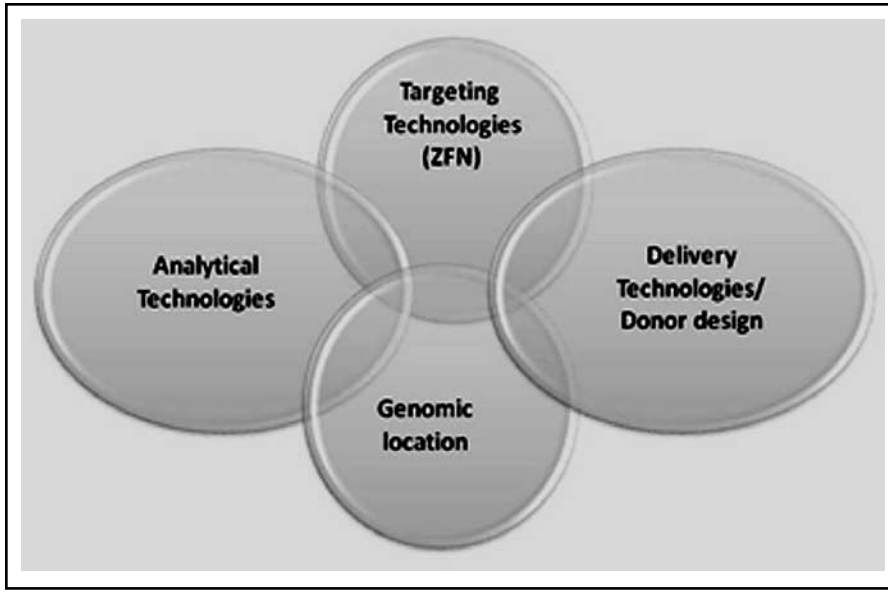

Figure 2. EXZACT™ Precision Technology platform. Donor design, delivery, analytics and genome-location information was coupled with DNA double-strand break generation ability to create an operable platform for trait applications.

EXZACT™ targeted gene addition offers several benefits for trait-product development compared to conventional methods. With current transformation methods, a GOI is randomly inserted in the plant genome and screening for events of interest is time-, resource- and cost-intensive. In contrast, targeted gene addition to a specific, desired locus, such as a safe harbor location (see *Genomic Locations* below) is expected to generate higher-quality events with minimal unintended side-effects and an increased probability of achieving the desired product. In addition, targeting to a specific locus simplifies analytics involved in event characterization and sorting, thus reducing trait-development cycle times.

An additional advantage of gene addition is that GOIs can repeatedly be targeted to the same safe-harbor location to generate new events, reducing costs over time through the reuse of targeting reagents, analytics and potentially regulatory and introgression processes. Gene addition also provides new options for stacking, to generate multi-gene, multi-trait products. Currently, stacking traits is primarily achieved by breeding together multiple traits from single events located at various loci in the plant genome. However, as the number of traits being bred or stacked into a single product increases, efficiency of stacking decreases due to the increased likelihood of the various loci segregating during the breeding process. Using ZFNs, it is possible to target multiple genes to the same location, which decreases the number of loci involved in breeding and facilitates multi-trait product development. It is also feasible to use ZFNs to add genes to existing commercial-grade events to generate new gene stacks to meet customer and market needs.

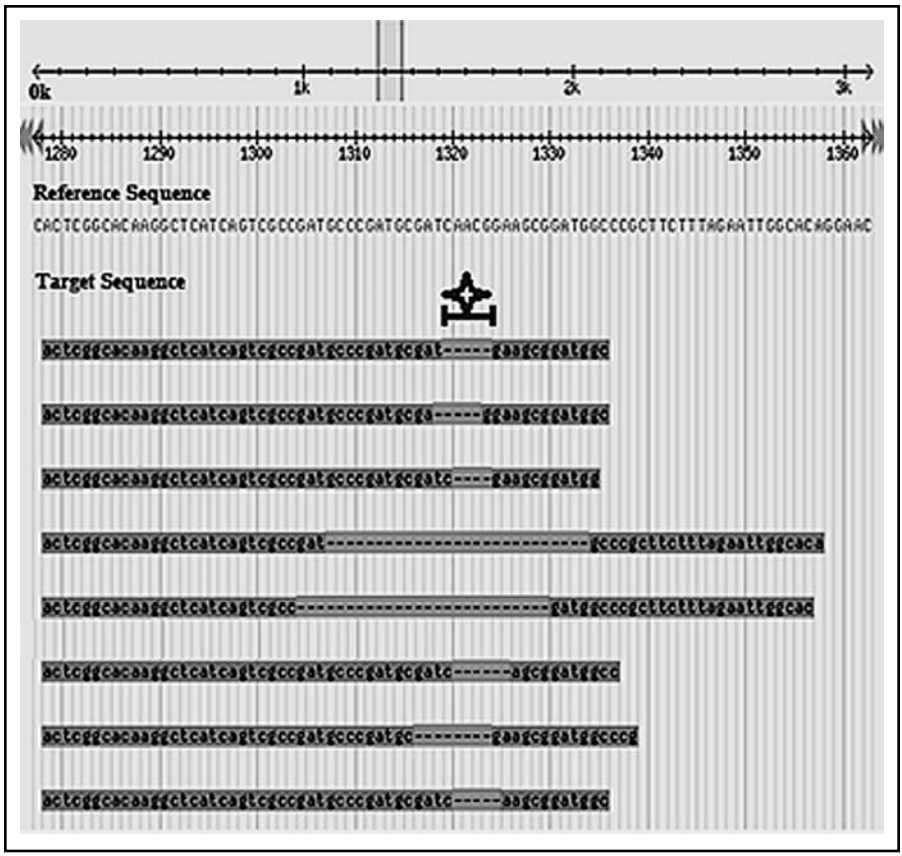

Figure 3. Activity of a ZFN in maize cells. DNA-cleavage activity of ZFNs is measured by the presence of INDELs (-----) using illumine sequencing. The bar with the star represents the cleavage site for the ZFN. The reference target sequence is shown at the top and sequences with INDELs from Illumina® are shown in dark gray.

EXZACT™ Precision Technology Platform

To optimize and enhance EXZACT™ technology for trait-product development, an EXZACT™ Precision Technology platform has been developed (Figure 2). This platform consists of companion technologies including DNA-donor design and delivery, analytics, identification and optimization of gene-targeting genomic locations and advancements in ZFN-targeting technology. The combined suite of technologies making up the EXZACT™ Precision Technology platform has been developed in several crop species including maize, soybean, canola and wheat.

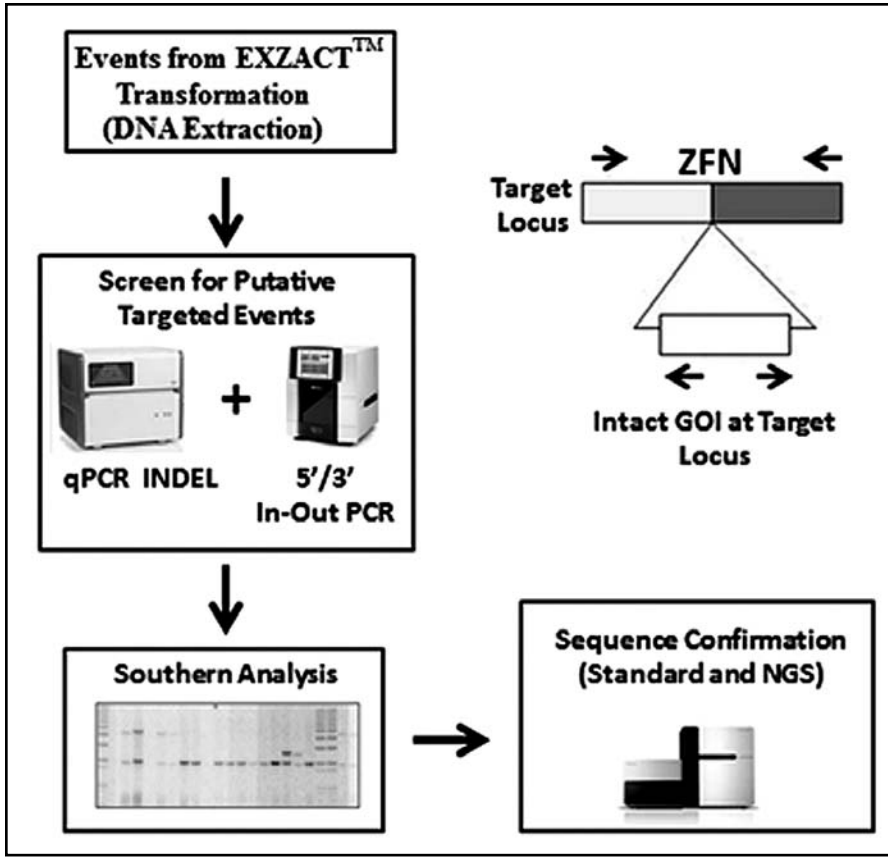

Figure 4. Analytical process for identifying targeted events.

Targeting Technologies: ZFN Design and Testing

ZFN design and testing capability is critical for a robust genome-editing program. Functional ZFNs have been developed for targets in a number of model and crop species for gene addition, deletion and editing applications (Cai *et al.*, 2009; Shukla *et al.*, 2009; Ainley *et al.*, 2013).

With advancements in ZF designs, ZFNs can now be effectively developed for DNA targets less than 100 bp and have been linked to enhanced (obligate heterodimeric) *Fok1* nuclease domains to reduce the potential of off-target cleavage (Doyon *et al.*, 2011). An agreement between Dow AgroSciences and Sigma Aldrich has been developed for plant-ZFN design and supply to meet product-scale needs.

In addition to improved ZFN designs, prior to deploying EXZACT™ ZFNs in plants, 3–16 ZFN designs are typically generated per DNA target and assayed for their ability to generate targeted DNA double-stranded breaks. Lead-candidate designs are identified by transient expression in plant cells followed by deep sequencing of genomic DNA from ZFN-treated and control materials. Delivery of ZFNs into cells leads to DNA double-

strand breaks that are repaired by NHEJ, which leaves an INDEL footprint at the break site. Presence of, and number of, sequences with INDELS is a measure of ZFN activity. A diverse INDEL footprint, as seen by the presence of different types of deletions, further indicates effectiveness of a ZFN. A representative activity profile for a maize target-specific ZFN is shown in Figure 3.

As a result of improved EXZACT™ ZF design, use of obligate heterodimeric *Fok1* nucleases and screening of designed ZFNs for DNA-binding and -cleavage activity, the resulting EXZACT™ ZFNs deployed for gene deletion, addition or editing result in highly efficacious ZFNs with minimal off-target cleavage and superior results.

Analytical Technologies

Analytical technologies are foundational to the success of the EXZACT™ technology. In most plant-cell-targeting studies, targeted gene integration occurs in conjunction with random integration. Therefore, there is a need for sensitive methods for rapid identification of only targeted events. A tiered molecular process has been developed that uses two PCR-based screens for rapid identification of putative targeted events that are then confirmed by higher resolution methods, including Southern analysis and next-generation sequencing (Figure 4). PCR screens are designed to measure both intact ZFN-binding sites at a target locus and also score for the presence of the GOI at the target site by junctional in-out PCR (Ainley *et al.*, 2013). The duplicate screens are done in parallel to reduce false positives and increase the robustness of targeted event identification.

Delivery and Donor-Design Technologies

Ability to effectively co-deliver ZFN and donor constructs is critical for achieving targeted genome addition. In maize, targeted gene addition has been achieved successfully by delivering ZFN and donor constructs using WHISKERS™ and microparticle bombardment (Shukla *et al.*, 2009; Ainley *et al.*, 2013). In addition to DNA delivery, donor constructs have been optimized for efficient and optimal HDR of the targeted double-stranded break through the use of 750- to 1,000-bp homology sequences flanking the GOI.

Genomic Location

In a plant genome, not all locations are suitable for targeted gene insertion. Safe-harbor locations are genomic regions where transgenes can be added with minimal unintended side effects and for consistent, reproducible transgene performance. Such safe-harbor locations can be identified through previously characterized transgenic plants. Event-32 is a Dow AgroSciences' pipeline event that was generated by standard transformation of maize with a corn-rootworm-resistant/herbicide-tolerant gene stack. The event performed well in the field as demonstrated by stable trait expression and efficacy, and exhibited neutral agronomics and good breeding characteristics. Because of these features, the transgene-insertion site of the event was classified as a potential safe-harbor location. This safe-harbor location has since been retargeted in a Hi-II maize background using a custom-designed ZFN (ZFN6) and a GOI by HDR (Figure 5). As a result of improved ZFN designs, screening and analytic methods, targeting frequencies of 2% have repeatedly been achieved at this safe-harbor location.

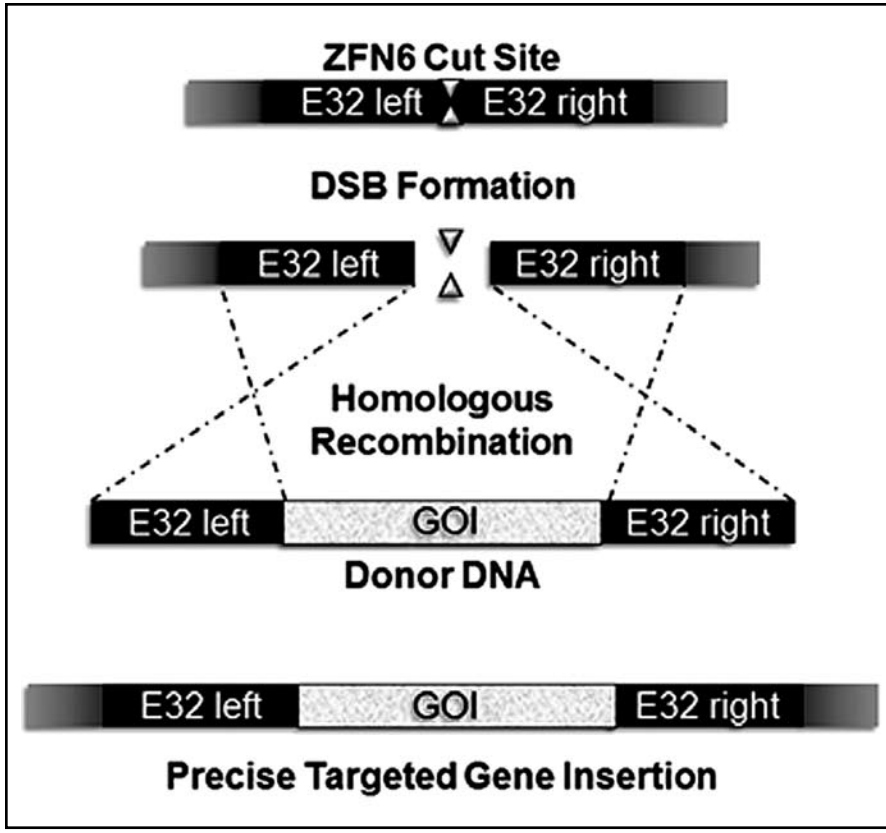

Figure 5. Targeted gene addition at the Event-32 locus.
A GOI was inserted at the E32 locus using EXZAT™ technology.
E32 left/right: sequences homologous to the E32 locus.
ZFN6: E32 locus-specific ZFN.

Gene Stacking

For stacking multiple genes at a single location using EXZACT™ technology, a suite of engineered ZFNs (eZFNs) and EXZACT™ landing pads (ELPs) have been developed (Ainley *et al.*, 2013) (Figure 6). ELPs are unique DNA sequences that carry an eZFN DNA-binding sequence flanked with DNA sequences homologous to an incoming donor DNA sequence. For stacking, an ELP with a first GOI is pre-integrated into the plant genome (*e.g.* E32 safe harbor); subsequent delivery of a donor carrying a second GOI and an appropriate eZFN leads to a DNA double-strand break at the ELP and insertion of the donor via HDR, resulting in stacking of the two genes of interest (Figure 6). Presence of a second ELP in the incoming donor allows addition of a third GOI, and so on. This strategy has been used to stack two herbicide-tolerant genes, PAT and aryloxyalkanoate dioxygenase 1 (AAD-1) in maize. The stacked genes were functional

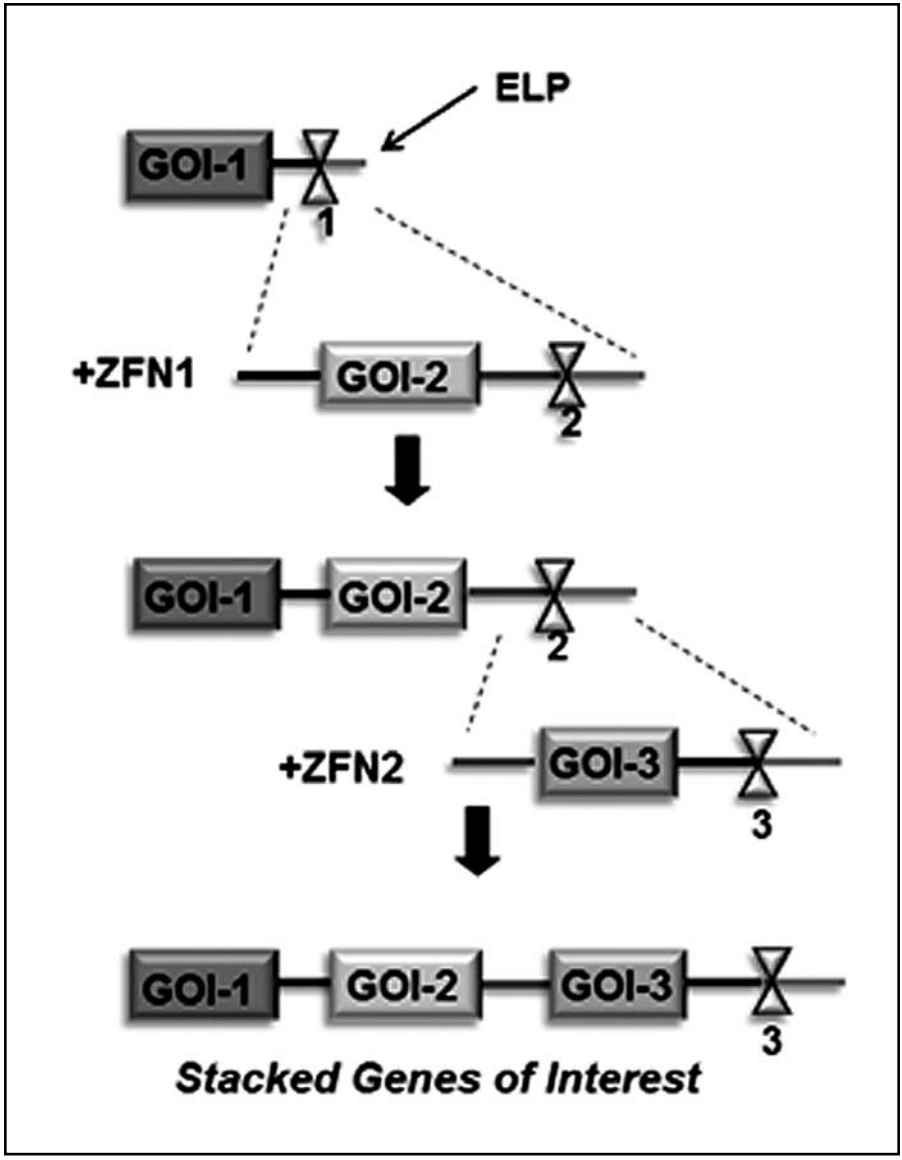

Figure 6. Transgene stacking with EXZACT™ landing pads.

at expression and phenotypic levels and co-segregated as expected (Ainley *et al.*, 2013). The stacking strategy allows insertion of multiple genes at a single location to facilitate multi-trait product development.

The EXZACT™ Precision Technology platform is a new way of thinking about agricultural biotechnology, and represents a departure from conventional breeding

methods. Targeted gene deletion, editing and addition have been demonstrated in tobacco, maize, canola, tomato and wheat. In maize, targeting to an endogenous locus (IPK1) as well as stacking of herbicide-tolerant traits at an engineered locus have been achieved (Shukla *et al.*, 2009; Ainley *et al.*, 2013).

External Collaborations

EXZACT™ Precision Technology is available and accessible both to the public and private sectors through a Dow AgroSciences' licensing agreement. Like the private sector, public-sector breeders and scientists have significant opportunities to employ EXZACT™ Precision Technology in their breeding programs, especially in minor crops. Dow AgroSciences has entered into several licensing agreements with partners around the world to develop targeted gene improvements ranging from deletions, edits and gene insertions in row, community and specialty crops such as maize, canola, cassava, wheat, tobacco, tomato and forestry trees.

As an example, the Department of Environment and Primary Industries (DEPI) of the State of Victory, Australia, through its commercial arm, Agriculture Victoria Services Pty Ltd. (AVS), strengthened a collaborative agreement to improve the performances of Australian canola varieties. The project uses the EXZACT™ Precision Genome Editing Technology platform to continue developing new varieties of canola with enhanced performance designed to benefit farmers in Australia and globally. In addition, AVS will also use the EXZACT™ Precision Genome Editing Technology platform to enhance the genetics of crops important to Australian primary producers.

Through valuable collaborative efforts, a variety of improved crop varieties is being developed with value-adding traits ranging from more-nutritious and insect-resistant cassava and higher-yielding tomatoes, to oil crops with healthier, improved oil profiles and crops with improved herbicide tolerance.

Advances in custom ZFN designs, high resolution analytics, novel donor designs, delivery technologies and genomics will continue to expand the utility of the EXZACT™ Precision Technology for trait discovery and product development. Benefits of targeting genes, genomic deletions and edits at desired locations in plant genomes will continue to be realized, resulting in reduced cycle times and costs for developers while resulting in improved, high-value crops for the farmer and consumer.

Regulatory Considerations

ZFN applications are simply innovative improvements and refinements of traditional breeding methods used to optimize plant health, nutritional quality and yield. Changes and variation in plant genomes (*e.g.* mutations, chromosome rearrangements) are ubiquitous and essential drivers for plants to adapt to their environment and have resulted in a wide variety of crops with a long history of safe use. In fact, over the past century, mutagenesis applications (*e.g.* chemical and radiation mutagenesis) have resulted in over 3,200 beneficial crop varieties that have been planted and safely consumed worldwide in over 175 species (IAEA, 2010). In addition, once developed, traditional breeding processes are deployed to efficiently breed desired mutations into elite plant varieties while minimizing the donors' genetic background.

Due to the controlled, precise manner of the EXZACT™ Precision Technology platform, only the desired genomic changes (gene introduction, deletion or edit) are introduced into the plant genome and the resulting trait can be bred into commercial crops more efficiently and rapidly compared to traditional breeding and mutational applications.

In addition to the considerable research and development advantages, EXZACT™ Precision Technology offers potentially significant regulatory advantages for products resulting from both targeted gene addition (ZFN-3) and targeted mutations (ZFN-1,-2). Globally, regulatory oversight of biotech products is a time-consuming and expensive endeavor estimated at $35 million with an average of six years for regulatory approval / deregulation (Phillips McDougall, 2011). As a result, most biotech crops developed to date have been limited to a handful of traits and high production-acre commodity crops where a return on the investment can be realized (James, 2013).

Through targeted precise addition (ZFN-3), unintended effects (yield drag, gene silencing) often associated with random gene integration, can be avoided. By precisely knowing the location of the introduced trait in the plant's genome, EXZACT™ will help researchers and regulators better understand the end product and help develop a more efficient, less costly and less time-consuming regulatory process (FSANZ, 2013).

Like traditional mutagenesis techniques, EXZACT™ Precision Technology delete (ZFN-1) and edit (ZFN-2), can induce changes in the DNA analogous to chemical mutagens, but with the benefit of targeting the mutation to a predetermined location in the plant genome. Traditional mutagenesis techniques are used to induce point mutations or small insertions / deletions and are intrinsically very non-specific, in which thousands of nucleotides are mutated instead of a desired single nucleotide. Additionally, traditional mutagenesis techniques can often result in genetic alterations at multiple genomic locations. As a result, years of breeding are required to eliminate undesired mutations and to cross the desired mutation into an elite commercial crop variety.

Through EXZACT™ Precision Technology delete (ZFN-1) and edit (ZFN-2) applications, breeders can now take advantage of a plant's own natural genetic variability, allowing precise mutations to be made at predefined locations in the genome, resulting in beneficial, desired mutational traits that can be bred into commercial crops more rapidly compared to traditional mutational applications. As with traditional mutational products, EXZACT™ Precision Technology mutational applications do not involve recombinant DNA and result in products that are similar, and in many cases indistinguishable, from conventionally bred or traditional mutagenesis products.

Based on these facts, it has been concluded by USDA-APHIS that EXZACT™ Precision Technology delete products fall outside their scope of regulation (USDA APHIS, 2014). In addition, an Australian scientific panel convened by Food Standards Australia New Zealand concluded that ZFN-1 and ZFN-2 do not present a significantly greater food-safety concern than other forms of mutagenesis. Such changes are small and definable with predictable outcomes and the panel concluded that food derived from such applications would be similar to food produced using traditional mutagenic techniques, and should, therefore, not be regarded as GM (FSANZ, 2013).

Efforts are underway to help facilitate and assist global regulatory decision making around products developed through various gene-editing applications. Gene-editing

techniques used to develop new plant varieties do not pose a specific safety hazard; rather, the characteristics of the plant determine its safety. Thus, the need to regulate plants developed through gene-editing techniques should be driven by the characteristics of the product (*i.e.* whether it is materially different from existing products present in food, feed or the environment) rather than by the method or process used to make that product. In addition, governments should avoid regulating products developed through gene-editing applications that are similar to, or indistinguishable from, products resulting from traditional breeding methods, since they do not differ in their safety (CropLife International, 2014).

Acknowledgments

We would like to thank Sangamo BioSciences, the Department of Environment & Primary Industries, Victoria, and all the individuals at Dow AgroSciences who have contributed to this work.

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GARY RUDGERS is the Global Regulatory Leader for New Business at Dow AgroSciences in Indianapolis. Over the past six years with the company, he has worked to develop, assess and coordinate global regulatory strategies for emerging and leading biotech crops developed through novel plant technologies.

In addition to his role at Dow AgroSciences, **DR. RUDGERS** chairs the CropLife International New Breeding Techniques (CLI-NBT) working group. Since 2013, this industry organization team has developed and distributed documentation on precision-breeding applications to governments and regulatory bodies worldwide. As chair, he has represented the CLI-NBT working group at various international organizations, on government agencies and at international conferences.

Prior to joining Dow AgroSciences in 2008, Rudgers was one of the leading scientists to help launch the biotechnology company, Chromatin, Inc., in Chicago. From 2002 to 2008 he led the Chromatin molecular biology research team in the development of the first autonomous plant mini-chromosomes. Rudgers held a post-doctoral fellowship at the University of Chicago from 2001 to 2002 and received his PhD in molecular microbiology and immunology from Baylor College of Medicine in Houston in 2001.

Potential for Selection of Beneficial Traits in Swine with Site-Specific Nucleases

Le Ann Blomberg
USDA-Agricultural Research Service
Beltsville, Maryland

leann.blomberg@ars.usda.gov

I am located in the Animal Biosciences and Biotechnology Laboratory of the Agricultural Research Service (ARS) in Beltsville, Maryland. Our lab falls under the purview of the national program or NP 101, which is food animal production, as well as NP 103, which is animal health. Within the lab we have five projects in three broad categories:

- Growth and reproduction,
- Health and alternatives to antibiotics, and
- Development of genome-editing tools.

A partner on campus, Dr. Bhanu Telugu with the University of Maryland, works closely with us and is a major contributor to this presentation.

As we prepare projects, it's important to recognize the interdependence of the health of all species and, furthermore, being part of the ARS, it's important that whatever knowledge that we acquire and intervention strategies that we develop should have global application when and wherever possible.

Our research falls under the USDA's priority *Global Food Supply and Security* and the focus is to maintain the efficient production of nutritious, affordable and safe food for human consumption. This will continue to be a priority, and, as we go forward, we anticipate challenges. For example, it is anticipated that, by 2050, there will be about a 70 percent increase in the demand for animal protein, not only due to an increase in the global population but also due to increased individual wealth in less-developed countries, particularly as those people adopt a more western lifestyle, they will demand more meat in their diets. Also, it is predicted that, by 2050, a large increase in urbanization will have

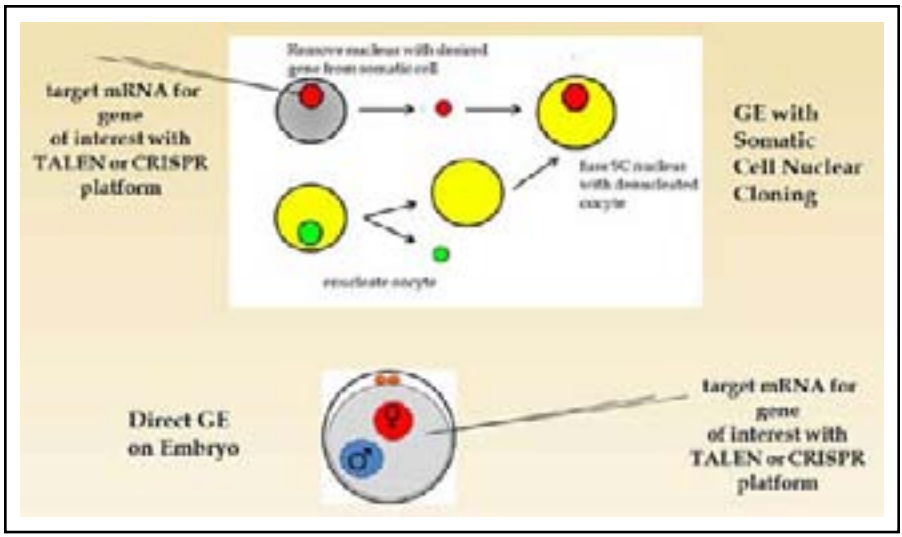

Figure 1. Genetic engineering.

occurred with decreases in rural populations, including the number of farmers. And it is expected that crop-farmland and animal-pasture "footprints" will be reduced in the next few decades.

Aside from challenges within the livestock-production system, another issue is animal disease as global travel increases. How do we mitigate current diseases as well as minimize the appearance of new diseases? We need to develop alternatives to antibiotics while fostering growth promotion. Feed efficiency is another salient issue; we have very efficient animals now, but can we boost their efficiency further? Other issues are animal welfare and the interactions of animals with the environment and how these affect production.

Breeding and Selection

Selective breeding has been beneficial for the agricultural community with the production of superior animals with desirable production traits including increased growth rate, increased feed efficiency, increased meat yield per animal as well as resistance to disease. On the other hand, frequently along with desirable traits, undesirable traits also segregate, such as the susceptibility to other diseases and, in litter-bearing species—particularly referring to swine—we've seen increased weight variability, and with attempts to increase muscle mass we may alter body structure, which has been known to affect reproductive capacity as well as raise animal-welfare concerns.

Another caveat for selective breeding is the length of time that it takes to achieve genotypic improvement; for cattle it can be about a quarter century. Selective breeding will continue, particularly considering increased genome information on animals, but the bigger question is whether there will be value in genetic engineering of livestock, particularly if we can guarantee precise targeting of an allele or quantitative trait nucleotides, improve traits and, therefore, produce healthy and safe animals.

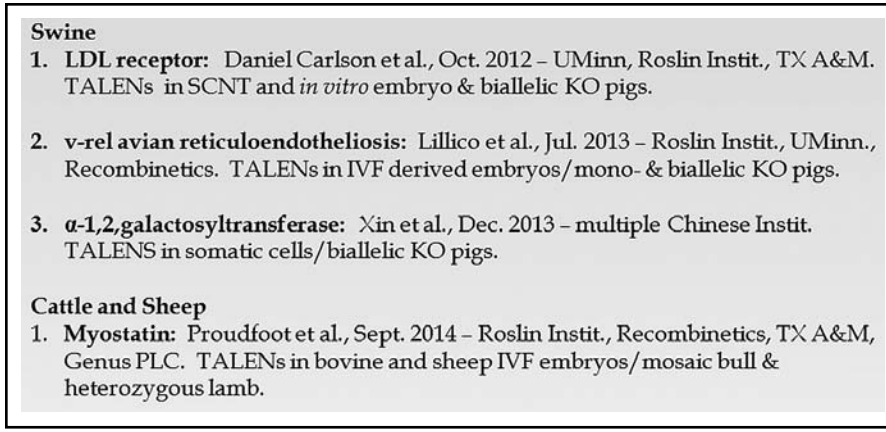

Figure 2. Reported gene targeting of livestock.

Gene Targeting

Primarily, the ARS's role in genetic engineering is development and refinement of tools. We are investigating technologies that utilize natural cellular mechanisms for genome repair that do not leave behind foreign DNA and precisely target genes of interest. And beyond development of tools, we seek opportunities for their implementation either through collaborative efforts or by passing on technologies to academia and industry. Like many present at this conference, our interests include TALENs and CRISPRs, investigating their utility primarily in swine and for addressing agricultural concerns such as animal well-being and zoonotic diseases.

We consider that there are two approaches for genetic engineering in farm animals (Figure 1). One is to modify the gene of interest in the genome of a somatic cell with either TALENs or CRISPRs and establish a cell line, and then use those cells for somatic nuclear cloning where you actually remove the genetically engineered (GE) nucleus fuse with an enucleated oocyte. Once the nucleus is in the oocyte, it is reprogrammed and a GE embryo generated. The second way is by direct modification of the gene of interest in the embryo genome again using TALENs and CRISPRs that are introduced directly into the embryo itself.

TALENs

Figure 2 shows some recent reports of success in genetically engineering swine, cattle, and sheep by *somatic cell nuclear transfer (*SCNT) as well as direct embryo modification.

Initially, we tried to use the TALEN platform to target a gene that was in a safe-harbor site for knock-out; for our proof-of-concept experiment, we selected the prion gene. Again we were interested in trying to use direct swine-embryo modification using *in vitro* matured oocytes that were *in vitro* fertilized to generate embryos. We chose direct embryo modification over SCNT primarily because, with SCNT, the efficiency had been low and, in addition, quite often there are developmental issues with offspring.

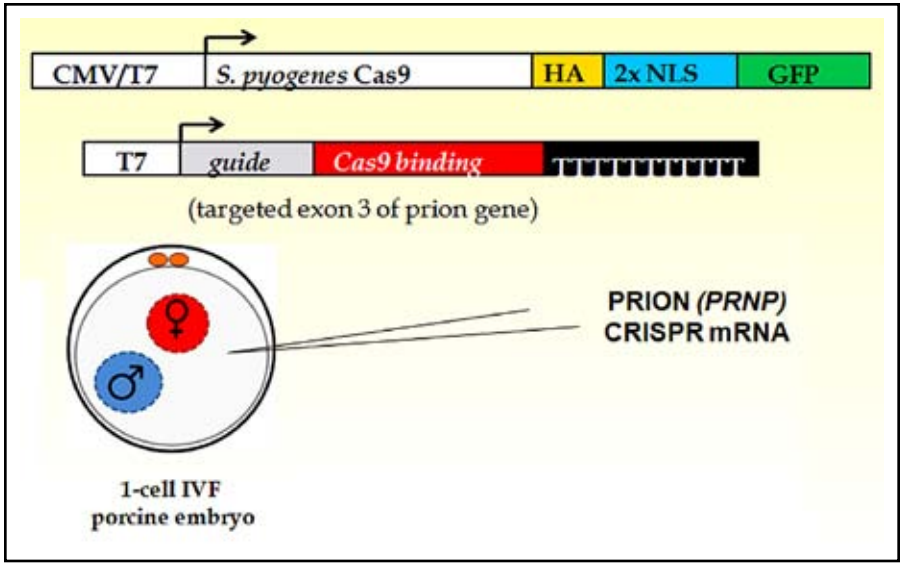

Figure 3. Gene knockouts in swine using the CRISPR/Cas NHEJ system.

Briefly wrapping up our experiments with TALENs, at least in our hands only a low percentage of the embryos developed to the blastocyst stage. Additionally, when we evaluated the sequences, many of our embryos had only a one-codon deletion. Accordingly, we moved on to CRISPRs because we had that technology operational at the same time, and, it seemed to work better in our hands.

CRISPRs

We chose the CRISPR-Cas9 system with which two approaches are possible, either through non-homologous end-joining, where you create an indel-mutation or, if you are interested in more-precise gene editing, you can adopt the homology-directed recombination method.

In our first approach, as stated, we wanted to target the prion gene and, particularly, modify exon 3 in the prion gene, so we used the Cas9 nuclease with a T7 promoter for *in vitro* transcription (Figure 3). The construct also had the nuclear localization signal as well as green fluorescent protein (GFP) as a marker; the guide RNA had the T7 promoter as well. These constructs were *in vitro* transcribed and then the RNA was injected directly into one cell each of *in vitro* fertilized porcine embryos. Those embryos were then cultured *in vitro* and, at 24 hours post-injection, we found that about 80 percent were GFP-positive. Also, we were able to identify their localization—within the cells of the embryo—which was good for us. Furthermore, we were able to mature 30 percent of those embryos to the blastocyst stage, superior to our experience with the TALEN technique.

Figure 4 shows sequences of clones obtained from the embryos that were analyzed to determine whether we had bi-allelic or mono-allelic or mosaic modifications. The bottom line is that we were successful in getting bi-allelic knock-outs or modifications in

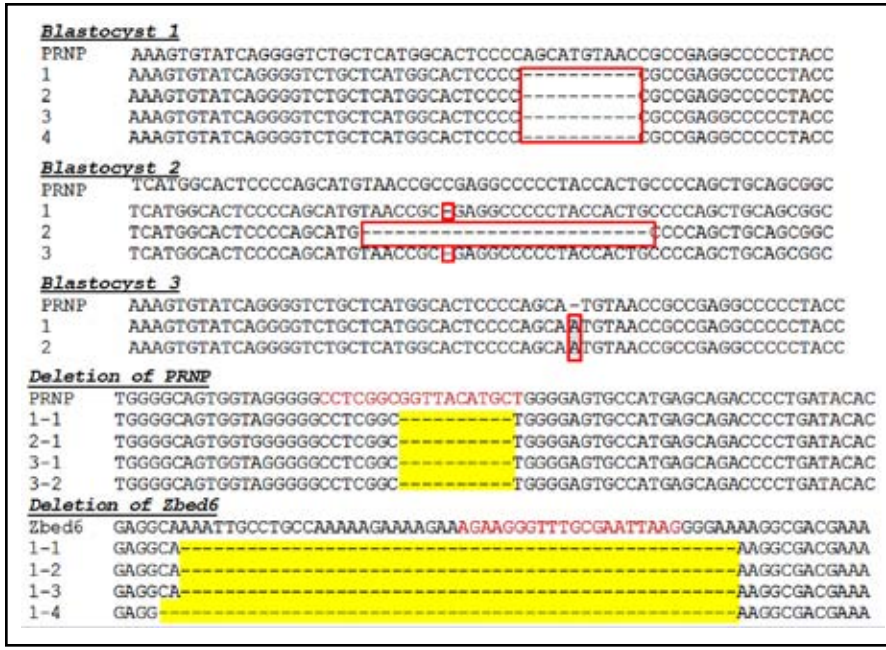

Figure 4. Successful single and double KOs.

about 80 percent of the embryos. Both deletions and insertions were observed between embryos, and, in some cases, we saw embryos that were mosaic. Because one of the targets that we were eventually going after is actually a bigenic disease, we were also interested in seeing if we could knock out two genes at one time. In addition to the prion gene, we chose the zinc-finger bed containing six transcription factors; the take-home message here is that we were able to create deletions in both genes simultaneously in the same embryo. That was good proof-of-concept, indicating that we could go ahead and target our genome of interest.

The next approach was to see if we could actually insert into or directly edit a particular sequence within a gene. In this case, we decided to insert a short sequence into the Zbed6 gene using the Cas nuclease with a guide tRNA, and a single-stranded DNA oligo that contained a loxP—*i.e.*, the sequence that we wanted to insert—the construct was flanked on the 5' end by an EcoR1 site (Figure 5). Again, the RNA constructs (nuclease and guide) plus the single-stranded DNA were injected into embryos, and, after we collected the embryos, a PCR was performed to see if we had actually been able to insert our short sequence. Figure 5 shows the banding patterns. The lower band is the wild-type allele that we expected. The upper band was of a size indicative of a 34-base-pair insertion. In addition, some embryos had the wild type and also had a lower band indicative of deletions in those embryos. The third pattern had a band of the same size as the wild type, but, because of its diffuse nature, we deduced that some other event had occurred in these embryos.

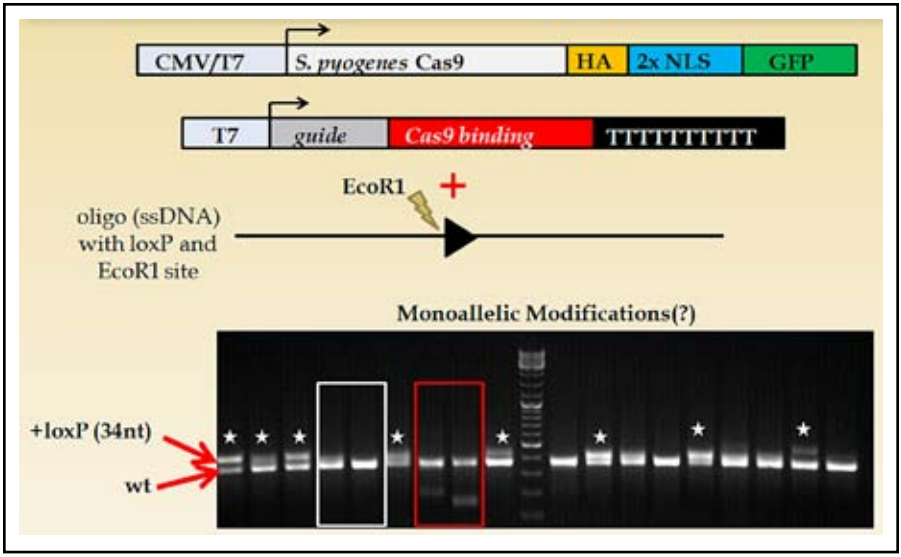

Figure 5. Gene-editing potential with HDR.

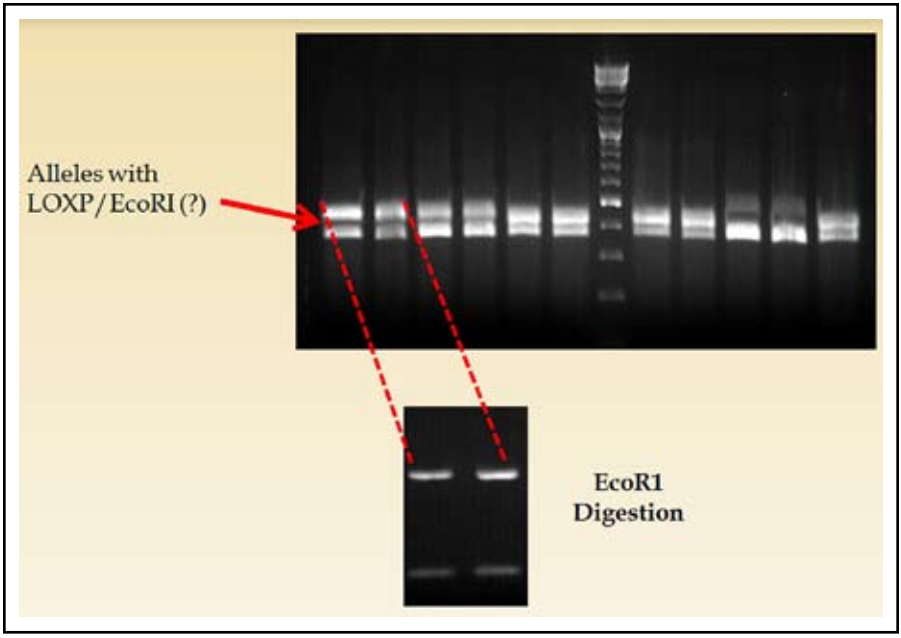

Figure 6. Confirmation of an EcoR1 site.

With the embryos that contained, what we thought was the loxP insertion, we did an EcoR1 digestion on the upper band; the allele that we thought contained loxP/EcoR1, and, sure enough, we were able to show that the EcoR1 site was inserted (Figure 6).

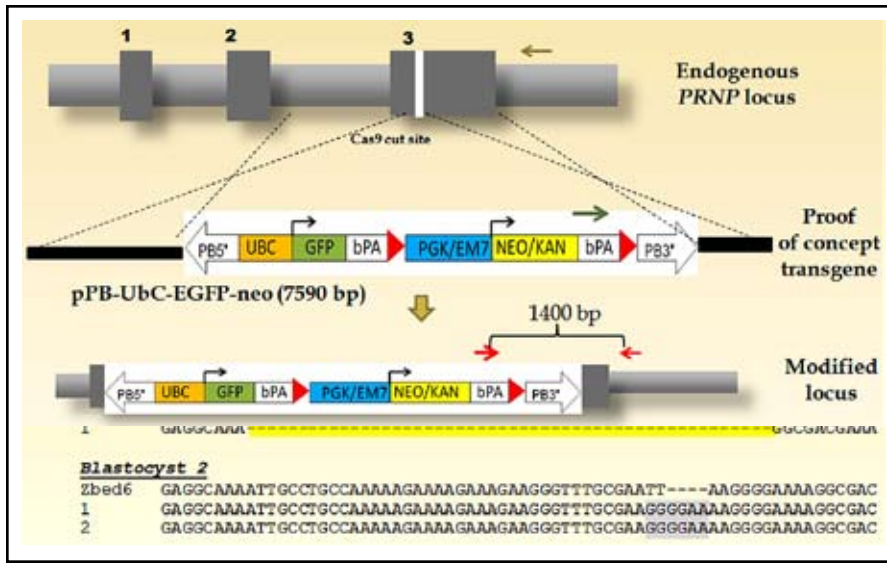

Figure 7. Mono-allelic modifications with precise gene editing.

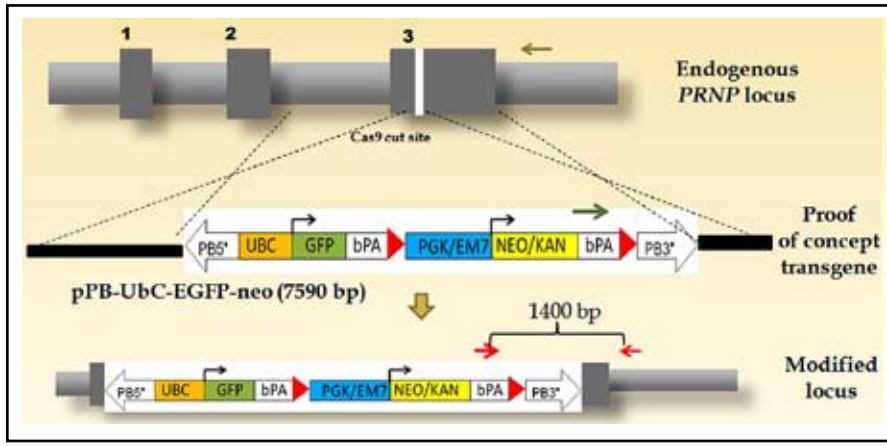

Figure 8. Gene insertion/replacement with Cas9 by homologous recombination.

Sequencing provided confirmation (Figure 7). We showed that we had the EcoR1 site plus the loxP gene inserted in a portion of the embryos, suggesting the ability to precisely target and modify a gene of interest; however, the sequence data also showed some random deletions and insertions. Although we need to work on this platform further, overall we were content that we were able to produce mono-allelic modifications.

Lastly, we were interested in determining if we could insert a transgene into a particular locus. In Figure 8, the upper schematic again shows the prion gene that we targeted for the introduction of a transgene into exon3. The middle schematic shows our proof-of-

concept transgene, which consisted of a ubiquitin promoter, GFP as a marker and then the phosphoglycerate kinase and EM7 promoters that flanked the gene providing neomycin and kanamycin resistance. The entire transgene construct was about 7.6 kb. The bottom schematic shows the modified locus subsequent to the insertion of the transgene; opposing arrows denote where we had designed PCR primers in the 3' end of the transgene construct and in the intron of the prion gene in order to amplify a 1,400-base-pair section to verify actual insertion of the transgene into the genome.

The construct along with the Cas9 guide RNA were injected into embryos, which we cultured to the blastocyst stage at day 7 (Figure 9) and, indeed, we were able to see GFP being expressed, indicating that we had successfully inserted our transgene. Furthermore, a PCR band appeared at the expected size subsequent to insertion of the transgene. Sequencing confirmed that many of the distinct embryos and the clones derived from those embryos also contained the transgene. On the other hand, we saw deletions in some of the embryos.

In Summary

We feel that we developed and successfully tested several CRISPR-based approaches for gene targeting in swine. We produced embryos and we were able to perform putative KOs using non-homologous end-joining and we plan to use this technology to address animal-welfare issues. We also feel that we can use the oligo-based insertion approaches to modify genes with homology-directed recombination, and we were also able to show that we could direct the targeting of expression cassettes into the genome of embryos as well.

On Going

We are probably a few years behind, but we are performing embryo transfers with our *in vitro* produced embryos in collaboration with the University of Maryland. We are trying to establish a dependable system to produce *in vivo* oocytes, so that we may actually go back and repeat this work, and then again try to refine the system by examining off-targeting and increasing efficiency.

Even though we have not produced animals, pigs in particular have been produced with the CRISPR/Cas 9 system using the SCNT for CD163 or direct embryo modification CD1D (Figure 9).

As an adjunct to our agricultural interests, when we focus on disease or welfare there is opportunity to study gene function particularly as more information is derived about the genome itself and as annotation is improved. There are also possibilities of dual benefit to agriculture and biomedicine where developmental issues and diseases are similar in swine and humans. Additionally, the pig has use as a model; for certain human diseases it is a better model than lab animals and it can also be applied to human transplantation.

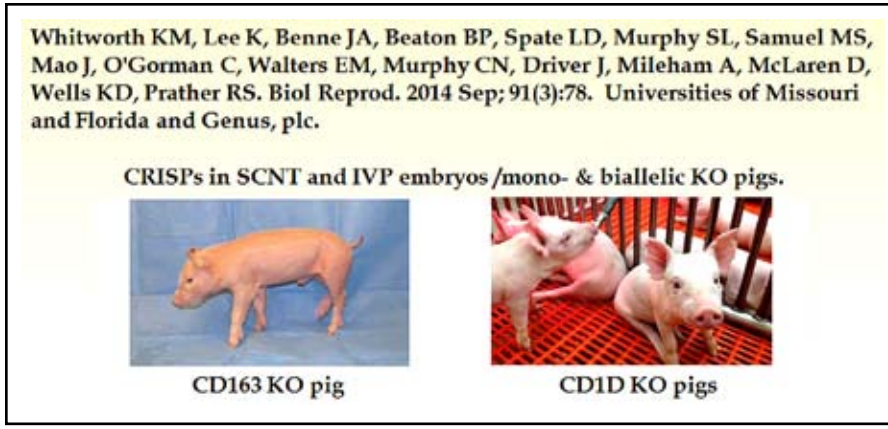

Figure 9. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from *in vitro*-derived oocytes and embryos.

LE ANN Blomberg is the research leader for the Animal Biosciences and Biotechnology Laboratory, Animal and Natural Resources Institute, US Department of Agriculture-Agricultural Research Service in Beltsville, MD. She received a BS in biology at John Brown University and subsequently received an MS and PhD from Georgetown University for her work in defining molecular cues regulating spontaneous postnatal alveoli formation in altricial animals and retinoic acid-induced alveolar regeneration in a rodent emphysema model. After joining the USDA as a postdoctoral fellow in 2002, she became a scientist and subsequent research leader for the unit.

DR. BLOMBERG'S work has focused on reproduction in swine with interests in understanding physiological aspects regulating embryo competence and the impact of uterine stress on fetal/neonatal development.

ISU Crop Bioengineering Consortium: Activities and Strategies

Martin Spalding
Iowa State University
Ames, Iowa

mspaldin@iastate.edu

The ISU Crop Bioengineering Consortium (CBC) was organized to address the urgent, grand challenge to provide sufficient food, feed, biofuels and biorenewable chemicals for the world's burgeoning population, through basic and applied research, to enable the bioengineering of valuable traits in a variety of crops. Importantly, the novel technologies employed can produce bioengineered crops that contain no transgenes and thus may face less-stringent regulation than classic genetically modified organisms (GMOs).

By 2050, the demand for staple food crops alone will require yield increases of nearly 80%. In addition, dwindling petroleum supplies, higher energy prices, dependence on energy imports, and the environmental consequences of fossil fuel use mandate development of renewable energy sources (including biofuels) and biorenewable chemicals. We need to increase food and biomass-crop productivity via both increased yield and expansion onto marginal lands. Historical yield increases have succeeded via improved management and intensive breeding, but we have begun to fall short of demand. Paradoxically, current crop varieties leverage only a fraction of the genetic potential available through natural and bioengineered alleles. Moreover, the discovery rate of potentially useful genes now clearly outstrips crop-testing capabilities. Therefore, technologies, as described here, that complement traditional management and breeding but dramatically accelerate production and testing of improved crops, are in critical demand.

Crop productivity is far from its yield potential due to losses from environmental and biological challenges, including water and nutrient limitations, non-optimal temperatures, diseases, pests, and weed competition. Many potentially ameliorating traits, such as increased photosynthesis or tolerance of environmental and biological challenges, have been recalcitrant to modern plant-improvement techniques. The CBC will try to overcome this intractability by establishing an innovative platform for the facile identification and incorporation of these beneficial traits based largely on new genome-editing technology. The transformative, novel genome-editing approaches known collectively as new breeding technologies (NBTs), developed in part through the pioneering efforts of ISU scientists including the CBC's Bing Yang[1], can make critical plant traits accessible to modern genetic tools and accelerate the production of improved crops. Just as improved plant breeding and crop management spawned the Green Revolution in the 1960s, so too could this new technology transform crop improvement in this generation.

Current crop-improvement efforts predominately emphasize transgenic technologies, which face public and regulatory challenges that dramatically increase both the time to market and the costs to obtain approval. Such hurdles are likely to increase for transgenics, so crop biotechnology companies are seeking alternative, non-transgenic approaches for the rapid engineering of crop traits. Because NBTs can generate small deletions or single-nucleotide changes in specific genes, and the tools that deliver them can be removed from an end product through traditional breeding, these innovative technologies provide an opportunity to make valuable genome changes without "leaving the tools at the worksite." Thus, NBTs offer a game-changing, non-transgenic approach to crop bioengineering. Because of the better regulatory prospects for NBTs, these technologies will have tremendous appeal, especially where transgenic products face strong public resistance. Nonetheless, the long-term sustainability of NBTs as the preferred approach to generate novel crop germplasm will depend on externalities to the technology itself, including the perceptions of interested and affected stakeholders through the technology's effect on regulation as well as anticipated demand.

Establishing the ISU Crop Bioengineering Consortium

The CBC was established July 1, 2013, with funding from the ISU Presidential Initiative for Interdisciplinary Research (PIIR). The CBC, which comprises 25 faculty (Table 1), 21 from ISU and 4 from other institutions, capitalizes on core strengths of ISU, including a long history of innovation in the area of plant-genome engineering, outstanding plant-transformation capabilities and world-class excellence in plant genetics and genomics. Establishment of the CBC promises to move ISU plant scientists in collaboration with selected researchers at other institutions to the next level through effective multi-disciplinary collaboration.

[1]See pp. 53–59.

Table 1. Outline of the CBC platform, illustrating the bidirectional interactions among the core research teams (gene/trait discovery, NBT/transformation, trait evaluation/integration, and regulatory, economic, environmental and societal impacts), the new germplasm developed and the beneficiaries of the germplasm.

Spalding, Martin H. (PI). Professor, Dept. of Genetics, Development, and Cell Biology, ISU.
Wang, Kan (CoI). Professor, Dept. of Agronomy, ISU.
Yang, Bing (CoI). Assoc. Professor, Dept. of Genetics, Development, and Cell Biology, ISU.
Baum, Thomas. Professor, Dept. of Plant Pathology and Microbiology, ISU.
Beavis, William. Professor & GFS Sprague Chair for Population Genetics, Dept. of Agronomy, ISU.
Becraft, Philip. Professor, Dept. of Genetics, Development and Cell Biology, ISU.
Bhattacharyya, Madan. Associate Professor, Dept. of Agronomy, ISU.
Hayes, Dermot. Professor and Pioneer Chair in Agribusiness, Dept. of Economics, ISU.
Howell, Stephen. Professor, Dept. of Genetics, Development, and Cell Biology, ISU.
Lamkey, Kendall. Professor and Pioneer Distinguished Chair in Maize Breeding, Dept. of Agronomy, ISU.
Lawrence, Carolyn. Associate Professor, Dept. of Genetics, Development, & Cell Biology, ISU.
Lubberstedt, Thomas. Professor and K.J. Frey Chair, Dept. of Agronomy, ISU.
Salas-Fernandez, Maria. Assistant Professor, Dept. of Agronomy, ISU.
Schnable, Patrick. Baker Professor of Agronomy, Dept. of Agronomy, ISU.
Vollbrecht, Erik, Associate Professor, Dept. of Genetics, Development, & Cell Biology, ISU.
Whitham, Steve. Professor, Dept. of Plant Pathology & Microbiology, ISU.
Wolf, Clark. Professor, Dept. of Philosophy & Religious Studies, ISU.
Wolt, Jeffrey. Professor, Dept. of Agronomy, ISU.
Wright, David. Associate Scientist, Dept. of Genetics, Development, & Cell Biology, ISU.
Yin, Yanhai. Associate Professor, Dept. of Genetics, Development, & Cell Biology, ISU.
Yu, Jianming. Professor and Pioneer Distinguished Chair in Maize Breeding, Dept. of Agronomy, ISU.
Brendel, Volker. Prof. of Biology and Computer Sci., Dept. Biol. & School of Informatics and Computing, Indiana U.
Huber, Steven. USDA-ARS Plant Physiologist and Professor, Depts. of Plant Biology & Crop Sciences, Univ. of Illinois.
Ladunga, Istvan. Professor, Dept. of Statistics, University of Nebraska-Lincoln.
Weeks, Donald. Maxcy Professor of Agriculture & Natural Resources, Dept. of Bioch., Univ. of Nebraska-Lincoln.

Therefore, the mission of the CBC is to:

> *deploy innovative, transformative genome-engineering technologies that identify, validate, and rapidly, but precisely, integrate strategically important traits and underlying genes into key crop plants.*

In practical terms, this means that the CBC will:

- Develop innovative, new technologies to enable and improve crop-genome engineering.
- Employ NBT genome-engineering approaches to enable basic research in plant biology, utilizing key crop plants, including maize, soybean, rice and sorghum, to facilitate identification of potentially beneficial genes and traits.
- Employ NBT genome-engineering approaches to incorporate and integrate potentially beneficial traits in important crop plants targeted by the CBC, maize, soybean, rice and sorghum, generating modified, improved lines (null segregants) containing no transgenes.
- Understand the regulatory, economic and societal implications of NBTs.

The core of the CBC comprises innovative genome-editing methods, including TALEN- (transcription activator-like effector nuclease) and CRISPR- (clustered regularly interspaced short palindromic repeats) based technologies, in building a platform for the identification and validation of strategically important plant genes/traits and for the subsequent rapid and precise integration of promising traits into important crop plants. These innovative technologies will accelerate both fundamental research that identifies genes controlling critical traits, and engineering of desired gene modifications that tests traits in crop plants. ISU has a catalog of attractive gene targets at various stages of verification, established capabilities for NBT-based genome editing, and the ability to transform target crops, all of which form the CBC framework. As illustrated in Figure 1, the CBC is establishing a platform comprising: active gene discovery and validation; incorporation of target gene modifications into crop plants using NBT approaches and novel delivery methods; trait verification and integration; and evaluation of regulatory, economic, environmental and societal impacts of the technology and the resulting traits.

Research at ISU and elsewhere has identified a surfeit of gene targets for crop improvement, including those influencing yield, photosynthesis, stress tolerance, and disease and pest resistance. The time is ripe to launch a public-sector infrastructure for rapid, precise crop bioengineering; ISU is ideally suited to lead this effort, and the CBC will serve this need. ISU has played key roles in development of the TALEN technology, one of the key approaches at the core of this endeavor, and it boasts international leadership in plant biology and genetics, plant transformation, plant breeding, crop production, crop-genome informatics, risk assessment, and agricultural economics. In addition to launching technology platforms, the CBC will establish four complementary, basic research foci to be addressed by the teams indicated in Figure 1:

- Core technology development and implementation;
- Gene/trait discovery;

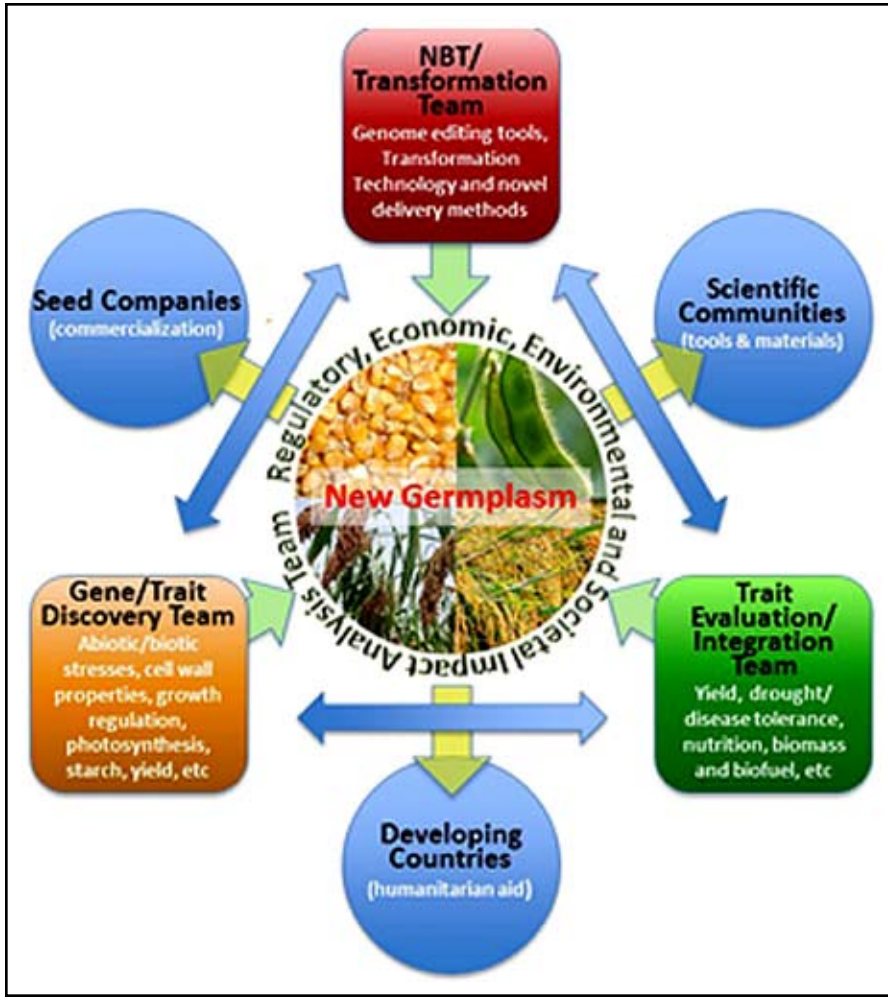

Figure 1. Outline of the CBC platform, illustrating the bidirectional interactions among the core research teams (gene/trait discovery, NBT/transformation, trait evaluation/integration, and regulatory, economic, environmental and societal impacts), the new germplasm developed and the beneficiaries of the germplasm.

- Trait verification and integration; and
- Regulatory, economic, environmental, and societal impacts.

Core-Technology Development and Implementation

The initial core enabling technology of the CBC was the highly innovative TALEN technique, based on the TAL effectors (TALEs) secreted by plant pathogenic *Xanthomonas* bacteria (Christian *et al.*, 2010; Li *et al.*, 2011; Voytas *et al.* 2011; Yang *et al.*, 2011; Bonus

et al., 2012; Joung and Sander, 2013; Wright *et al.*, 2014). TALEs bind to specific plant-DNA sequences and modify gene expression. TALE proteins use a simple recognition "code" to bind specific, target DNA in their host plant. Within the central region of the TALE protein are several repeats, each recognizing one of the four DNA nucleotides. This simple code can be used to engineer designer TALENs, which are fusion proteins comprising custom TALE DNA-binding domains and the DNA-cleavage domain of the endonuclease FokI, that have specificities for preselected DNA sequences. TALENs produce DNA double-strand breaks that lead to mutagenic insertions/deletions at the gene target sites or, in the presence of a donor DNA template, integration of new DNA at the gene-target sites by homologous recombination.

In addition to improving TALEN technology and demonstrating its application in targeted crop plants (see below), the CBC also has expanded its capabilities for genome engineering by incorporating the newer CRISPR/CAS9 technology and demonstrating its effectiveness in crop plants targeted by the CBC. The prokaryotic CRISPR/Cas9 system (Cong *et al.*, 2013; Mali *et al.*, 2013), in its simplest form, consists of only two genes: one encoding Cas9 and one encoding a "guide" RNA that enables Cas9 to identify and cleave a "target" DNA sequence. As with TALEN-based DNA breaks, Cas9-based breaks lead to either insertions/deletions at the target sites or homologous-recombination-based integration of sequences from a template. CRISPR/Cas9 has been functionally demonstrated in a number of eukaryotes, but had not been demonstrated in photosynthetic organisms, so the CBC adapted this new system for use in plants (Jiang *et al.*, 2013, 2014; Zhou *et al.*, 2014).

The CBC also is pursuing other novel, enabling technologies, including breakthrough protein-delivery technologies and software development, as well as developing and incorporating high-throughput processes across the whole pipeline from construct design and construction to identification of edited plants. Direct delivery of proteins into plant tissues has recently been demonstrated by CBC members (Martin-Ortigosa *et al.*, 2012, 2014). This innovative approach should enable delivery of genome-editing proteins rather than having to integrate transgenes encoding the editing proteins. This technology will completely bypass DNA (transgene) integration into the plant genome, while generating precisely modified and truly "non-transgenic" plants. It will also shorten the time from lab to field testing by avoiding the need to remove the editing transgene DNA from the engineered plants.

The ability to engineer genomes is extremely powerful, but the ability to engineer genomes in a high-throughput platform, to rapidly make large numbers of edits in large numbers of plants, greatly amplifies the power of the technology. The CBC is developing high-throughput processes for all stages of the genome-engineering pathway, beginning with development of software for the prediction of CRISPR-editing targets for any gene in a variety of genomes, including the ability to target one or more specific members of a highly similar gene family. This software is currently undergoing beta-testing by CBC members, but will be made publicly available on the CBC website (cropbioengineering.iastate.edu/). Efforts also are underway to improve the throughput for transformation and screening using a variety of process-automation approaches.

Enabling Basic Plant-Biology Research

A major strength of the CBC is the enabling of exploratory basic research to reveal the function of key plant genes, thus identifying potential targets for modification. Numerous, potentially beneficial candidate genes have been identified already by CBC members, and vast numbers of additional candidates can be identified from analyses of transcriptional profiling, proteomics, and genomics data. However, verification of any candidate gene function requires direct gene manipulation, as by using genome-editing technology to precisely inactivate or alter the expression of the candidate gene to reveal any resulting mutant phenotype.

The CBC has invested much of the past year demonstrating the efficacy of genome-editing technology in the target crop plants. Initial work using TALEN technology for genome editing in plants focused on rice (Li *et al.*, 2011, 2012, 2013). Proof-of-concept demonstration of TALEN function in maize and soybean has been successful, including the demonstration of including inheritance and segregation of edited genes in maize (Char *et al.*, 2015). Demonstration of genome editing has been expanded to include the CRISPR platform, which has proven highly efficient in rice (Kitaokaa *et al.*, 2014; Zhou *et al.*, 2014) and maize (CBC, unpublished).

Gene/Trait Discovery and Incorporation of Beneficial Traits

Once identified, desirable gene modifications can be rapidly, precisely and efficiently integrated into cultivars using genome-editing technology. For example, the use of TALEN technology by the Yang[2] lab to modify the regulatory region of a specific rice gene verified that the targeted regulatory sequences facilitated pathogenicity of the rice-blight pathogen; furthermore, disruption of this region conferred resistance to the rice-blight pathogen (Li *et al.*, 2012). We have disrupted a variety of specific genes in maize based on hypotheses that their disruption may, in some cases, increase growth or, in other cases, make beneficial changes in the properties of starch. Similarly, we hypothesized that disruption or modification of specific soybean genes involved in carbon assimilation will result in increased carbon assimilation and yield. We are testing this hypothesis using genome-editing technology to disrupt or modify the genes in question. Because these gene manipulations in maize and soybean are predicted to improve plant growth, yield or starch characteristics, verification of the hypothesis could lead to the integration of a valuable trait into these key crops.

Verification of predicted traits or phenotypes resulting from targeted genome modification is an essential part of the CBC mission, as is verifying that the integrated genome modifications are inherited in a simple, predictable manner. Because of the high precision of genome-editing technologies, once a predictable genomic location is altered, confirming the inheritance pattern of the modified locus is straightforward. Self-pollination of a heterozygous initial transformant or of a non-transgenic, but edited, plant will generate a segregating, second-generation population in which inheritance patterns can be verified

[1]See pp. 53–59.

by genotyping multiple individuals and any editing transgene locus can be eliminated. Moreover, phenotyping of the trait of interest in a segregating population will establish a genotype-phenotype relationship, if it exists, and confirm or refute the predicted phenotypic benefit.

The strategy developed by the CBC for commercialization of beneficial traits is based on partnering with the crop-biotechnology industry. The intellectual property landscape for genome-editing technologies is not clear at present, but as an academic entity, the CBC enjoys relative freedom of operation for academic research. Companies operating in the crop-biotechnology industry are likely to be licensed and pursuing use of NBT approaches for crop improvement. Crop-biotechnology companies are undoubtedly using genome-editing technologies to generate gene modifications predicted to produce potentially beneficial traits. However, many potentially beneficial traits either will not be predicted or will be judged as too risky for pursuit. Therefore, demonstrated and verified beneficial traits are expected to be of interest for further investigation and potential commercialization by industrial partners.

Regulatory, Economic, Environmental, and Societal Impacts

The possibility that plants modified by genome-editing technologies, *i.e.* using NBTs, may qualify as non-GMO is one of the most intriguing aspects of this new approach to crop improvement. If NBT approaches carry a dramatically lower regulatory burden, their advantages in bringing traits to market could be extraordinary. Public concerns regarding bioengineered crops and products have been acutely focused on the transgenic technologies used to produce them. Because NBT approaches result in non-transgenic products, it is plausible that they may carry less perceived and actual risk, and that regulatory concerns will be minimal.

The regulatory, economic, environmental and societal impacts team is charged with addressing the anticipated market potential, and economic sustainability of improved varieties developed using NBT approaches. This team also will examine (1) whether the public perceives NBT products to be *risky* either to human interests or to the environment, and (2) whether existing regulatory measures will apply to crop varieties produced with NBT approaches. This research examines existing regulatory regimes, as well as evaluates the reasons used to justify and defend them. Association with the CBC facilitates study of TALEN- and CRISPR-derived products including comparative risk analysis, study of perceived risk and the basis for perceived risk, and review of existing regulations.

The CBC is already a significant participant in the discussion of NBT regulatory issues, having hosted an international workshop, *Science and Opportunities in Using Site-Directed Mutagenesis for Plant and Animal Improvement*, November 4–5, 2013, and was invited to help plan NABC 26, *New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture*, hosted by Cornell University and the Boyce Thompson Institute.

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MARTIN SPALDING received a BS degree in both environmental science and biochemistry in 1974, and an MS in botany in 1976, from Washington State University. He received his PhD in plant physiology from the University of Wisconsin-Madison in 1979, was a postdoctoral associate at the University of Illinois-Urbana-Champaign until 1982 and was a visiting postdoctoral fellow at the Australian National University later that year. From 1982 to 1984, he was a postdoctoral associate and a research assistant professor in the US Department of Energy's Plant Research Laboratory at Michigan State University.

DR. SPALDING joined Iowa State University in 1984 as an assistant professor in the Department of Botany. He served as chair of the Interdepartmental Plant Physiology program at ISU from 1992 until 2000. In July 2003, he assumed the position of chair of the newly formed Department of Genetics, Development and Cell Biology, and served as chair of the Department of GDCB until July 2011, when he became interim associate dean for research and graduate studies in the College of Liberal Arts and Sciences, and formally accepted the position of associate dean in January 2013. Spalding also became the director of the ISU Crop Bioengineering Consortium in July 2013.

Plenary Session 2–Uses

Q&A

Moderator: Alan Collmer
Cornell University
Ithaca, New York

Steve Pueppke (Michigan State University): This is a deep background question, and I'll be interested to have the panelists' thoughts about it. It's a question of balance, between giving people what they need in terms of food and giving them what they want. And how do we balance the use of technologies with other applications, other things we can do to provide food. What got me thinking about this was that almost every speaker today has shown the same slide, which is the increase in the global population and our obligation to feed all those new mouths. I know that our focus today is on how we may use wonderful technology to do that. Some other people suggest alternative approaches, such as reducing food waste. The numbers I have seen indicate that we could feed another 4 billion people if we could figure out how not to waste the food we produce today. Another issue is whether we grow vegetables and fruits that people eat directly as food or use the land to grow crops for other uses. How do we balance these complexities as scientists and as a society?

Martin Spalding: I can say something about that, although I can't really answer the question. I don't disagree that eliminating food waste is something that we ought to focus some effort on. And there's a valid discussion to have about how much energy should go into various agricultural products. Our group plays a small role in this—to make it possible to produce more food—but I don't think we can have much impact on those other two processes.

Gary Rudgers: Part of that, too, is that there is no single solution. There's a number of solutions, including irrigation, pesticide and farming-equipment availability, along with improved breeding techniques. There are many small parts to the big picture and how each plays in the big picture is difficult to say. Part of it is needing to identify the other solutions, bring them all together and try to more efficiently use technologies.

Le Ann Blomberg: Opportunities for improvements in developed countries are not necessarily applicable to developing countries. Where food is wasted, would it otherwise be possible to transport it to developing countries? What can we do to improve conditions where people need it most? We need to focus on how to address their needs in their environments. It's a complex issue. Part of it is educational in terms of sharing science across the world.

Heather Shearer (Canadian Food Inspection Agency): This question is for Gary. Many speakers have referred to the high cost of preparing a data package for a regulatory submission. However, I think we can agree that a lot of these data would be collected anyway during the course of developing a new submission—molecular characterization, verifying that the plant has desirable agronomics, and so on. I am interested in your perspective on what portions of the data packages may be excessive, or where there might be opportunities to be more efficient.

Rudgers: It depends whether you are looking at mutations versus addition of genes. Part of my concern—especially with mutations—is harmonization in terms of what regulatory agencies are looking for. I am concerned that one country will ask for one type of data, another country will require other types of data and another country will ask for different types of data and the data needs increase over time. When it comes to mutational products, clearly what needs to be done is to put them into the perspective of natural mutations in environments. I don't know if there's going to be a way of having no regulatory oversight, but I would hope that if there is some type of regulatory oversight of mutational products developed with these technologies that they be harmonious and that the data are very basic to address questions of product and not process. It concerns me that countries are trying to regulate the process because it's not the process that results in any safety hazards. The evaluation really needs to be based on the safety of the product and if no risk is attendant on the product there should be little to no regulatory oversight. When it comes to gene addition, hopefully by introducing a trait at a precise location on the genome you can address some regulatory questions upfront. Salient issues are where the gene is located and some of the molecular analysis is straightforward. It depends on the country you are approaching what regulatory data you need. There seems to be general agreement among regulatory agencies internationally that regulatory oversight should be reduced, but no one has proposed what the necessary data might be. I would hope to change the trend of requirement of more and more data and reduce the amount of data required.

Audience Member: Gary, you mentioned CropLife International. Should the crop side and the animal side be working together on CropLife International's mission?

Rudgers: Yes and no. Yes, we should be working together. I think that the regulation of any of these technologies will have impact on the others, whether with animals or with plants. But, they fall into different regulations in different countries and their oversight is very different. I don't know if CropLife International is part of CropLife America. I don't know if there's a similar type of international organization for animals. But, I do feel that, in a sense, we are on the same page of understanding of how these products should be viewed and potentially regulated or not regulated. But, I don't think that any great conversation is going on between the two.

Perry Hackett (University of Minnesota): Gary, something you said earlier—because you use oligos, and they can go almost anywhere, that's considered random mutagenesis and not falling under some of the regulatory guidelines it sounds like. I am thinking, "Why in the world are we breaking our necks to be specific, when what I should be claiming is that we have off-targeting—God know where the off-targeting is—therefore, it's at least semi-random mutagenesis, and don't bother us anymore!"

Rudgers: I agree. An agency asked, "Why do you even come to us if you have mutations and they aren't regulated"? The other problem is, I can guarantee that if, say, Dow Agrosciences generated a mutation in a crop plant and made it available to the public, and Greenpeace found out about it, it would be viewed as a gigantic hazard. It's something that needs to be addressed by the agencies to provide regulatory clarity. But, it is a question of, "Why are we even talking about this?" Then the question becomes, "Do you consider that oligo use should be a regulated technique?", and that's where we seek clarity. At the same time that raises the concern, "Now, do we regulate mutations?"

Ralph Hardy (North American Agricultural Biotechnology Council): We've heard of a number of targets on the animal side, and of a number of targets on the plant side. Some are novel. Many we have heard about in the past. What about the microbial side? Is there opportunity here to do some things? Let's take nitrogen fixation. Problems prevail—high cost of fertilizer input, inefficient use of fertilizer, and so on. There may be bigger impacts on the microbial side of agriculture than on the animal and crop sides we talked about previously.

Spalding: One of my areas of particular interest is algal biotechnology. These technologies could have a big impact on the ability to manipulate eukaryotic algae with regard to their products, their growth potential and any number of opportunities in that arena. The field is largely in its infancy in terms of ability to modify algal genomes, especially in production-type algae. These techniques may facilitate entry into that arena and make a big splash.

Adam Bogdanove (Cornell University): Le Ann, when it comes to genome editing with animals, there's an additional "layer" to regulatory considerations—which you touched on—and that is animal welfare and the ethics of the targets you go after. I was intrigued to hear that some of the target-selection criteria are to improve animal welfare, bringing in favorable traits without dragging along those unfavorable traits that may cause undue suffering. Can you expand on that topic? I don't have a well formed question, but one thing that occurs to me is designer pets. What would the American Kennel Association think about fast-tracking new breeds, such as German Shepherds free of hip dysplasia?

Blomberg: From my own perception, activists don't want certain things to happen, but, on the other hand, it really comes down to the perception of their eating a GMO and that creates another problem. The need is to educate the public and let them know exactly what is being done: is it natural or is it unnatural? What we are trying to address—which I can't talk about as it's in association with a collaborator—I think would be acceptable to industry, but we have to see how it plays out with the public. It's a touchy subject because of people's perception of Frankenfoods, but we need to help people understand what we are trying to do, and the attendant long-term benefits.

Reuben Tayengwa (Washington State University): I am curious as to the reactions of Greenpeace and other anti-GMO people to these new technologies.

Rudgers: On the plant side, it has been relatively quiet, which is good. A group in France has stated that they are against any mutations, implying that we can't eat anything. As products get closer to market there is more conversation about the new technologies, but the objections are not as loud as I anticipated them to be. We have enough time to educate the public. Greenpeace has been good at scaring the public about such products; they can say anything without proof and it's believed. However, on our side—the scientific side—we have a more difficult time relating the science and conveying the importance of science and the role it plays in food. As said, we still have the opportunity to do that, but it's just a matter of time before we are confronted by NGOs.

Alan Collmer: The public's problem lies in dealing rationally with risk versus benefit—every time we get in a plane, a car or an elevator, there it is: a potential risk. But we freely use these vehicles because of the vast benefits. I am wondering if we can shift the public discussion to traits and the benefits that are close to being delivered, particularly with focus on those that are consumer-related, involving improved nutrition and other health benefits.

Rudgers: That's a good point. Recently, while in New Zealand, a government representative asked me, "When will you market a product that's beneficial to the public?" I said that this has been bounded by Greenpeace and individuals who have protested it for years, and despite tremendous potential benefits there hasn't be much success. I agree that we do need to talk about such benefits; it's something that the public will need to accept the technology.

William Serson (University of Kentucky): Do you see the possibility of less opposition from NGOs for the use of these technologies with biofuel crops, which circumvent the "Frankenfood" issue? There has been less resistance to *Bt* cotton because it isn't consumed.

Rudgers: Some say that growing crops that aren't to be consumed is a waste of land, but, yes, there is less opposition to genetically engineered biofuel crops.

Patrick Di Bello (University of Arkansas): Regarding manipulating genes to benefit human nutrition or the environment, how do you sell those to the farmer? Can he market them as specialty crops?

Rudgers: The farmer will grow what will make money. If there is no potential to make money, she or he won't grow it. So, the consumer has to educate the farmer by building demand and including the farmer as part of the process.

Hackett: I want to address two points. Number one, I'll repeat, you can't educate the public. That's what academics talk about—that's what we are all about. We don't do it—deal with that. The second aspect is that a massive experiment has been run here in the United States, wherein a few people stood up to order the bullying and intimidation that we are talking about here. It has to do, actually, with transgenic animals that are on sale in forty-nine out of fifty states; they're called GloFish®. You can't buy then in California because they are genetically engineered pets, and you know how Californians feel about their pets. Apparently, they saved the fresh-water aquarium industry. Walmart, PETSMART, PETCO, these giant chains, wouldn't touch them for the first year or year and a half because they didn't want Greenpeace and others protesting outside their doors. No one protested. You can buy these fish in Walmart, PETSMART and PETCO. It wasn't a big thing. It turned out that these animals had something to offer the consumer who would buy them. They had something to offer the people who were selling them. If you have a competitive product, it will sell. People won't come out and protest; they may buy it.

Hardy: Regarding benefits to consumers—NABC's conference in 2013, on fruits and vegetables, focused in on three or four examples of consumer benefits. The non-browning Arctic® apple was one of those and it seemed to be moving fairly quickly through the regulatory system; it's out for comment at the moment. Another example was *Bt* sweet corn, which doesn't require spraying with insecticide. Simplot's Innate™ potato technology is another good story, as is the means of tackling citrus-greening disease. Will US consumers want orange juice from Brazil, produced without much oversight or from the United States, free of citrus greening thanks to genetic engineering? NABC produced a brief white paper on these examples[1].

[1]http://nabc.cals.cornell.edu/Publications/WhitePapers/SpecialtyCrops.pdf.

Can Opportunities Lost Be Regained? Reframing Genetic Engineering for Crop and Livestock Improvement

Jeffrey D. Wolt
Iowa State University
Ames, Iowa

jdwolt@iastate.edu

The rapid and continuing global adoption of modern agricultural biotechnology has been encumbered by steadily increasing public anxiety. Scientists and regulators continually point to the weight of evidence showing that genetically-engineered (GE) crops pose negligible risks to human health and the environment. Interestingly, this very argument may lead to the counterintuitive result of the public viewing the technology as unsafe. Unraveling this quandary requires consideration of the relationship of risk to safety, the effects of public questions on regulatory processes, and the problem of risk stigma. And critically, we must ask if the correct messages have been framed and the appropriate messengers tasked to communicate the key importance of modern biotechnology to a safe, secure and sustainable food supply. As we look to the special opportunities afforded by new breeding technologies, especially new DNA-editing approaches, it is essential to identify and understand where opportunities for effectively addressing modern biotechnology have been lost in dealing with transgenic crops. And we must ask whether there is the ability to reframe genetic engineering using these new technologies in agriculture in a way that more effectively connects with the public.

Judgment of Food Safety

Despite nearly two decades of safe use worldwide, large segments of the public continue to express concerns regarding foods derived from modern biotechnology. As Europeans became aware of GE-derived foods late in the 1990s, their initial concern that they were "risky" rapidly declined (Gaskell *et al.*, 2006), but the judgment that they are unsafe remains (Gaskell *et al.*, 2010). During this same period, US consumers became increasingly aware of the reality of GE products in the food supply. Concerns about these products have increased to the point where, today, significant percentages of Europeans and Americans alike view GE-derived foods as unsafe (Hallman *et al.*, 2003; Bonny, 2008; Gaskell *et al.*, 2010; Langer, 2014). Consumer concerns relate less to the risks of these foods, which are broadly recognized as very low, and rather to uncertainties regarding the nature of the risks they may pose. Since risk represents the probability of an unwanted outcome as balanced against safety, which reflects willingness to accept a given level of risk (Lowrance, 1976), uncertainties in the nature of the risk lead many to deem these products as unsafe.

Consumer Anxiety

Biosafety specialists continue to answer concerns regarding transgenic crops and derived foods by pointing to the weight of evidence showing negligible risk (European Commission, 2010; Nicolia *et al.*, 2013) as well as to the elaborate regulatory processes that have been established to evaluate GE crops prior to their release to the marketplace [see, for instance, the history and description of the US coordinated framework (McHughen and Smyth, 2008)]. The complexity of current regulatory systems and the continuing questioning of regulatory processes and decisions have led to delays in decision-making (Smyth *et al.*, 2014). Complex and prolonged regulatory assessments create public uncertainty surrounding what are deemed very low probability risks. Further uncertainty is engendered by activists who challenge scientific consensus and regulatory findings based on the association of biosafety information and specialists with industry [see, for instance, Bauer-Panskus and Then (2014)]. Additionally, these technology opponents use words and images to great semiotic effect to create risk stigma (Slovic *et al.*, 2001). The uncertainty represented in regulatory complexity and delayed decision-making, in addition to the stigma of GE-derived foods generated in public debate, stimulates consumer anxiety.

Scenarios of very-low-probability risk with uncertainty create anxiety for individuals, which economists describe in terms of second order risk (Seo, 2009). Under such conditions, there is a strong tendency to reject low-probability risk with uncertainty in favor of an outcome with greater certainty. This has been shown for food choices where the consumer will pay more to avoid uncertainties surrounding what are very-low-probability risks (Kivi and Shogren, 2010) and appears to be the situation with regard to public attitudes toward foods derived from GE crops. Increasingly complex regulatory processes and delays in decision-making feed into uncertainties regarding the very-low-probability risks associated with GE-derived foods. Avoiding the anxiety due to the uncertainties surrounding risk leads to a willingness to consider a low-probability risk as unsafe. The desire to avoid uncertainties is reflected in actions such as support for labelling of GE-derived foods.

Window of Opportunity

Consumer preferences for unambiguous food choices are best addressed by information that reduces ambiguity (Kivi and Shogren, 2010) and so effective public communication to build knowledge and trust should lead to more well-reasoned judgments as to safety. As we encounter new breeding technologies, especially site-directed gene-editing techniques, there would appear to be a window of opportunity to reframe public understanding of genetic engineering in agriculture to reduce ambiguity in individual choice. Key to this will be the presence of trusted sources of information.

One upshot of modern agricultural biotechnology and the questions it has engendered is the establishment of a large cadre of biosafety specialists in government, industry and, to a smaller degree, in the public sphere. As scientists, regulators and risk assessors, these individuals evaluate the risks of GE crops and derived foods. Meeting at venues throughout the world to discuss the sad state of public opinion and the regulatory process, these experts continue developing more nuanced approaches for risk assessments and are training an ever-expanding universe of biosafety specialists. It is largely this body of expertise that we call upon to communicate the risks and safety of products of modern biotechnology, but the very presence of these specialists and the growth of their discipline suggests to the public that uncertain consequences of genetic engineering are worthy of concern.

What are lost here are spokespersons who can counter this risk-focused view of modern agricultural biotechnology and who can communicate, not only in words but through their actions, the benefits to food safety, security and sustainability. Much has been said about the decline in public-sector plant breeders and the shift in agriculture to more fundamental research (*e.g.*, Thirtle *et al.*, 2001). Much of this occurred late in the 20th century and has been linked to the rise in genetic engineering (Murphy, 2007). What bears repeating is the key importance of public-sector agricultural scientists as recognized experts connecting genetic engineering to food production and the public good (Thro, 2003). As public-sector plant breeders have become fewer in number and the emphasis of agricultural scientists has shifted to more fundamental considerations, consumers have lost site of the relationship of the research and development enterprise—both public and private—to food sustenance and sustainability. With limited public voices attesting to benefits of modern agricultural biotechnology balanced against their costs, we are left with polarized views that argue points of risk and uncertainty that leave the public increasingly anxious. Offsetting this anxiety requires the cultivation of public-sector agricultural scientists who, by virtue of the work they do, can be trusted voices in communicating the practice of genetic engineering as a key benefit to our modern food-productions systems.

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JEFFREY WOLT is a risk analyst with Iowa State University where he specializes in research, teaching, and outreach activities relating to risks and benefits of modern plant agriculture. He obtained his doctorate at Auburn University and spent his early career on the faculty of the University of Tennessee and in industry. At ISU, he is a member of the faculties of agronomy, toxicology and environmental sciences, and he maintains an adjunct appointment in epidemiology at the University of Iowa.

DR. WOLT has extensive experience in global and national risk assessment and issues management in relation to the commercialization and use of genetically engineered crops. His diverse research interests encompass environmental fate and effects, food safety and non-target risk assessment in relation to crop production and use. Also, he is interested in globally harmonized principles for biotechnology-risk assessment. Wolt currently serves on the US National Academy of Sciences committee to evaluate pesticide risk-assessment methods for the state of California. He is a fellow of the American Society of Agronomy and the author of the text *Soil Solution Chemistry.*

Regulatory Paradigms for Modern Breeding

Drew L. Kershen[1]
[1]*University of Oklahoma*
Norman, Oklahoma

Wayne A. Parrott[2]
[2]*University of Georgia*
Athens, Georgia

dkershen@ou.edu

Modern breeding, based upon molecular biology using genetic information, has made rapid advances. Breeders using rDNA techniques can properly think of this technique as traditional biotechnology. Within the past ten years, breeders have begun to use newer techniques [site-directed nuclease techniques (SDNs), RNAi, and synthetic biology] to create and to develop plants and animals with desired genetic traits.

Traditional breeding, whether by farmers or by scientists, has been either unregulated or lightly regulated, primarily to assure seed purity and efficacy. The rDNA techniques have been carefully regulated domestically and internationally. The regulatory classifications of the newer techniques of the past ten years are still in debate and have much uncertainty.

In this chapter, the authors address the question: What is an appropriate regulatory paradigm for modern breeding?

At the Beginning

Questions about the appropriate regulatory paradigm for modern breeding emerged concurrently with the breeding techniques themselves—specifically rDNA breeding. It is very helpful and instructive to read anew the conclusions reached at the beginning of rDNA breeding.

The US National Academy of Science (NAS, 1987) concluded: "There is no evidence that unique hazards exist either in the use of R-DNA techniques or in the movement of genes between unrelated organisms." And further, "Assessment of the risks of introducing R-DNA engineered organisms into the environment should be based on the nature of the organism and the environment into which it is introduced, not on the method by which it was produced."

The US Office of Science and Technology Policy (OSTP, 1992) wrote, "Exercise of oversight in the scope of discretion afforded by statute should be based on the risk posed by the introduction and should not turn on the fact that an organism has been modified by a particular process or technique. ... [O]versight will be exercised only where the risk posed by the introduction is unreasonable, that is, when the value of the reduction in risk obtained by additional oversight is greater than the cost thereby imposed."

Similarly, the Organisation for Economic Co-Operation and Development (OECD, 1986) recommended:

> *2. There is no scientific basis for specific legislation for the implementation of rDNA techniques and applications. Member countries should examine their existing oversight and review mechanisms to ensure that adequate review and control may be applied while avoiding any undue burdens that may hamper technological development in this field.*
>
> *3. Any approach to implementing guidelines should not impede future developments in rDNA techniques. International harmonization should recognise this need.*
>
> *6. .... For certain industrial applications and for environmental and agricultural applications of rDNA organisms, countries may wish to have a notification scheme.*

The regulatory paradigm recommended in the three quoted documents urged a focus on the organism (product) and not the process of breeding, while understanding that the empirical evidence does not show any unique hazards, and proposed the use of general legislation with the OECD suggesting an amendment limited to requiring a notification scheme. It is evident that NAS, OSTP, and OECD concluded that modern breeding should be regulated like traditional breeding—*i.e.* unregulated or lightly regulated.

The Regulatory Reality

The United States did not adopt biotechnology-specific legislation. Rather, the US government developed a coordinated framework (OSTP, 1986) allowing the three primary administrative agencies [Department of Agriculture (USDA), Environmental Protection Agency (EPA), and Food and Drug Administration (FDA)] to develop policies under existing statutory authorities about regulating rDNA techniques.

The USDA Animal Plant Health Inspection Service (APHIS) created a category called a "regulated article" under the Plant Protection Act. EPA created a category called a "plant-incorporated protectant" (PIP) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). FDA created a voluntary consultation process for foods derived from biotechnology and later declared that all animals derived from biotechnology are "new drugs" using the Federal Food Drug & Cosmetic Act (FDA, 2009). What each of these regulatory approaches have in common is that these newly created categories captured rDNA breeding as the trigger for extensive regulation. Extensive regulation means an application process requiring much data and regulatory filings, public comments and hearings, and prior approval (permission) from the agency before any biotechnological

product can enter the market. No other breeding techniques (or their crop and animal offspring) face anything close to this level of regulatory scrutiny[1].

Beginning in 1990, the European Union (EU) enacted a series of biotechnology-specific directives and regulations. In 1998, after the "mad-cow" events, the EU undertook revision of the 1990 laws, culminating in three of particular applicability to agricultural biotechnology: directive 2001/18/EC (on the deliberate release into the environment of GMOs), regulation 1829/2003 (on GM food and feed), and regulation 1830/2003 (on the traceability and labeling of GMOs and the traceability of food and feed products produced from GMOs). The EU directives and regulations focused specifically on the process by which a product came into existence and enmeshed these processes and products in extensive risk analyses related to health and environmental concerns (EU, 2013).

New Zealand enacted biotechnology-specific legislation titled "The Hazardous Substances and New Organisms (HSNO) Act of 1996." By associating biotechnology with hazardous substances, New Zealand created extensive regulatory scrutiny just for agricultural biotechnology.

At the international level, 168 countries have ratified the Cartagena Protocol on Biosafety governing the transboundary movement of "living modified organisms" from "modern biotechnology." Although the Cartagena Protocol is a very complicated and intricate document, it is fair to say that it created and continues to create extensive regulatory controls over the international trade in crops and animals from agricultural biotechnology.

The EU, New Zealand, and Cartagena Protocol all share several features: a focus on process, not product; detailed and rigorous risk analyses without requiring evidence of any unreasonable risks or unique hazards; prior approval before commercial use that is subject to political influences; and a plodding decision-making process. Moreover, each of these three regulatory approaches explicitly adopts a precautionary stance towards agricultural biotechnology, implicitly communicating through laws or regulations that society should "[b]e afraid, be very afraid of agricultural biotechnology."

Surveying the regulatory reality, it becomes clear that agricultural biotechnology faces extensive regulation wherever created or located. Governments and agencies around the world have not adopted the regulatory paradigm recommended by NAS, OSTP, and OECD. Just the opposite, governments and agencies have adopted a regulatory paradigm that expressly and purposefully burdens agricultural biotechnology. In light of these regulatory burdens, one can be amazed that a few products of agricultural biotechnology have achieved the level of adoption that the International Service for the Acquisition of Agri-Biotech Applications annually reports (ISAAA, 2014).

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[1]The FDA voluntary consultation process uses the concept of "comparative safety analysis," sometimes known as substantial equivalence, whereby foods derived from biotechnology considered substantially equivalent to comparable conventional foods, in that they lack any novel substances that can cause harm, can enter the market without FDA prior approval. No developer of biotech-food products has been sufficiently courageous or foolhardy to interpret the "voluntary" consultation process as embodying the phrase, "It is better to ask forgiveness than permission." The FDA voluntary consultation process has verified the safety of food, and FDA has responded in a timely manner without excessive burdens or barriers to foods from crop biotechnology.

Past and Present Impact of the Regulatory Reality

What has been the impact of these excessive and stigmatizing regulations upon agricultural biotechnology?

- USDA-APHIS has approved 96 petitions for non-regulated status. Farmers have adopted these approved (and improved) traits immediately and widely. For corn, soybean, canola, sugarbeet, and cotton, farmers have adopted GM varieties at 90 percent or above of acres planted (Fernandez-Cornejo, 2014). Yet, USDA-APHIS now takes nearly 5 years to make a decision with a regulatory cost per trait up to $34 million.

  The impact of this cumbersome, expensive, and labor-intensive process has been stultifying to research and competition. Despite many successful and needed crop transformations by public-sector scientists, only one public-sector crop has ever achieved regulatory approval and commercial release—the virus-resistant papaya for Hawaii. APHIS has also approved a USDA-ARS virus-resistant plum, but EPA pesticide-labeling requirements have prevented its commercial release.
- In 1997, EPA defined genetically-modified microorganisms (GMMs) as regulated "new chemicals" under the Toxic Substances Control Act (TSCA). Since then, EPA has approved one GMM for commercial use.
- FDA has not approved a single commercial release of animal agricultural biotechnology. In possibly the most egregious example, a genetically-engineered fast-growing salmon from AquaBounty, using a Pacific (Chinook) salmon gene transferred to an Atlantic salmon, has been in the regulatory system for almost 20 years with costs above $78 million. FDA has fully cleared the salmon as safe for food and having no, or minimal, risk to the environment. Despite the findings, FDA has not issued a decision, giving rise to a petition letter, initiated by scientists, bemoaning the delay, cost, and suspected political interference (Letter, 2014).
- The EU, in 25 years of its biotechnology-specific regulatory system, has approved only two traits for commercial release to European farmers. Several dozen crop traits have been approved for import as food and feed, but its own farmers cannot grow what farmers in other countries grow and supply to the EU.
- New Zealand has approved a few confined field trials of genetically-modified traits for plants and animals. However, New Zealand has not approved any broad-scale field trials and has never even considered a petition to approve the commercial release of an agricultural trait derived from biotechnology.
- For most countries signatory to the Cartagena Protocol, the Protocol has proven to be an almost impassable barrier to the growing of genetically-modified crops and animals. The most troubling example of the Protocol's impact has been on Golden Rice, engineered to have precursor beta-carotene, a biofortified public

good to reduce blindness and death from vitamin-A deficiency. Ingo Potrykus, a co-inventor and donor of Golden Rice for humanitarian use, has written clearly and passionately about this regulatory blockage (Potrykus, 2012, 2013).

Forecasting the Future Impact of Regulatory Regimes

The forecasted impact of the present regulatory systems on future agricultural biotechnology ranges from cloudy to devastating. These pessimistic forecasts arise from the fact that newer techniques of molecular breeding—SDNs (such as MNs, ZFNs, TALENs and CRISPRs-Cas9), RNAi, and synthetic biology—have not been classified clearly and explicitly as subject to the existing regulatory regimes or not subject. Uncertainty about regulatory classification serves as a disincentive to engage in research and development and an even greater disincentive to investment in these techniques for commercial release (Smyth, 2014).

The authors have previously analyzed the specific definitions and provisions of regulatory schemes (except for New Zealand) in an attempt to make informed predictions about the application of present regulations to these newer breeding techniques (Kershen and Parrott, 2013). We now present a brief summary of this legal analysis.

USDA-APHIS regulates biotechnology through the Plant Protection Act. Consequently, APHIS focuses on whether the biotechnological technique involves the use of a plant pest at any stage of the genetic engineering. APHIS allows developers to query whether a particular engineered plant is or is not regulated (USDA-APHIS, 2014).

As of September 2014, APHIS has not been queried specifically about TALENS and CRISPRs-Cas9. However, in two letters—one on ZFN-1 and one on MN-1 breeding—APHIS stated that such plants were not subject to regulation because the techniques did not involve use of any plant pest at any stage. In these two letters, APHIS cautioned that SDN-2 and SDN-3 techniques would be dealt with on a situation-by-situation basis.

APHIS also responded to two letters about the Bioglow plants from synthetic biology, concluding that they are outside its regulatory authority because the glowing plants did not involve any plant pest at any stage of their engineering.

Finally, APHIS affirmed that null segregant plants (*i.e.* offspring plants, in which the plant-pest element used to engineer the parent plant has been removed through conventional breeding) are outside its regulatory authority.

In light of these responses to letters of inquiry, USDA-APHIS appears poised to declare many—but not all—plants developed by the newer breeding techniques to be beyond its regulatory authority.

EPA uses FIFRA to regulate plants with traits inserted for the purpose of "preventing, destroying, repelling or mitigating any pest." EPA has asserted FIFRA authority over an RNAi plant created to be virus resistant. Moreover, in a recent scientific advisory panel (SAP) report, the SAP took a very precautionary approach to RNAi breeding and an affirmative view of the need for EPA to assert regulatory authority through FIFRA, including using the FIFRA term "plant regulator" to expand its regulatory reach (FIFRA SAP, 2014).

FDA has asserted that all genetically modified animals are "new animal drugs." FDA appears likely to assert that it will consider any animal modified by these newer breeding techniques also to be "new animal drugs," entailing extensive pre-market scrutiny and approval. FDA's likely regulatory stance is evident in its claim that a "polled" Holstein (dairy) cow, using the "polled gene" from the Angus (beef) breed, created by TALENs, is a "new animal drug" (Regalado, 2014).

As for synthetic biology, the J. Craig Venter Institute released a report in May 2014 setting forth options for regulatory approaches. The minimum option presented was to apply the present regulatory system for rDNA breeding to synthetic biology. All other options proposed enhanced agency power and regulatory scrutiny. The basic message of this Venter report was that nothing in synthetic biology should avoid regulation. Extensive regulation was the default approach (J. Craig Venter Institute, 2014).

The EU has not officially discussed how its extensive regulatory regime on rDNA breeding applies to newer breeding techniques. However, there are several reasons to believe that the EU regulatory system will capture all newer breeding techniques. First, the EU uses a "precautionary principle" as its underlying attitude towards molecular breeding. Extensive regulation is the preferred and default approach. Second, the EU regulations focus specifically on the process, and expressly exempt listed techniques from regulation. The implication appears to be that those not expressly exempted are within the coverage of the regulatory regime. Third, the European Food Safety Authority (EFSA) has opined that the SDN-3 technique does not differ from rDNA breeding (EFSA, 2012). Fourth, the EU regime also covers products of covered techniques, meaning null-segregant plants also would be regulated (EU Working Group, 2013).

In New Zealand, the Environmental Protection Agency (NZ-EPA) decided that ZFN-1 and comparable TALEN techniques were outside the regulatory reach of the HSNO law. The New Zealand Sustainability Council challenged the NZ-EPA decision in a lawsuit. In an opinion issued in May 2014, the High Court (trial court/first level court) of Wellington agreed with the Sustainability Council. The High Court interpreted the statutory list of exempt techniques as exhaustive. As ZFN-1 and TALEN techniques were not expressly exempted, the High Court ruled the HSNO law applied. The High Court stated that the precautionary principle colored its interpretive analysis and opined that the New Zealand Parliament should be the governmental authority to exempt these techniques, not an administrative agency, if doing so is deemed socially desirable (NZ-High Court, 2014).

The Cartagena Protocol on Biosafety has provisions and language that closely resemble the EU and New Zealand regulatory regimes. Thus, it can be predicted that the Cartagena Protocol too is very likely to cover newer breeding techniques as regulated technologies. Moreover, groups antagonistic to rDNA breeding have launched a campaign against the newer breeding techniques, especially synthetic biology, similar to their campaign against rDNA breeding. These groups call for a moratorium on newer breeding techniques until their demands for extensive and stifling regulations exist at all levels of governance—local, federal, and international (FOE, 2013).

Regulatory Paradigms—Proposed Regulatory Reforms

In the United States, rDNA agricultural biotechnology has moved forward in farmers' fields, but at a slow, costly, and halting pace and has not come close to fulfilling its potential mainly because of the impact of excessive regulatory regimes. Despite 30 years of experiences evidencing that agricultural biotechnology has verified the favorable conclusions of NAS, OSTP, and OECD, the US regulatory system has not responded to this real-world evidence of benefits without novel harms. The US regulatory system could be improved through several efforts within the power of the regulatory agencies such as:

- adopting categorical exclusions for those traits that have been already reviewed and proven safe and beneficial;
- focusing anew on product, not process, and on identified unreasonable risk, not imaginable hazards, so as to regulate a particular genetically-engineered crop or animal only if there is a scientific need, not just a default for regulation based on technique used;
- exercising agency discretion to decline invoking new terms and new definitions that expand regulatory power;
- creating a culture of facilitating innovation, science and technology in agriculture to meet the challenges agriculture faces from population growth and climate change.

As for Europe, New Zealand, and the Cartagena Protocol, it is worth quoting from a 2013 report issued by the United Kingdom Advisory Committee on Releases into the Environment (ACRE):

> *Our understanding of genomes does not support a process-based approach to regulation. The continuing adoption of this approach has led to, and will increasingly lead to, problems. This includes problems of consistency,* i.e. *regulating organisms produced by some techniques and not others irrespective of their capacity to cause environmental harm.*
>
> *Our conclusion, that the EU's regulatory approach is not fit for purpose for organisms generated by new technologies, also applies to transgenic organisms produced by 'traditional' GM technology. … the potential for inconsistency is inherent because they may be phenotypically identical to organisms that are not regulated* (ACRE, 2013).

The ACRE report provides a bookend that reconfirms what NAS, OSTP, and OECD stated at the beginning. Agricultural biotechnology does not present unique hazards. Regulation should be about the product, not the process. Oversight should occur when the risk posed is unreasonable. There is no need for biotechnology-specific regulatory regimes. Regulation should not hamper and burden scientific discovery and technological adoption.

At the root of a regulatory paradigm is an attitude. In the 1970s and early 1980s, molecular breeding was viewed as new and different from what occured in plants and

conventional breeding. From the genomic perspective, that "new and different" view has long been gone—since the late 1980s as the quoted reports of NAS, NRC, OSTP, and OECD evidence. But the regulatory attitude has remained unchanged.

Consequently, the present regulatory paradigm in the United States, the European Union, New Zealand, and the Cartagena Protocol is an attitude of mistrust of science and scientists and an unwarranted, unsubstantiated, and non-empirical aversion to agricultural biotechnology. This attitude is fueled and promoted by a protest industry that thrives by spreading misinformation, promoting scientific ignorance, and creating fear. Regulatory agencies should not be allies of misinformation, ignorance, and fear.

Regulatory agencies should return to the paradigm set forth at the beginning of the biotechnological era. Regulatory agencies should adopt a paradigm of confidence in science and scientists, and an openness to agricultural biotechnology rooted in the biological sciences and the favorable empirical results of biotechnology in farmers' fields, industrial enzymes, and medicines. Regulatory agencies must adopt this benevolent paradigm, not only for rDNA breeding but also for the newer breeding techniques. Without this paradigm change, rDNA breeding will continue to be impaired, rather than stimulated. Without this paradigm change, newer breeding techniques likely will be imprisoned in laboratories and exiled from agriculture.

Most importantly, the authors are profoundly concerned that, without a paradigm change, poor and vulnerable populations will not have access to genetic-engineering technologies to enable them to raise their standards of living, improve their health and protect their environments (PAS, 2009). For good or ill, regulatory paradigms matter in the real world.

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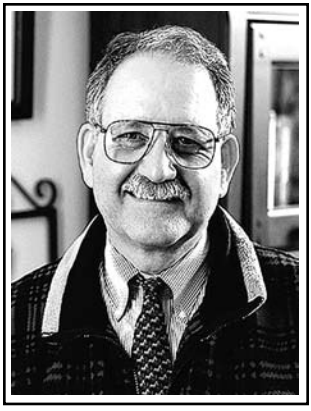

DREW KERSHEN is the Earl Sneed Centennial Professor of Law (Emeritus) at the University of Oklahoma. He has a BA from Notre Dame, a JD from the University of Texas, and an LL.M from Harvard. Professor Kershen taught at the University of Oklahoma from 1971 to 2012. He has served as a visiting professor at the University of Illinois Urbana-Champagne and the University of Kansas, and as a Fulbright teaching fellow to the Universidad José Cecilio del Valle in Tegucigalpa, Honduras.

In the past sixteen years, **DR. KERSHEN** has focused his teaching, writing, and speaking on agricultural biotechnology law and policy issues. He has written extensively on legal liability, intellectual property, international trade, and regulatory issues in agricultural biotechnology. He has been a speaker at biotechnology conferences in many nations and has participated in international negotiations concerning agricultural biotechnology.

He is admitted to the Oklahoma Bar and the Bar of the United States Court for the Western District of Oklahoma.

Consumer Issues Relating to Products from New DNA-Editing Techniques

Gregory Jaffe
Center for Science in the Public Interest
Washington, DC

gjaffe@cspinet.org

I run the Biotechnology Project[1,2] at the Center for Science in the Public Interest. I've been with CSPI for over 13 years, during which time the Biotechnology Project has issued a number of statements about genetically engineered (GE) crops and animals. CSPI has said that we have looked at the data behind the crops that are grown in the United States and that foods made from those crops are safe to eat. I don't know of too many consumer organizations in the United States or around the world that have said that, but this is what the scientific evidence shows. Also there are benefits from those crops, to farmers and to the environment, although not necessarily any direct benefits to consumers as of now. However, CSPI does advocate for each GE product to be assessed on a case-by-case basis. The Biotechnology Project works to make sure that we have functional regulatory systems that ensure safety but allow safe products to be marketed. Also, it is important that biotechnology products are used in a sustainable manner so that they are available to future generations of farmers.

Consumers

When you talk about food and consumers, the primary concern is safety. As a parent, I want to know that the food I choose at the supermarket is safe. But safety is not the only consideration that consumers have about food: it must also be healthy and nutritious, and taste is important. Also, food serves other objectives for many people. Cultural forces help define our interests in certain food. You tend to eat what you were brought up

[1]https://www.cspinet.org/biotech/.
[2]http://cspinet.org/images/biotechbrochure.pdf.

eating. Tradition is important. So too is religion. Food is used in many religious celebrations. There is a strong social aspect to food—we don't eat just for calories and nutrition. I mention these considerations because sometimes scientists think that they can rely simply on rational, factual, and scientific arguments when talking to consumers about food. Recently, I attended a meeting at which it was suggested that *in vitro*-cultured meat will be attractive to consumers in the future. Scientifically, it may be the best thing for us, but consumers may have reasons to avoid it. It's important to realize that consumers do not always make food choices that are rational or based on science. For example, a consumer may buy organic produce for health reasons, but also purchase a 6-pack of soda or a huge piece of beef.

I do think that consumers—some more than others—care about the impact their choices, including what they eat, may have on the environment. Some young people are expressing that they are choosing agriculture as a career for environmental reasons. But, when discussing the environment, it is important to appraise new technology in comparison with current practices in a comparative analysis, as opposed to a definitive determination of whether it is good or bad for the environment.

Some consumers know a lot of about science, whereas some don't know much at all. I mention this because, again, scientists and others involved in scientific careers sometimes think that, if they just talk about science more clearly, people will understand them better. Another consideration is that various sources of scientific and non-scientific information are available, notably from the Internet, government, and scientific institutions. Consumers also receive information from opinion leaders whose viewpoints they consider important. Those opinion leaders may be with NGOs or universities. They could be politicians or academically qualified neighbors. That is important to understand because a lot of people will rely on opinion leaders to tell them what they think about these new technologies.

For some consumers, if they strongly believe something, scientific data and reasoned argument may not change their minds. You can talk to them in-depth about the evidence but it may make no difference to them, especially if it addresses a particular food preference they have.

These are all things to think about as these new DNA-editing techniques move forward to produce products and as you communicate with the general public about them.

New and Old DNA-Manipulation Techniques

CSPI has stated that current GE plants are safe and beneficial. That is the international consensus among scientific institutions and government agencies who have looked closely at the issue. Those GE crops have been met with farmer acceptance worldwide, but not necessarily with consumer acceptance.

A couple of new GE crops are on the horizon. The Arctic® apple is non-browning and the Innate™ potato is also non-browning and has a low acrylamide content when fried. The developers of both of those products used "endogenous" DNA, which means they used the crops' own genetic material instead of creating a transgenic organism by using DNA from a different species. The developers believe that there will be much more of a positive acceptance by consumers due to this difference. However, when one looks at the

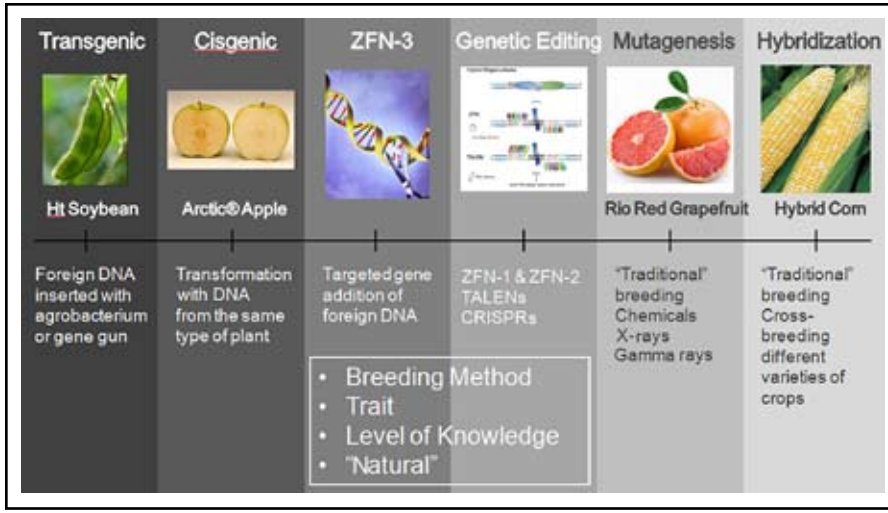

Figure 1. Selected methods of improving crops.

comments sent to USDA, many NGOs and academics who have been against transgenic crops are not making that distinction. Their comments are just as negative for the GE apple or potato as they are for transgenic corn or soybean plants.

The question is, "What will consumers do?" Will they believe those academics and NGOs as their opinion leaders? Will they listen to the producers and others who say these are different from transgenic products and are less risky? I don't know the answer.

A few articles have been published in scientific journals about gene-editing techniques and how they might be perceived by consumers and regulated by the government. The consensus of those articles is that it would depend on whether exogenous DNA or endogenous DNA is involved. The authors honed in on this idea that as long as you are working within the plant's own genome it should be regulated differently and potentially have greater consumer acceptance. I don't think it's so simple; I don't think it's going to be so binary.

Figure 1 shows a number of techniques for crop improvement that scientists currently use on a continuum.

On the far left of Figure 1, there are transgenic plants. Then come cisgenic plants (such as the GE apple and potato). In the middle, there are zinc-finger nuclease (ZFN) -3, and other DNA-editing techniques, including ZFN-1 and -2, TALENs and CRSPRs. Then, on the right, there are "traditional" forms of breeding, including mutagenesis—chemical and radiation—and hybridization. I believe that when consumers look at these technologies they are not going to appraise them simply on whether they contain introduced DNA from a different species. I think it is more complicated. They are going to consider, *inter alia*, the breeding method, the specifics of the trait, and the level of knowledge about the technique. Most consumers know nothing about mutagenesis, for example; if an organization decided to inform consumers about the crops they eat that were developed

with mutagenesis, many consumers might end up being concerned. CSPI conducted a consumer opinion poll some years ago asking consumers if they wanted "hybridized" on the label of foods containing corn and more than 50 percent said "yes." Most consumers do not say "no" to questions asking if they want more information. What the answer shows, however, is that people didn't know what hybridized corn is, because virtually all the corn we eat comes from hybrid seeds. So, whether something is perceived as "natural" could also determine consumer perspectives.

Factors for Consumer Acceptance

What, then, are the factors that influence consumer acceptance when looking at new technologies to produce food crops? Number one is safety. Consumers want to know who is ensuring safety. And they are likely to seek information about safety from opinion leaders they find credible, which could be government officials, representatives of trusted NGOs, or academics. Universities like Cornell and their faculty have a role to play as trusted opinion leaders on issues like this. Consumers want to understand how much is known about the process and the product; accordingly, the scientific knowledge of the consumer is important, particularly in terms of how the product compares to other similar products.

Many consumers may consider who benefits from the products. Corporate control is a factor that has adversely affected acceptance of GE crops, not necessarily in terms of safety but because multinational corporations, like Monsanto, own and benefit from applications of the technology. Accordingly, intellectual property issues may also be an important factor for some consumers. A product in the public domain is a lot more acceptable to many people than if it's patent-protected. Intellectual property can be an important criterion for acceptability by some consumers.

What questions will the public ask that scientists and developers need to answer? Consumers will need to know what you are doing and you need to have answers that are scientifically accurate, but also understandable to the public. Scientists need to find a way to be true to the science yet provide understandable information that explains the product and clarifies their motivations. What are the potential benefits and who benefits—who are the winners and who are the losers? As scientists, you may not think certain questions are important, but consumers may ask, "What's in it for me? Do I or do others gain anything from this?" Also, "Who is overseeing this, to make sure that adverse effects don't materialize?" Answers to those questions could determine their opinion on many new products.

Transparency and engagement will be crucial to consumer perceptions. Developers and scientists need to be transparent with stakeholders and the public. They need to engage early in the development process and be honest regarding both benefits and risks, if, indeed, there are any associated risks or other externalities. It isn't necessary to be balanced, but all of the information must be provided. You are not necessarily an advocate but you are somebody providing information. You are not like a lawyer in a courtroom trying to prove one side is correct and the other is wrong. If a scientist does this, he or she can lose credibility very fast.

Natural?

Another issue that may come up when discussing the new DNA-editing techniques is, "What is natural?" There may not be a specific scientific definition, but, clearly, the public's perception of what is natural could come into play with their acceptance of DNA-editing techniques. The public may say that some things are natural that scientists would disagree with. As an example, I ask consumers if they have eaten Rio Red grapefruits, and, if so, whether they think that particular fruit is natural. They often say "yes," not realizing that this particular variety of grapefruit was developed by mutation breeding, whereby DNA was broken with X-rays. Some consumers even think that they are organic. Another example is the pluot,[3] which wasn't available in supermarkets when I was growing up. Now they are broadly for sale, yet many people don't realize that it's a plum-apricot hybrid that would not normally arise in nature. It is not a "natural" fruit, but was developed by a biologist through field trials involving simple cross-pollination and isolation of plants in greenhouses.

Addressing Safety and Oversight

There may not be enough information yet to have an opinion on what risks, if any, are associated with particular products made using new DNA-editing techniques. However, if there are potential risks, then those products should have some form of federal oversight. The questions that need to be asked are:

- What are the potential risks, if any, from the process used?
- What are the potential risks, if any, from the products made from that process?
- How does the risk profile compare to other agricultural breeding techniques and products?

The third question is particularly important because risk is not absolute, it's relative.

Regarding government oversight, consumers generally want decisions that are risk/science-based. As stated earlier in this paper, transparency and public participation also will be important. We heard from Peter Whitfield[4] that many federal laws could oversee these new products. If there is a perceived risk or a potential for risk that doesn't fit into the exact legal mandates provided by current laws, however, consumers will expect legal modifications. Consumers believe that the government is there to protect them. On the other hand, the public does not favor overregulation any more than it favors underregulation.

Some of the discussion at this conference has surrounded whether new DNA-editing techniques will lead to new regulations or whether they will be treated under the existing regulations for GE crops. If potential risks are found from using gene-editing technologies that need federal oversight through regulation, it would not be good to apply the current regulatory process used for GE crops. Instead, it would be better to adopt a process that corrects the problems of the current GE-crop regulatory structure in the United States.

[3]Pluots/plumcots and apriums/apriplums are hybrids of the *Prunus* genus.
[4]Pages 217–222.

If there are potential food-safety risks, we should not adopt the FDA's voluntary process. Instead, let's establish a mandatory process to assure the public that crops made from DNA-editing techniques are safe as determined by an independent FDA review. This change is needed for GE crops and if there is a need to regulate products from these new DNA-editing technologies, they should also require a mandatory FDA review process. Similarly, the USDA regulates based on whether a crop is a potential "plant-pest." However, one could not find three scientists in the country, who, as scientific experts, would say that adding one new gene to a corn plant makes that variety a plant pest just because *Agrobacterium* was used as the transformation vector. The reality is that nobody thinks that current GE crops could be "plant pests" and yet we use that legal hook to regulate those GE crops. The USDA regulatory system wastes a lot of time trying to verify something that everybody knows is already a fact. I do think, though, that there are potential risks around using GE crops in a sustainable manner—and perhaps with products made from these new DNA-editing techniques—that need some oversight by USDA and EPA. But let's look at real "potential" risks, such as development of resistant weeds or pests, not whether something could be a "plant pest."

Conclusions

In the end, consumers want safe food. And consumers will adopt food-related technologies if they think they are safe and beneficial. A new ice-cream product involves slow churning, which leads to less fat, and those products fly off the shelf. Consumers are not against technology applied to food as long as they are assured that it is safe. However, I do believe that nonscientific considerations are important to many consumers when talking about food. The factors for acceptance are multidimensional and beyond "Where did the DNA come from?" Consumer viewpoints will continue to be influenced by opinion leaders, some of whom have been against GE crops and may be opposed to gene-editing. So, I call on all of you, to the extent that you are opinion leaders, to be vocal. My organization, CSPI, is an opinion leader, but there are lots of others, including academic institutions, who need to be part of the public discussion.

If there are potential risks from products made with DNA-editing techniques, then we should have regulation to ensure safety. For now, the answer to that question is not yet known. If there are potential risks that need oversight, the goal should be not to treat them like GE crops but instead develop a better risk-based and science-based regulatory process.

GREGORY JAFFE is the director of the Project on Biotechnology for the Center for Science in the Public Interest (CSPI), a non-profit consumer organization. He joined CSPI in 2001 after a long and distinguished career in government service as a trial attorney for the U.S. Department of Justice's Environmental and Natural Resources Division, and as senior counsel with the U.S. Environmental Protection Agency's Air Enforcement Division.

MR. JAFFE is a recognized expert on agricultural biotechnology and biosafety, on which topics he has published numerous articles and reports. He has worked on biosafety regulatory issues in the United States and throughout the world, including in Kenya, Uganda, Tanzania, Ghana, Nigeria, Malawi, South Africa, Indonesia, the Philippines, and Vietnam. He was a member of the secretary of agriculture's Advisory Committee on Agricultural Biotechnology and 21st Century Agriculture from 2003 to 2008, and was reappointed in 2011. He was also a member of the Food and Drug Administration's Veterinary Medicine Advisory Committee from 2004 to 2008. Jaffe earned his BA with high honors from Wesleyan University in biology and government and then received a law degree from Harvard Law School.

Plenary Session 3–Non-Governmental Regulatory Aspects

Q&A

Moderator: Steve Pueppke
Michigan State University
East Lansing, Michigan

Perry Hackett (University of Minnesota): Greg, what's your take on Golden Rice, because you made a point that, so far, there's nothing to benefit consumers versus the producers or farmers.

Gregory Jaffe: I was in the Philippines in July, visited IRRI[1] and talked to some of the researchers. I saw some of the Golden Rice there. I don't anticipate that there is any food-safety risk from that and I don't think there are any major environmental concerns. The bigger issues are how you get it into the proper communities and make sure they adopt it and use it in their diets. The most important thing is getting it into varieties that yield well and those farmers want to grow. The project seems to be moving forward, and it will be a benefit for those consumers. For consumers in the United States, some high-oleic-oil soybean and other things are beginning to come out and those may be perceived by consumer as benefits, including my organization[2]. But, to date, most of the crops around the world have not been seen as beneficial.

Michael Kahn (Washington State University): A question for Jeff, but the others may want to comment too. With regard to risk, there is one smoking gun out there—that I am aware of—and that is the Showa Denko tryptophan nutritional supplement that caused

[1]The International Rice Research Institute.
[2]Center for Science in the Public Interest (CSPI).

a lot of problems in the early 1980s. The supplement, sold over the counter, in some way generated high levels of neurotoxic compounds. They eventually settled and paid a lot of damages. The irony is that this is still a possibility, because nutritional supplements are not regulated by FDA, except after the fact. After the damage is done, you pull a harmful compound off the market. So, with regard to Jeff, yes there could be risks out there, but the focus of the system on the process of getting to market as opposed to the risks that exist in the market—or the quality of the product—is a problem that is not being addressed. Would you comment on that, from the standpoint of risk?

Jeff Wolt: I will try to. I think you are saying that we need to be cautious. We cannot be cavalier in our approach to these products as they come to the market. You pointed out a good example of where, from a societal standpoint, with our process focus we are missing the perspective of what we are responsible for in seeking out risk. Why do we look so closely at genetically engineered crops when food supplements are largely ignored? There's a disconnect within our regulatory process. It's due to the complexity in the way we regulate things and the fact that they are dispersed over so many agencies and subparts of agencies. We need to always be open to the potential for risks, but we are so often constrained by the regulatory remits within our governmental agencies that we misdirect our attention.

Brian Larkins (University of Nebraska): I'll preface this by saying that I am not an economist and I'm not a sociologist. I am ignorant of a lot of things, but it strikes me that we need a new strategy. It might be simpler just to label things. Many things appear on labels on packages and for the kinds of ingredients that we are talking about—principally corn, soy, and maybe cotton—they are refined products. Why not just put something on there such as, *This food contains ingredients from genetically modified crops that are generally recognized as safe*? I think that, within a very short time, consumers who bother to read labels would not be so concerned. Is the cost of not labelling worth what we are paying in terms of regulation and delay in future technologies that could be very useful to society?

Wolt: This is problematic for me because, as a risk assessor and scientist, I have long adhered to FDA's position that the information on food labels should have a bearing with regard to consumer safety. They are not always able to do that, but the intent is to label in that way. Once we open the floodgates and allow information on the label that is broader than safety, we distract the consumer and we weaken the strength of the label. However, I do have mixed feelings on this because labeling of GM foods has been reshaped by the protest industry over time, from a science issue to a choice issue. All of us are sympathetic to the idea of choice, and so, having labeling as a choice issue, tends to resonate. On the other hand, I'm afraid that labeling and the advent of labeling—which I believe is going to happen—won't be the last salvo in this war. It will be just one step along the way. Those who are aligned against GM technology and foods derived from GM crops will simply use it as the next step in their campaign to denigrate and stigmatize this technology.

Drew Kershen: I think they are quite distinct issues. Labelling really has no impact on the regulatory system as a practical matter. Labeling laws are not going to affect, in any way, the ability to move these crops to market in a quicker or more efficient or less costly way. In Europe, the advent of labeling meant that the processor simply stopped accessing any food that had an ingredient that required the food to be labeled, and it has had a tremendously detrimental impact. It was perceived to be a worry about loss of market and a worry about stigma. I think the United States would likely be very similar to that; it would be used by the protest industry as a way of stigmatizing and a way of then making certain that it was a market-share issue. Of course, you can do this anyway in terms of pressure. You may know that there is pressure on all sorts of food companies to drop ingredients that come from genetically modified crops or genetically modified animals. And so, it's unlikely to solve anything and I agree with Jeff that it would be used as another hammer to stigmatize agricultural biotechnology. Food companies are very worried about that. There are several issues along that same line. You could consider trade issues in similar fashion. The issue is *what would be the impact on these crops?* The protest industry is intent on using this to drive agricultural biotechnology out of the market, by making it impossible to sell these foods. That's their goal. That's what they want to do. That is why they are labeling. They are allied with the organic industry, which sees this as a way of increasing their market share significantly. With those two allies doing that, I don't favor labeling. Along the lines Jeff has said, *what is the purpose of the label?* The goal is to provide effective, clear information to consumers so that they have safe foods.

Jaffe: I agree with Jeff and Drew. At CSPI, we don't support mandatory government-imposed labels except in situations where a safety or nutritional issue dictates it. Labeling shouldn't be a surrogate for safety; if there is an issue of safety we should have a regulatory process to determine that the food is safe before it goes into the supermarket. As a parent, I want to know that everything in the supermarket is safe to eat. I can then choose among different labeled foods for different philosophies or religion or other reasons I have, but I don't say, "We're not going to have a regulatory process, but we'll label it and people can choose whether or not they want to eat it, whether they think it is safe or not." I don't think that is the proper public policy. With that in mind, though, I do advocate to all in the food chain that transparency is very important. Consumers have a point when they ask, "If this is safe why are you hiding it from me?" Whether it's voluntary labeling on the package, or it's on a website on the internet, or it's in a barcode you can read with your smartphone, I do think that, for the person who wants the information, it shouldn't be hidden. One way forward is better transparency.

Kershen: Let me add to that. Transparency might in fact work the way Greg has just described it, if, in fact, we are willing to be transparent about the reality of genetically modified organisms. For example, we would have to drop the distinctions Europe makes between a food made *with* a GMO and a food made *from* a GMO, and that prepositional difference matters tremendously. A food made *from* requires a label. A *from* food is, for

example, a canola oil that has no trace of the transgenic DNA or chemistry. That has to be labeled because it's *from*. However, many cheeses, and wines are made *with* enzymes that are genetically modified. The leading enzyme company in the world is Novozymes of Denmark, which produces many enzymes and if you were to label every food produced with those enzymes, it would be almost 100% and then it would become irrelevant.

Steve Pueppke: We've had good discussion on this. Let's try to get a couple more questions in.

Tom Turpen (Citrus Research and Development Foundation): I'd like the panel to continue to throw out ideas on how to reframe the discussion for public opinion, because that seems to be so key to regulatory reform. It has two parts. First, who is the voice? Who speaks for this? I think the NABC has a unique role to play, particularly if on the same page with the NRC study that is in process. The National Academy, the USDA and NABC would provide a powerful voice. But then, you still have to address the content. What positive content will sway public opinion? For citrus greening, we need a sustainable solution and it's got to be genetic in the long term. However, it's not at all clear who is going to pay for tree replacement. It's not just the public sector that is priced out of that equation. It's also the private sector. That's bad enough for seed crops, but for permanent crops it doesn't make financial sense which means it won't get done. This means that food is going to be more scarce and more expensive, and yet cost always seems to be way down the list of anyone's themes to talk about. I want to hear ideas about how to communicate for public opinion with positive themes that are going to resonate. We need an anti-Frankenfood poster; what is the counter opinion that is equally effective to that messaging? We have poverty and hunger in our country too. It's not just a developing-world problem. Why isn't that part of the discussion?

Jaffe: Citrus greening, if that ends up being a genetically engineered solution will be viewed as a tremendous consumer benefit, if it is properly presented to the public. The major question, when you are talking about this technology is, "Why are you doing this?" People haven't talked about cost because these are commodity crops, and the cost of the cornflakes in the box isn't relevant. I think if consumers have the choice of American orange juice versus Brazilian orange juice and Brazilian orange juice is twice as expensive, I think they will choose American orange juice—no question about that. In addition to cost, they care about "American." A new thing now is labeling things that are not imported. Nobody wants stuff from China and that's another selling point. Properly answering *why are you doing this* is important for the public because many suspect that somebody is tinkering with something because they can do it rather than for a good reason. Secondly is the public aspect of it. If things are done by a multinational corporation, consumers are more hesitant than if they are done by a small company or by a public university. I was sorry when everybody was saying *we're going to do the public stuff but then we are goingto license the IP to the big companies*. I think that is not advantageous. Why not license to

some small companies? I think that small companies may be perceived differently. And the third thing I'd mention is education, such as provided by NABC members: education about agriculture in general. Where does our food come from? I think most Americans are like me; I grew up in the suburbs of New York City and now I live in the suburbs of Virginia, far away from farms. Many consumers don't understand what scientists do for agriculture and what farmers do and what their work involves. One of my suggestions was going to be *put things in context*, but the public has the context of "Old McDonald had a farm" and everything else sounds scary by comparison. Context is really important.

B.J. Haun (Cellectis Plant Sciences): An interesting thing was brought up by Drew about USDA having an exempt category, and an interesting point was made about people thinking that the ruby red grapefruit is organic. There's a double-edged sword there which I would like the panel's opinion on. If you make these exempt categories you would actually reduce transparency, whereas transparency is what allowed the ruby red grapefruit to be considered natural. Going down the path of listing exempt categories, is that a good thing or a bad thing? Is that going to make certain things that industry and the public sector make more readily received? Will less transparency give the NGO's even more ammunition against us?

Kershen: The theory has been that if the government regulates it, people have confidence in it. I think that that has been proven incorrect. You regulate because it's not safe and you are dealing with safety. So, there is no need to regulate when there is not a safety issue. In fact, to do so miss-educates the public, miss-educates them in a fundamental way because it is really against transparency. It's like the headlines you get on the internet; many are simply incorrect and biased. So, I think the answer is, we've gone down that route, it's been tested, it's been proven that if you regulate that, in fact, you are giving the wrong message, you educate the public incorrectly. Therefore, one way to deal with this is, in fact, to say, "It's time to change the regulatory paradigm."

Donald Weeks (University of Nebraska): Greg, you indicated that you thought that there could be a chance for redoing the regulatory system. Some of us are of the opinion that, once the regulatory system has been put in place, it's not going to be displaced because of its—I don't know the right word to use—it's permanency. Are there chances for redoing the regulatory system, or are we fantasizing if we think about that?

Jaffe: Changing regulatory systems is tough to do, but it does happen. The Food Safety Modernization Act was signed into law in 2011 to revise what were outdated food-safety laws and to change the power that FDA has and how they regulate different things, with emphasis on produce, which was causing lots of outbreaks, and less emphasis on other things that weren't causing outbreaks but were maybe covering food-safety problems in the 1950s. Whether that's likely to happen in the immediate future for biotech, I'm not sure there is sufficient interest. But, I think it would be better.

Wolt: A quick word of caution—be careful what you wish for. I think that most of us who deal in regulatory-related arenas are cautious in encouraging change in regulations, because you really don't know what the repercussions are going to be down the road. It might be better to live with what you've got than to wish for a new day. The Food Quality Protection Act is a good example. There was a big push by a lot of parties to move that through to, essentially, update our regulatory approach to pesticides, and unintended consequences have made things somewhat more difficult in terms of our ability to use modern chemical pesticides. So, I think there is an opportunity to change regulations, but what you wish for and what you get may be two different things.

Pueppke: Let's give Mike Kahn the last question.

Kahn: With regard to changing the regulatory climate, one can go back a few years to the Delaney Amendment, which was aimed at keeping all cancer-causing compounds out of food. However, with improvements in analytical technologies, it turns out that cancer-causing compounds are in all food at some level. And so, that particular regulatory paradigm had to be broken because it didn't make any sense. In fact, it was essentially redefined as *there's a minimum kind of background, cosmic rays or something like that, that creates a hazard and you are not going to get below that. You shouldn't be regulating to the nth degree when there are these other things present.* Somebody pointed out that some of the results of this editing technology are indistinguishable from natural mutations. If you knock four bases out of a promoter, you can't tell whether that was done with CRISPR or TALEN or a zinc-finger nuclease or it just happened to a plant that was growing out in the field. We are getting to the point where this kind of product distinction makes no sense—to distinguish recombinant DNA from natural products. I think there is a need for redefinition to incorporate that kind of realization.

Kershen: I agree, but I don't think that the law will necessarily make the same distinction that you just made. And I say that because when you look at the EU regulations, particularly regulation 1830/2003 on labeling and traceability, it requires that anything that meets the definition of genetic modification—even though it is not in the final product—you've got to provide a paper trail for labeling purposes. If you use a technique that is regulated within that system, the fact that you won't be able to detect it at the end will be irrelevant because the food people and the food developers who put this on the market are required to provide a paper trail that comes with an enforcing mechanism with both civil and criminal penalties. As a lawyer, I would be very adverse to advising my client *they'll never find it* because, I guarantee you, there are NGOs who are looking every day to find it to punish you and they will find it, in which case I'll be in the European court with my client. I have a theory about representing clients: they go to jail, I go home. I'll be trying to keep my client out of jail, but if I fail, I'll say, "I hope you find good wine in your French cell."

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USDA Regulation of Organisms Developed Through Modern Breeding Technologies

Neil E. Hoffman
USDA-Animal and Plant Health Inspection Service
Riverdale, Maryland

neil.e.hoffman@aphis.usda.gov

The federal government has a coordinated, risk-based system to ensure that new biotechnology products are safe for the environment and for human and animal health. Established as a formal policy in 1986 (US-OSTP, 1986), the coordinated framework for regulation of biotechnology describes the federal system for evaluating products developed using modern biotechnology. The coordinated framework is based upon existing laws designed to protect public health and the environment. The US government has written new regulations, policies, and guidance to apply these laws to biotechnology-derived products.

The US government agencies responsible for oversight of the products of modern agricultural biotechnology are the USDA's Animal and Plant Health Inspection Service (USDA-APHIS), the US Environmental Protection Agency (EPA), and the Department of Health and Human Services' Food and Drug Administration (FDA). Depending on its characteristics, a product may be subject to the jurisdiction of one or more of these agencies. Regulatory officials from the three agencies regularly communicate and exchange information to ensure that any safety or regulatory issues that may arise are appropriately resolved.

Roles of US Regulatory Agencies

APHIS

Within USDA, APHIS is responsible for protecting agriculture from pests and diseases. Under the Plant Protection Act (7 USCC 104, 7701), USDA-APHIS has regulatory oversight over products of modern biotechnology that could pose a risk to plant or animal health. Accordingly, USDA-APHIS regulates organisms and products that are known or suspected to be plant pests or to pose a plant-pest risk, including those that have been altered or produced through genetic engineering. These are called "regulated articles." USDA-APHIS regulates the import, handling, interstate movement, and release into the environment of regulated organisms that are products of biotechnology, including organisms undergoing confined experimental use or field trials. Regulated articles are reviewed with regard to appropriate handling, confinement, and disposal to ensure that, under the proposed conditions of use, they do not present a plant-pest risk.

USDA-APHIS biotech regulations provide a petition process for the determination of nonregulated status. If a petition is granted, that organism will no longer be considered a regulated article and will no longer be subject to oversight by USDA-APHIS biotech regulations. The petitioner must supply information such as the biology of the recipient plant, experimental data and publications, genotypic and phenotypic descriptions of the genetically engineered organism, and field-test reports. The agency evaluates a variety of issues including the potential for plant-pest risk; disease and pest susceptibilities; the expression of gene products, new enzymes, or changes to plant metabolism; weediness and impact on sexually compatible plants; agricultural or cultivation practices; effects on non-target organisms; and the potential for gene transfer to other types of organisms. A notice is filed in the *Federal Register* and public comments are considered on the environmental assessment and determination written for the decision on granting the petition. Copies of the USDA-APHIS documents are available to the public.

APHIS employs the term "biotechnology" to mean the use of recombinant-DNA technology, or genetic engineering (GE) to modify living organisms (7 CFR 340.1). APHIS regulates certain GE organisms that may pose a risk to plant or animal health.

FDA and EPA

FDA has primary responsibility for ensuring the safety of human food and animal feed, as well as proper labeling and safety of all plant-derived foods and feeds. EPA regulates pesticides, including plants with plant-incorporated protectants (pesticides intended to be produced and used in a living plant), to ensure public safety. That agency also regulates pesticide residue on food and animal feed. APHIS, through its Biotechnology Regulatory Services (BRS) program, regulates the introduction of certain GE organisms that may pose a risk to plant health.

APHIS Authority

Federal Statutes

Congress authorizes USDA agencies, including APHIS, to regulate specified areas of

US agriculture under federal statutes. The federal statute from which APHIS derives its authority to write regulations is the Plant Protection Act (7 USC, 7701).

Federal Regulations
APHIS describes in their regulations what (importation, interstate movement and confined release) and how (time frames, permitting processes, penalties) certain GE organisms may be regulated. All formal federal regulations are published in the *Federal Register* and also in the *Code of Federal Regulations* (CFR). Regulations for agriculture and the USDA comprise fifteen volumes and those governing biotechnology as overseen by APHIS are found in Title 7 of the CFR part 340.

Regulatory Trigger Under Current 7 CFR 340.

Organisms that were created using recombinant-DNA techniques and are plant pests are regulated under the current version of 7 CFR 340. The definition of a plant pest found in the regulations (7 CFR 340.1) is:

> *Any living stage (including active and dormant forms) of insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof; viruses; or any organisms similar to or allied with any of the foregoing; or any infectious agents or substances, which can directly or indirectly injure or cause disease or damage in or to any plants or parts thereof, or any processed, manufactured, or other products of plants.*

Organisms such as plants that are not normally considered to be plant pests also fall under the regulation if they were engineered with DNA from a donor organism, recipient organism, vector, or vector agent that is listed as a plant pest in 7 CFR 340.2, such as *Agrobacterium tumefaciens*. If a GE organism meets the definition of a regulated article, APHIS authorization is required for its importation, interstate movement, or confined release into the environment.

Petition Process for a determination of Nonregulated Status

Engineered organisms that meet the definition of a regulated article represent a potential plant-pest risk until the agency determines that it does not pose a plant-pest risk. The petitioner is required to provide information under § 340.6(c)(4) related to plant-pest risk that the agency may use to determine whether the regulated article is unlikely to present a greater plant-pest risk than the unmodified organism. A GE organism is no longer subject to the regulatory requirements of 7 CFR part 340 or the plant-pest provisions of the Plant Protection Act when APHIS determines that it is unlikely to post a plant-pest risk.

Am I Regulated?

The current regulations under 7 CFR 340 do not apply to all GE organisms or even all GE plants. For example, plants transformed by particle bombardment with DNA that is not derived from a plant pest do not trigger the regulations under 7 CFR 340. APHIS may be consulted as to whether a specific organism falls under the regulation through a process named *Am I regulated?* APHIS' response specifically addresses whether or not the

GE organism is regulated under 7CFR 340; however, APHIS does consider if other US agencies or other APHIS regulations are triggered and indicates such in the response as appropriate. The APHIS website lists the types of information that should be included in a letter of inquiry at the following link: *Am I Regulated*[1]?

Previous letters and responses are posted at the following link: Regulated Letters of Inquiry[2].

APHIS has made several decisions on whether GE plants are regulated under 7 CFR 340. Cases where APHIS concluded that the engineered plants were not regulated under CFR 340 include:

- Null segregants from genetically engineered plants (6/6/2012; 10/27/12)
- Deletion of the IPK1 gene in maize using zinc finger nuclease (5/26/12)
- Targeted gene deletions using I-CreI meganuclease (1/6/12)
- Centromere-mediated chromosome elimination (10/27/11)

On two occasions, APHIS responded to letters of inquiry that DNA insertion or editing would be handled on a case by case basis:

- For zinc-finger nuclease (3/8/12)
- For I-CreI meganuclease (5/16/11).

Efforts to Revise 7 CFR 340

Beginning in 2004, APHIS began a process to thoroughly revise the regulations under 7 CFR 340. Stakeholder scoping meetings were held in February and March of that year. In August 2007, a draft EIS was published and a public comment period was held (USDA-APHIS, 2007). About 23,000 comments were received on the draft EIS (USDA-APHIS, 2008). The proposed rule was published four years later, on October 9, 2008. Two meetings to discuss proposed regulations were held, in November 2008 and April 2009.

APHIS has two options to close out the proposed rule. It can withdraw it or it can finalize it in whole or in part. Because APHIS is still in active rulemaking, APHIS is not

[1]http://www.aphis.usda.gov/wps/portal/aphis/ourfocus/biotechnology/sa_regulations/!ut/p/a1/rZLLcoIwFIafpksmEYSEpYjKRWqntlXYMCFySYsEIXaqT99Au7Ez3maa3Zl85_L_54AIrE-FUkU-WE8F4RcoujozYWzjqwIKqO5tNLOg-TudPyPdUqOoSCE-ApTmRwNs0wHOk-R46zcfIg-dCdLjzfspcatAdgBSIQ0UrUogAhqQvWxpRXIq1EXLKkIc3hAbYk5vsmzjdt32UMC5SWlS85PnPf5Pm-7lfuu0K1pRtQJgmEOl-GoiumgTfKkGpEwYjoCtpQqGEzo6au_gqAZ94I3mvAKeDrt-WPZyNniOay4xCr0LUtx0ZmIL0yruX3Bl5ZQQ9cknixSaehBy-4MGUoV6GwFCSzvXIt3g2_sfbeLRvJ8uoP5EmD9P_cjK6tNMA5yOTARhcKqJlP1H6jevm6xdmBM-Xh2ji_ZdoXbbzOWZ0U!/?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_biotechnology%2Fsa_regulations%2Fct_am_i_reg

[2]http://www.aphis.usda.gov/wps/portal/aphis/ourfocus/biotechnology/sa_regulations/ct_am_i_reg/!ut/p/a1/tVLLVoMwFPyWLlxykgbawBJKWx7FeqxaYMMJEUqUVyH1WL_egC5k0dfC7G4yk3tn7oAQ-CAsyQfbEc6qkuRdHU4jZ22hsQGRvVzODWjfL1YP2HUQRBMBCAaAjTYXgJeFp66w7-Dp4yFexBaG9WDuuYW5kaI7BFoQgpCWveQYCUmesjWhV8qTkUc7ihjTHO9iSqDo0UVrR-Q9tXMat4QrOyyqvdz3uT7A55P7RAUB6RImLdZfd7TdkrCFIliRVMqaQpqiYpJIVSDGVFo-imBcIJThMbTXzXwxNHhrW4MAe7kOv5sqVsKXomOioqgbRqWiTVPGDe9xO_dvLCPHnBO4tk-mnYYecGbIQKjAJ38QgM2Na3Gu8I297fehLrLUpeeTA_8fwiTaoMabeSJWNeGZxMq0Av6QAfy_jL-p4LlT5yJj0_mh9PaXFVm11fTT6BmBMwKo!/?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_biotechnology%2Fsa_regulations%2Fct_reg_loi

at liberty to discuss the ensuing agency deliberations that have occurred since April 2009. APHIS may discuss the fundamental revisions that were proposed in the rule in 2008.

Noxious-Weed Authority

When 7 CFR 340 was codified in 1987, it derived its authority from the Federal Plant Pest Act of 1957 (PL 85-36) and the Plant Quarantine Act of 1912 (7 USC 151), which provide APHIS authority to regulate plant pests. In 2000, the Federal Plant Pest Act and Plant Quarantine Act were subsumed into the Plant Protection Act (7 USC 7701). In addition, the PPA incorporates authority that previously was under the Noxious Weed Act of 1974 (PL 93-629), which gave APHIS additional authority to regulate noxious weeds. One of the most fundamental changes APHIS proposed for the new 7 CFR 340 was to include a provision for noxious-weed authority. As defined under the current regulations and the PPA, most plants are not plant pests, with the exception of a few parasitic species, such as striga, witchweed, and dodder. Hence the noxious-weed authority may be more appropriate for regulation of genetically engineered plants that may pose a weed risk.

Risk-Based Regulation/Regulatory Trigger.

As noted in the proposed rule (USDA-APHIS 2008 p. 60011):

> *[T]echnological advances have led to the possibility of developing GE organisms that do not fit within the plant pest definition, but may cause plant pest or weed damage covered by the definition of noxious weed in the PPA.*

One objective of the proposed rule was to make the regulatory trigger consistent with current science and to provide regulatory oversight commensurate with the risk due to the organism. Under such a change, APHIS could conclude that organisms that were not previously regulated under the old 7 CFR 340 regulation might fall under the oversight of the new regulation.

Reduced Regulatory Burden

Just as there is the possibility that organisms that do not fall under the current regulations at 7 CFR 340 may cause plant-pest or weed damage covered by the definition of noxious weed in the PPA, there are many examples of organisms that do not cause such harm, but, nevertheless, are regulated under 7 CFR 340. The proposed rule sought to reduce regulatory burden by applying regulatory oversight commensurate with risk. For example, to quote the proposed rule (USDA-APHIS 2008 at p. 60012);

> *APHIS would subject a GE organism to regulatory oversight based upon known plant pest and noxious weed risks of the parent organisms, or based upon the traits of the GE organism, or based upon the possibility of unknown risks as a plant pest or noxious weed when insufficient information is available.*

Principles for the Oversight of Emerging Technologies

Although APHIS is not in a position, as mentioned above, to discuss agency deliberations on the proposed rule until closeout, there is a relevant document that describes overarching principles for the regulation and oversight of the products of emerging technologies.

These principles are described in a memo by the White House Office of Science and Technology Policy (US-OSTP, 2011) that APHIS has been adhering to in its deliberations on the new 7 CFR 340. These principles are listed below:

- *[T]o ensure the fulfillment of legitimate objectives such as of the protection of safety, health, and the environment while avoiding unjustifiably inhibiting innovation, stigmatizing new technologies, and creating trade barriers. … When no significant oversight issue based on a sufficiently distinguishing attribute of the technology or the relevant application can be identified, agencies should consider the option not to regulate.*
- *Scientific Integrity—Federal regulation and oversight of emerging technologies should be based on the best available scientific evidence. Adequate information should be sought and developed, and new knowledge should be taken into account when it becomes available. To the extent feasible, purely scientific judgments should be separated from judgments of policy. … Decisions should be based on the best reasonably obtainable scientific, technical, economic, and other information, within the boundaries of the authorities and mandates of each agency.*
- *Public Participation—To the extent feasible and subject to valid constraints (involving, for example, national security and confidential business information), relevant information should be developed with ample opportunities for stakeholder involvement and public participation. Public participation is important for promoting accountability, for improving decisions, for increasing trust, and for ensuring that officials have access to widely dispersed information.*
- *Communication—The Federal Government should actively communicate information to the public regarding the potential benefits and risks associated with new technologies.*
- *Benefits and Costs—Federal regulation and oversight of emerging technologies should be based on an awareness of the potential benefits and the potential costs of such regulation and oversight, including recognition of the role of limited information and risk in decision making. … The benefits of regulation should justify the costs (to the extent permitted by law and recognizing the relevance of uncertainty and the limits of quantification and monetary equivalents).*
- *Flexibility—To the extent practicable, Federal regulation and oversight should provide sufficient flexibility to accommodate new evidence and learning and to take into account the evolving nature of information related to emerging technologies and their applications.*
- *Risk Assessment and Risk Management—Risk assessment should be distinguished from risk management. The Federal Government should strive to reach an appropriate level of consistency in risk assessment and risk management across various agencies and offices and across various technologies. Federally mandated risk management actions should be appropriate to, and commensurate with, the degree of risk identified in an assessment.*

- *Coordination—Federal agencies should seek to coordinate with one another, with state authorities, and with stakeholders to address the breadth of issues, including health and safety, economic, environmental, and ethical issues (where applicable) associated with the commercialization of an emerging technology, in an effort to craft a coherent approach. There should be a clear recognition of the statutory limitations of each Federal and state agency and an effort to defer to appropriate entities when attempting to address the breadth of issues.*

In Conclusion

At the present time, APHIS considers products of emerging technologies under the current 7 CFR 340. Organisms engineered without plant-pest sequences may not fall under the 7 CFR 340 regulations. The *Am I Regulated?* process is specifically used to ascertain whether the agency concludes that the organism is regulated under 7 CFR 340 or whether other APHIS regulations are triggered. APHIS is still in active rule making having proposed changes to 7 CFR 340 such as including a noxious-weed provision, changing the regulatory trigger, implementing oversight commensurate with risk, and reducing regulatory burden. A revised 7 CFR 340 may fundamentally change the oversight of the products created with emerging technologies compared to the current regulations.

References

USDA-APHIS (2007) Draft Environmental Impact Statement-Introduction of Genetically Engineered Organisms. Regulations.gov Docket 2006-0112.

USDA-APHIS (2008) 7 CFR 340. Proposed Rule. Importation, Interstate Movement, and Release into the Environment of Certain Genetically Engineered Organisms. FR 73 60008.

US-OSTP (1986) The Coordinated Framework for Regulation of Biotechnology. FR_51: 23302.

US-OSTP (2011) Memorandum for the Heads of Executive Departments and Agencies: Principles for Regulation and Oversight of Emerging Technologies. http://www.whitehouse.gov/sites/default/files/microsites/ostp/etipc-memo-3-11-2011.pdf.

NEIL HOFFMAN is the chief scientific advisor in the Office of the Deputy Administrator in Biotechnology Regulatory Services, USDA/APHIS. Prior to this role, he was director of the Environmental Risk Analysis Division in BRS from 2003 to 2008. Prior to joining BRS in 2003, he spent a year as a program director in the Molecular and Cellular Biology Division at the National Science Foundation. From 1999 to 2002, he was a research manager at Paradigm Genetics where he directed the research and development activities for the establishment of high-throughput platforms for discovery of stress genes, new herbicide targets, and the mode of action of herbicides. From 1988 to 1999, **DR. HOFFMAN** was a faculty member of the Department of Plant Biology in the Carnegie Institution of Science where he worked in the area of chloroplast protein targeting.

He received his BS degree in biology from Cornell University and a PhD in plant physiology from the University of California, Davis.

Regulation of Plants with Novel Traits: Canadian Perspectives on the "Novelty" Trigger

Heather Shearer
Canadian Food Inspection Agency
Ottawa, Ontario

Heather.Shearer@inspection.gc.ca

In determining whether a plant that is the product of gene editing would be regulated in Canada, it is important to consider whether the product would be considered to be novel. The following discussion will focus on Canada's product-based approach to assessing plants with novel traits (PNTs) for use as food, as feed, and for release into the Canadian environment. As the author works in the area of environmental release of PNTs, this will be the main emphasis.

Background Information on the Canadian Regulatory Authorities with Regard to Plants with Novel Traits and Products Derived from Them

Canada's product-focused system for regulating agricultural products of biotechnology relies on science-based safety assessments and risk management, with the overall goal of protecting human and animal health as well as the environment. This product-focused framework employs regulatory triggers to distinguish PNTs and novel plant products from their conventional counterparts.

The *Canadian Environmental Protection Act* (CEPA) requires that a person who wishes to import, manufacture, or sell any new substance must notify the appropriate Canadian regulatory authority, so that the new substance can be evaluated for potential effects on the environment and human health. To avoid duplication of regulatory oversight, CEPA exempts those products of biotechnology regulated under certain other acts and regulations

(*e.g.* the *Seeds Act, Feeds Act, Fertilizers Act*) from the requirement to notify Environment Canada. However, Environment Canada retains residual powers under CEPA to regulate any products or end-uses that other acts do not regulate.

Each act describes the powers held by the minister responsible for that act. Regulations are made under the authority of the enabling act, and define the application and enforcement of that act. For example, in the case of the *Seeds Act*, the responsible minister is the minister of Agriculture and Agri-Food. The minister's authority to authorize the environmental release of seed is defined in the *Seeds Regulations*, Part V, paragraph 111. To paraphrase the authority in that paragraph: after receiving and assessing all requisite information, and with consideration of risk to the environment, the minister will authorize release, imposing any conditions necessary to manage environmental risk. Refusing to authorize a release is within the minister's power only when the proposed release poses an unacceptable risk to the environment, or when the minister has reasonable grounds to believe the proponent will not respect the conditions imposed upon the release.

To provide guidance in the interpretation of the relevant acts and regulations, departmental documents, such as directives and guidelines, are often available. These documents are based on the legislation, but do not have the force of law.

Steps in the Regulatory Process in Canada

Regulatory Trigger

Canada takes a product-based rather than a process-based approach to regulation of products of biotechnology. The trigger for regulation in all cases is based on novelty. The responsibility to determine that a product may be novel rests with the proponent, while the final decision on novelty rests with the appropriate regulatory authority. A proponent may be unsure whether a product would be considered "novel." In these cases, a consultation with regulators to determine novelty is often a useful step. A full description of this process can be found here:
http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/pre-submission-consultation/eng/1368394145255/1368394206548.

In brief, a novelty determination can involve a meeting between the proponent and regulators from Health Canada and the Canadian Food Inspection Agency (CFIA). The proponent will provide a description of the product. After evaluating the information provided, each regulatory authority will provide the proponent with their novelty determination.

The regulatory trigger is not identical for novel foods, novel feeds, and PNTs. It is, therefore, necessary to consider whether a product may be novel under each relevant set of regulations:

- *Food and Drugs Act and Regulations:* Health Canada
  More information on the assessment and regulation of novel foods can be found at: http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/nf-an/guidelines-lignesdirectrices-eng.php.

Definition of Novel Food from the Food and Drugs Regulations:

- *"Novel food" means*

  (a) a substance, including a microorganism, that does not have a history of safe use as a food;

  (b) a food that has been manufactured, prepared, preserved or packaged by a process that

  (i) has not been previously applied to that food, and

  (ii) causes the food to undergo a major change; and

  (c) a food that is derived from a plant, animal or micro-organism that has been genetically modified such that

  (i) the plant, animal or microorganism exhibits characteristics that were not previously observed in that plant, animal or microorganism,

  (ii) the plant, animal or microorganism no longer exhibits characteristics that were previously observed in that plant, animal or microorganism, or

  (iii) one or more characteristics of the plant, animal or microorganism no longer fall within the anticipated range for that plant, animal or microorganism.

- *"Genetically modify" means*

  to change the heritable traits of a plant, animal or microorganism by means of intentional manipulation.

- *"Major change" means*

  in respect of a food, a change in the food that, based on the manufacturer's experience or generally accepted nutritional or food-science theory, places the modified food outside the accepted limits of natural variations for that food with regard to

  (a) the composition, structure or nutritional quality of the food or its generally recognized physiological effects;

  (b) the manner in which the food is metabolized in the body; or

  (c) the microbiological safety, the chemical safety or the safe use of the food.

o *Feeds Act and Regulations: CFIA Animal Feed Division (AFD)*
The CFIA provides more information on the regulation of novel feeds at: http://www.inspection.gc.ca/animals/feeds/novel-feeds/eng/1370227088259/1370227136675

Definition of Novel Trait from the Feeds Regulations:

- "Novel trait," in respect of a feed, means a characteristic of the feed that

  (a) has been intentionally selected, created or introduced into the feed through a specific genetic change, and

  (b) based on valid scientific rationale, is not substantially equivalent, in terms of its specific use and safety both for the environment and for human and animal health, to any characteristic of a similar feed that is set out in Schedule IV or V.

Novelty determination guidance for feed is provided at:
http://www.inspection.gc.ca/animals/feeds/regulatory-guidance/rg-1/chapter-2/eng/1329298059609/1329298179464?chap=6#s25c6

- *Seeds Act and Regulations: CFIA Plant Biosafety Office (PBO) and Plant Biotechnology Risk Assessment Unit (PBRA)*

  These two groups work closely to manage the environmental release of plants with novel traits (PNTs). PBO is responsible for decision making surrounding novelty and authorizations of PNTs, and for establishing and implementing policy and programs for PNTs. PBO operates based on the science advice of the risk assessors in PBRA. For more information on the regulation of the environmental release of plants with novel traits, visit:
  http://www.inspection.gc.ca/plants/plants-with-novel-traits/eng/1300137887237/1300137939635

Definition of Novel Trait from the Seeds Regulations:

- "Novel trait," in respect of seed, means a characteristic of the seed that

  (a) has been intentionally selected, created or introduced into a distinct, stable population of cultivated seed of the same species through a specific genetic change, and

  (b) based on valid scientific rationale, is not substantially equivalent, in terms of its specific use and safety both for the environment and for human health, to any characteristic of a distinct, stable population of cultivated seed of the same species in Canada, having regard to weediness potential, gene flow, plant-pest potential, impact on non-target organisms and impact on biodiversity

To provide proponents with additional guidance on the determination of novelty, PBO provides Directive 2009-09: *Plants with novel traits regulated under Part V of the Seeds Regulations: Guidelines for determining when to notify the CFIA*
http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-2009-09/eng/1304466419931/1304466812439.

Regulatory Trigger: Special Cases

1) *A product does not trigger all three regulatory authorities:* Not all products will trigger regulation as novel foods, novel feeds, and for environmental release. For example, a herbicide-resistant turfgrass would not be expected to trigger regulation as a novel food if the turf species is not used as food. Similarly, a virus-resistant citrus cultivar would not be considered to be a PNT in Canada if the crop is not capable of surviving in this climate, even though approval for use as food and feed would still be required. When a novel product triggers the requirement for regulatory approval under more than one piece of legislation, Canada's "no split approvals" policy specifies that it will be authorized only once all implicated regulatory authorities are prepared to proceed.

2) *Retransformation/remutation:* It is noteworthy that, in certain cases, even if regulation is triggered, a full risk assessment may not be required by all three assessment groups (novel foods, novel feeds, and environmental release). One example of this is "retransformation." For the purposes of environmental release, retransformation is defined as the transformation of a plant with a DNA construct that has already been authorized in another variety of that species, provided that the intended uses are similar, and that the plant is known to be similar to the authorized PNT. (For more details, consult CFIA Directive 94-08.) A related policy applies to remutation events. This is particularly relevant to vegetatively propagated crops such as potato, with which incorporating a novel trait through conventional breeding methods is impractical.

In these cases, the plant is still considered to be novel, and is, therefore, subject to the same regulatory requirements as the original event. However, since a risk assessment would not be required, its authorization for environmental release could be greatly simplified. In principle, this concept would be equally applicable to some products of gene-editing technologies, although, with no formal policy in place at the time of this writing, consultation with regulatory authorities is encouraged early in the development process. Please note that, since Canada is a Codex signatory, Health Canada adheres to international guidance regarding recombinant-DNA technologies, and may, therefore, differ from the CFIA in decisions on whether assessment of retransformation events is required.

3) *A history of use in Canada:* Part V of the *Seeds Regulations* was drafted in such a manner that it grandfathered in potentially novel products of biotechnology that had already been released into the Canadian environment prior to its enactment. This means that, if a crop and trait were present in Canadian agriculture prior to 1996, it would not be considered to be a PNT. However, food or feed products derived from plants with an historic use exemption under the *Seeds Regulations* would not necessarily be exempt under the *Food and Drugs Regulations* or *Feeds Regulations*.

If this Part-V exemption had not been implemented, many products that fall within the "novel" category would have required assessment even though they may have already been safely grown for many years in Canada. Some examples of such products include triticale (released in Canada in 1969), canola (substantially equivalent to rapeseed), and triazine-tolerant canola (displaying novel herbicide tolerance).

Similarly, this concept of "new to Canada" continues to apply in novelty assessments. If a proponent can demonstrate that a trait was already present in that species in Canadian agriculture prior to 1996, then the trait is not novel for the purpose of environmental release. For example, if a plum cultivar with resistance to plum-pox virus had been cultivated in Canada prior to 1996, the genome of a different cultivar could be modified using gene-editing techniques to possess the same sequences and demonstrate the same resistance. Since the trait is not new to the species, a reasonable case could be made in some situations that this is not a PNT.

Pre-Submission Consultation

A pre-submission consultation is available to proponents who wish to discuss their products with regulators prior to making a submission. This consultation provides the proponent with an opportunity to present an overview of the submission and to ask specific questions regarding the content of the submission. Assessors will provide guidance on the information requirements specific to the individual product, explain regulatory requirements, and clarify expectations for data quality.

This practice often reduces the number of requests from regulators for either clarification or additional information that might otherwise have been required in order to complete a safety assessment and reach a decision. Health Canada and the CFIA have developed a guidance document for pre-submission consultation that is intended to provide new applicants with more information. It is available at:
http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/pre-submission-consultation/eng/1368394145255/1368394206548.

Data Submission and Review

Where a safety assessment is required, the proponent must make applications to satisfy the data requirements of each regulatory group. In the case of novel foods, evaluators will perform a nutritional assessment and a toxicology assessment (which considers the chemical, toxicological, and allergenicity of the novel food) to determine whether the novel product is equivalent to its conventional counterpart, as well as a molecular characterization of the genetic change. The novel feed assessment includes nutrition, toxicology, and molecular reviews, but considers this information in the distinct context of use as feed. With respect to environmental safety, evaluators perform a molecular characterization, and assess the PNT against its conventional counterparts by reviewing information addressing: weediness, gene flow, plant-pest potential, impacts on non-target organisms, and impacts on biodiversity. There are many similarities in these reviews (for example, all three groups perform a molecular characterization); in recognition of this, evaluators are in regular communication with each other to maximize efficiency.

If, following a review of all submitted information, the evaluators have questions or require clarification of information submitted, a letter will be sent directly to the proponent outlining these questions and/or requests for clarification. Information requirements have been met when requests for further information and/or clarification have been satisfied. At this point, the science review is complete, and the regulatory decision will be made.

Regulatory Decision

When a plant is considered to be a PNT and a source of a novel food and/or a novel feed, regulatory decisions regarding the use as a novel feed, novel food and environmental release will be coordinated and harmonized to minimize the potential for unapproved products to enter the Canadian environment or food or feed supplies. Once regulatory decisions have been harmonized, the CFIA and Health Canada will send decision letters to the proponent and post a decision document on their respective websites. The decision document summarizes the information that was assessed, and the evaluators' findings.

Furthermore, risk management of certain PNTs may be required as a condition of authorization. Risk management imposes conditions on the use of the PNT such that identified potential risks to the environment are mitigated. Risk management may not be necessary or appropriate for all PNTs, but some (particularly insect-resistant and herbicide-tolerant PNTs) warrant a stewardship plan.

Considerations that may Impact Future Policy Development Relating to the Environmental Release of Products of Gene Editing

Advancements in molecular analysis techniques continue to contribute to our understanding of plant genomes and genetic change. Also, after nearly two decades with novel plant products available in the marketplace, a high degree of familiarity with these products has developed. In keeping with the comparative approach that Canada takes to assessing novel products, regulators from the CFIA and Health Canada undertook a literature review to compare the insertional effects that could arise during the creation of a PNT to other types of spontaneously occurring genetic changes in plants (Schnell *et al.*, 2014). The findings of this review will help to inform future policy direction, as the CFIA and Health Canada work towards ensuring that regulators are focusing their efforts on assessing novel products of biotechnology in a manner that is suited to the expected potential for risk.

The product-based approach to regulation allows the Canadian regulatory system to effectively adjust to any new developments in the science of plant breeding. Policy work is ongoing to help to ensure that guidance documents are available as products of gene editing are brought forward for assessment. The CFIA and Health Canada are committed to providing an efficient and appropriate level of regulatory oversight that encourages innovation while allowing Canadians to benefit from the advances brought by new technologies.

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HEATHER SHEARER is a regulator with the Canadian Food Inspection Agency's Plant Biosafety Office (CFIA-PBO), which oversees the environmental release of plants with novel traits. She joined the PBO after completing postdoctoral studies at the National Research Council Canada Plant Biotechnology Institute. Her educational background includes a PhD in plant agriculture from the University of Guelph, and an MSc in plant molecular biology from Queen's University.

DR. SHEARER particularly enjoys the aspects of her work that involve keeping policy relevant to rapidly-developing technology and knowledge.

EU Perspectives on New Plant-Breeding Techniques

Joachim Schiemann and Frank Hartung[1]
Julius Kühn-Institut
Quedlinburg, Germany

joachim.schiemann@jki.bund.de

In the last two decades, numerous new biotechnological methods have greatly accelerated the efficiency of plant breeding, referred to as new plant-breeding techniques (NPBTs). These techniques are heterogeneous and may or may not involve modification of the plant genome[2]. In the former case, the end product may not possess the genetic modification, *e.g.* fruits and some vegetables. By the European definition, a genetically modified organism (GMO) is one "in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (The Council of the European Communities, 1990a, 1990b). These two directives, aimed at covering the legal issues of contained use and intentional release of GMOs, were passed not long after the first field trials with transgenic plants in the late 1980s. The intention was to protect human and animal health and the environment against possible risks from organisms created by recombinant-DNA technology. Both directives have been revised several times, resulting in 2001/18/EC and 2009/41/EC (European Parliament and European Council, 2001; 2009). They list techniques that:

- give rise to genetic modification (annex I, part A of directive 2009/41/EC and annex IA part 1 of directive 2001/18/EC);
- are not considered to result in genetic modification (annex I, part B of directive 2009/41/EC and annex IA, part 2 of directive 2001/18/EC); and
- yield organisms that are excluded from the directive (annex II, part A of directive 2009/41/EC and annex IB of directive 2001/18/EC).

[1]Corresponding author: frank.hartung@jki.bund.de.
[2]In the context of this manuscript, genetic modification means that a recombinant DNA sequence has been introduced.

However, the definition of what a GMO constitutes, or the use of which techniques will result in a GMO, still originate from 1990 and, consequently, do not match the development of modern biotechnologies in plant breeding.

Since the vociferous discussions and subsequent formulation of GMO legislation in 1990, numerous new techniques in plant breeding have been developed for which it is not clear if they have to be covered by GMO-legislation or not. Therefore, in 2007, the European Commission and the European National Competent Authorities (NCAs) initiated a review of eight of these plant-breeding techniques defined as NPBTs. At the request of the NCAs, a working group—the new techniques working group (NTWG)—was established with the aim of clarifying the legal status of these new techniques in line with the GMO legislation. The final report of the NTWG was not officially published, but was distributed to the NCAs in 2012 and will be discussed below (New Techniques Working Group, 2012). In 2009, another working group was established on request of the directorate-general for the Environment of the European Commission with the mandate to summarize the state of science for adoption, economic impact and possibility of detection of these new techniques. The study, led by the Joint Research Center (JRC), was finished in 2011 and published as a comprehensive public JRC report and later in summarized form as a peer-reviewed article (Lusser *et al.*, 2011, 2012).

Report of the NTWG to the European Commission

The preamble of the final report includes the statement that the views expressed are those of an expert working group and do not necessarily represent those of the European Commission or the Member States Competent Authorities (New Techniques Working Group, 2012). The NTWG, consisting of two experts per European Member State, at first compiled a list of eight techniques that had to be discussed. This list included: oligo-directed mutagenesis (ODM); zinc finger nuclease (ZFN) technology; cisgenesis; grafting; agro-infiltration; RNA-dependent DNA methylation (RdDM); reverse breeding; and synthetic genomics (SG). Next, the NTWG defined and interpreted terms important for their analyses and then examined the techniques one by one in relation to directives 2001/18/EC and 2009/41/EC.

ODM

Concerning ODM (Beetham *et al.*, 1999) the NTWG came to the conclusion that the oligonucleotides cannot be considered as recombinant nucleic acids. The reasoning for this majority opinion was as follows. Oligonucleotides introduced into the cell by ODM are not capable of replication and are not heritable. Furthermore, the resulting organism itself is captured by annex IB because the technique entails mutagenesis. This view is shared by several competent authorities in Europe [*e.g.* the German Central Biosafety Commission (ZKBS, 2012)]. However, a minority of NTWG experts suggested that ODM leads to a new combination of genetic material resulting in a heritable change in the DNA sequence and that the oligonucleotide has been prepared outside the organism. Both are criteria listed in annex IA, part 1 of directive 2001/18/EC.

ZFN

The ZFN technique (Bibikowa *et al.*, 2001) was divided into subcategories named ZFN-1, -2 and -3. In the case of ZFN-1, the nuclease is applied without integration of the respective gene and no additional template DNA is added. ZFN-2 resembles ZFN-1 but, in addition, an added template DNA for guided DNA-repair, which is not integrated into the genome of the host, is explored. Considering the non-replicative template DNA and a not-integrated ZFN construct, the experts agreed by majority that ZFN-1 and -2 are captured by annex IB (directive 2001/18/EC) or annex IIA (directive 2009/41/EC). In the view of the experts, ZFN-1 and -2 result in a GMO that should be excluded from GMO regulation because the provoked genetic change is a mutation that can also be introduced by other forms of mutagenesis and cannot be distinguished from a mutation introduced by other techniques. ZFN-3 is characterized by the addition of a larger stretch of homologous DNA as a repair template for homologous recombination, aiming for a site-specific integration of transgenes (gene targeting). ZFN-3 was considered by all experts of the NTWG to fall within the scope of directive 2001/18/EC and to be covered by annex IA, part 1 as the resulting plants would be transgenic. However, some cases may meet the criterion of self-cloning and might be considered to fall outside of the scope of annex IIB of directive 2009/41/EC. The opinions expressed by the experts of the NTWG concerning ZFN-1 to -3 are shared by the German ZKBS and, concerning ZFN-3, also by the European Food Safety Authority (EFSA).

Cisgenesis

In the case of cisgenesis/intragenesis, recombinant DNA is introduced into the genome of the recipient plant (Belfanti *et al.*, 2004). The difference from transgenesis is that the recombinant DNA originates from a crossable plant (*i.e.* the same or a closely related species). All experts came to the conclusion that this technique falls under the scope of directive 2001/18/EC and is sufficiently covered by annex IA, part 1. This conclusion is shared by the ZKBS and EFSA. In addition, experts of the different working groups expressed the opinion that, in some cases, cisgenesis may be equivalent to self-cloning (EFSA, 2012a; New Techniques Working Group, 2012).

Grafting

Applying a non-GM scion to a GM rootstock (MacKenzie *et al.*, 1991) results in a chimeric plant that falls within the scope of directive 2001/18/EC, whereas the fruits, seeds or progeny should not be regulated as GMOs. For the converse (*i.e.* non-GM rootstock and GM scion), the chimera, as well as the fruits, seeds or progeny from the scion, are transgenic and, therefore, fall under the scope of directive 2001/18/EC (New Techniques Working Group, 2012). This view is agreed with by other expert bodies (*e.g.* ZKBS).

Agro-Infiltration

Agro-infiltration (Lee *et al.*, 2001) was divided into two subcategories by the NTWG experts. In the case of agro-infiltration *sensu stricto*, only non-germline tissue is infiltrated and the T-DNA of the *Agrobacterium* accumulates but does not replicate in the cells. The aim of this method is not to produce offspring in which the T-DNA is integrated into

the genome, which would result in a transgenic plant. As recombinant *Agrobacterium* is a genetically modified microorganism, the infiltrated plants containing recombinant *Agrobacterium* would fall within the scope of directive 2009/41/EC. In rare cases, the T-DNA might integrate into the genome, but as there is no selection on these integration events, a transfer of the recombinant DNA to the progeny, albeit highly unlikely, has to be controlled. The majority of the experts concluded that progenies of these plants, in which it is proven that no recombinant DNA is integrated into the genome, should be considered to fall outside the scope of directive 2001/18/EC. The second category is agro-infiltration of germline tissues, called "floral dip." This method is usually employed to generate offspring that do contain recombinant DNA in the genome. Therefore, all experts agreed that offspring that do harbor a stable integration event fall within annex IA, part 1 of directive 2001/18/EC. The German ZKBS agreed with this view, but expressed the opinion that multicellular organisms (including plants) that do contain some cells with a recombinant genome should not be defined as GMO. This point was not explicitly discussed by the NTWG experts.

RdDM

Regarding RdDM (Mette *et al.*, 2000), all experts agreed that, in cases where any DNA that encodes the effector RNA is integrated into the genome, the resulting plants are GMOs. In cases where the DNA is only transiently present and not stably integrated, the intermediate plant is a GMO but not the fruit, seeds or other progeny. In cases in which the RNA is directly delivered into the cell without being able to replicate, the experts agreed that such a plant should not be defined as GMO. Summarizing the different scenarios that are possible using RdDM, the conclusion of the NTWG was that all plants containing RNA-dependent DNA methylations only are not genetically modified. Therefore, such plants would not fall under the scope of directive 2001/18/EC (New Techniques Working Group, 2012). This view is shared by the German ZKBS.

Reverse Breeding

During reverse breeding (Dirks *et al.*, 2009), a number of steps are taken that transiently might involve genetic modification. In line with conclusions drawn for other NPBTs such intermediate plants would fall under the scope of directive 2001/18/EC, but not their fruits, seeds or other progeny if they do not contain recombinant DNA. As the goal of RB is to produce parental genomes of superior F1-hybrids in a controlled manner, the screening procedure is on genomes containing no genetic modification at all. Therefore, the NTWG experts agreed that plants resulting from RB do not fall under the scope of directive 2001/18/EC. This view is in agreement with the opinion of the German ZKBS.

Synthetic Genomics

Synthetic genomics (Smith *et al.*, 2003) is a rapidly evolving field within synthetic biology, which may include techniques of genetic modification. Because of the breadth of SG, the NTWG did not discuss it in general, but only some aspects of it (New Techniques Working Group, 2012). The view of the experts is that, in most cases, SG would fall under the scope of directive 2001/18/EC and/or 2009/41/EC if a living (micro-)organ-

ism is the recipient of the synthetic genome. As most of the work done so far has been basic research in microorganisms, this falls under the scope of directive 2009/41/EC. The NTWG offers two possible interpretations of how this technique might be covered in future, either with emphasis on the recipient, which is not considered as a (micro-)organism, or with emphasis on the resulting entity which is considered to be a (micro-)organism (New Techniques Working Group, 2012). In the first case, the technique falls outside of GMO legislation, in the latter case it falls under the scope of the respective directive 2001/18/EC or 2009/41/EC.

Report of the Joint Research Center Working Group

The JRC working group—established in 2009 on request of the Directorate-General for the Environment—analyzed various aspects of the NPBTs which were the state of science for adoption, the economic impact and the possibility of detection of these new techniques. The main conclusions have been published and are as follows:

- a significant part of R&D on NPBTs was done in public research institutes in Europe, but most of the patents are held by US-based companies;
- some of the NPBTs are already in the late stages of commercial breeding programs and will appear on the market in the near future; and
- many regulatory jurisdictions around the world will make decisions on the legislation of NPBTs (Lusser *et al.*, 2011; 2012).

The last point has implications for the adoption of these techniques; regulation of them as GMOs will hamper the adoption of new crops. Furthermore, differences in the regulation of new crops in different parts of the world will cause severe asynchrony in the approval of such crops. Consequently, Lusser *et al.* (2012) have demanded that global discussion concerning governance of the NPBTs is necessary to achieve synchronized and evidence-based governance.

Opinion of the EFSA Concerning the Safety Assessment of Cisgenesis and ZFN-3

The EFSA expressed its scientific opinion in 2012 upon request by the European Commission to address the question of whether the existing guidelines to assess the safety of GMOs can be applied also to NPBTs. To perform an analysis of selected NPBTs and their potential risks, the EFSA established two working groups consisting of GMO panel members and external experts. At first, cisgenesis and intragenesis were evaluated and the results published in 2012 (EFSA, 2012a). The working group came to the conclusion that the existing guidance documents for GMO-risk assessment are applicable to NPBTs and do not need to be developed further (EFSA, 2012a). Furthermore, the experts expressed the opinion that, in some cases of cisgenic plants, fewer event-specific data may be needed to perform the risk assessment.

In the case of ZFN-3, the EFSA working group at first changed the term ZFN to SDN (site-directed nuclease) as in recent years in total four different nuclease systems have been developed that are applicable for the introduction of sequence-specific DNA-

strand breaks and the specific incorporation or exchange of genetic material. The expert group came to the conclusion that the aim of the SDN-3 technique is to integrate or exchange recombinant DNA and, therefore, it is comparable to transgenesis but more precise (EFSA, 2012b). Therefore, the existing guidance documents for transgenic plants are applicable also for plants derived from SDN-3 but, again, in some cases (*e.g.* SDN-3 combined with cisgenesis) fewer event-specific data might be needed for the risk assessment (EFSA, 2012b).

Report of the European Academies Science Advisory Council

The EASAC has provided an extensive report on the risks and benefits of so-called "crop genetic improvement technologies," a term that includes NPBTs, gene technology and techniques that will evolve in the future (European Academies Science Advisory Council, 2013). The report did not find evidence of intrinsic higher risk of GM technology in comparison to conventional breeding. This finding is based on solid science conducted in several thousand research projects and published in the last 20 years. Therefore, EASAC came to the following key conclusion and recommendation (European Academies Science Advisory Council, 2013):

> *The trait and product not the technology in agriculture should be regulated, and the regulatory framework should be evidence-based.*

This request for a trait/product-based regulation reflects the scientific evidence that is very solidly based on GMO-safety research and risk analyses accumulated in the last two decades (Heap, 2012; Swiss National Science Foundation, 2012; Hartung and Schiemann, 2014). The EASAC report was recently endorsed by Anne Glover, chief scientific adviser to the president of the European Commission. Besides the EASAC statement mentioned above that intrinsic risks of gene technologies do not exist, concerning NPBTs, she stated that "… we shouldn´t make the mistake of regulating them to death as we have done with GM" (Glover, 2013).

Additional European Activities to Discuss the Legal Status of NPBTs

In Europe, NPBTs continue to be discussed vociferously. In 2012, in Alnarp, Sweden, Mistra-Biotech organized an international workshop,—*Future of Plant Biotechnology in Europe*—involving various stakeholders, to discuss the governance of NPBTs (Lehrman and Alexandersson, 2012). Experts from EPSO, EFSA, the Swedish Gene Technology Advisory Board, and other competent authorities as well as the Federation of Swedish Farmers and journalists discussed the implications of the currently unclear regulatory status of NPBTs.

In June, 2014, several meetings dealing with the legal uncertainty of NPBTs took place. A symposium in Quedlinburg, Germany, was dedicated to explaining these techniques to stakeholders from the national government, national NGOs and to farmers and representatives of the national press. The urgent need to clarify the legal status of NPBTs in Europe and the negative effects of the current legal uncertainty were expressed (Julius-Kühn-Symposium, 2014).

A similar workshop was organized in London by the Biotechnology and Biological Science Research Council (BBSRC). Members of the European Commission, NCAs, farmers and scientists from all over Europe discussed the opportunities of NPBTs, their uncertain legal status and, especially, what may be done to ameliorate this unsatisfactory situation. As a result of the workshop, a position statement of the BBSRC—to be disseminated and discussed in the UK and Europe—will be published later in 2014 (BBSRC, 2014).

The Position of the European Technology Platform "Plants for the Future"

The ETP published a statement on NPBTs—"Plants for the Future"—in which it welcomed the conclusions described above that the legal definition of a GMO does not apply to most of the NPBTs (European Technology Platform, 2012). To provide the plant and agricultural sectors with legal certainty concerning NPBTs, the ETP requested a move of the existing GMO legislation towards a more suitable science-based and transparent regulation system (European Technology Platform, 2012):

> *It is thus crucial for companies to be certain now that their investments will not be in vain and that their future products will not be subject to the uncertain outcome of politicized regulatory procedures, as is the case with GMOs.*

To provide European Member State representatives with an overview on the technical and legal interpretations of the private and public plant-breeding sectors as regards possible regulatory requirements for the individual techniques as well to show the socio-economic importance of these techniques for industry and society at large, the ETP "Plants for the Future" and New Breeding Technologies (NBTs) platforms jointly hosted an informational meeting on NPBTs, "The Future of Plant Breeding Techniques in the European Union," in June, 2014, attended by representatives from Member State national governments, the European Commission, industry, academia, and the farming community. A key message was that the European Commission's delays in clarifying the legal status of the NPBTs weakens the competitiveness of, and hinders job creation in, the EU agro-food sector. It is important that the European Commission creates favorable regulatory conditions for European plant breeders to maintain leadership in research and innovation. European policymakers are facing difficulties in modifying the existing legislation, due to the absence of consensus amongst the main political EU actors, and the strong divergence in views amongst Member States and stakeholders. This situation mainly reflects broad hostility to GMOs amongst EU citizens.

Summarizing the discussions described above, one can conclude that there is general agreement amongst experts to define a GMO on the presence of foreign recombinant DNA. When an organism does not contain recombinant DNA, it should not be risk assessed and regulated as a GMO. This view also includes techniques that involve creation of GMOs as intermediate steps, but in which the end product does not contain recombinant DNA (*e.g.* ZFN-1, -2, agro-infiltration *sensu stricto*, and reverse breeding). Furthermore, this would include other, so far not extensively discussed, techniques like fast breeding, in which an intermediate plant contains a transgene to accelerate the breed-

ing process, but which is subsequently crossed out and only the null-segregants are used for further breeding. Such an approach concerning GMO legislation would support the further development and adoption of NPBTs in Europe, but it can only be the first step towards a more flexible evidence-based and transparent regulatory system for crop genetic improvement technologies. The future regulatory framework to allow international harmonization and to avoid trade disruptions between countries and continents should take the new trait/product into account and not the technology to generate it.

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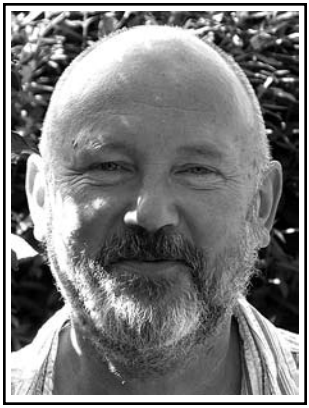

JOACHIM SCHIEMANN is director of the Institute for Biosafety in Plant Biotechnology at the Julius Kühn Institute in the Federal Research Centre for Cultivated Plants and is an honorary professor at the University of Lüneburg. He received a PhD in biochemistry from Martin-Luther-University, Halle, in 1977. From 1976 to 1991 he worked as senior scientist at the Institute for Plant Biochemistry, Halle, and the Central Institute for Genetics and Crop Plants Research, Gatersleben. From 1991 to 2007 he was senior scientist at the Institute for Plant Virology, Microbiology and Biosafety in the Federal Biological Research Centre for Agriculture and Forestry, and was head of the Genetechnology and Biosafety Division.

DR. SCHIEMANN has been coordinating several national and EU-funded cluster projects on biosafety research. From 2000 to 2003 he was a member of the Scientific Committee on Plants of the European Commission at the Health & Consumer Protection Directorate-General, and from 2003 to 2009 was a member of the Panel on Genetically Modified Organisms of the European Food Safety Authority. From 2002 to 2012 he served on the Executive Committee of the International Society for Biosafety Research (ISBR), and from 2004 to 2008 was president of ISBR. Since 2004, he has been a member of the Steering Council of the European Technology Platform, *Plants for the Future*.

Plenary Session 4–Governmental Regulatory Aspects

Q&A

Moderator: Kay Walker Simmons
USDA-Agricultural Research Service
Beltsville, Maryland

Dana Carroll (University of Utah): Dr. Shearer, how long does it take for the regulatory process to be completed in Canada?

Heather Shearer: It depends very much on the complexity of the file—sometimes quite quickly if it's something we are familiar with and if the package is well presented by a more experienced proponent. With first-timers it can take a bit longer just because they are not so familiar with our process and requirements. Also, it depends on the length of our queue; right now it's very short. At times we have questions about a product and require more information. It may take some time for them to get back to us with the answers. There can be a back and forth.

Carroll: What's the range?

Shearer: Twenty months is the average. The range—I would say, as a rough guess, probably twelve to twenty-four months would be fairly typical.

Perry Hackett (University of Minnesota): Given that large companies have no real incentive for making large changes in the *status quo*, is it realistic to expect that small companies, which do want to make significant changes in the areas that you regulate, really have a chance to do so with the current regulations?

Shearer: I'm an optimist. I'm going to say *yes*, and it's not because of the advocacy of a large or small company, it's honestly coming out of the regulators ourselves. We are

motivated to make this system efficient. Now, there's a lot of inertia there. It's a big system, there is interconnectedness around the globe. We interact with lots of other countries. Political considerations impinge when you want to change a law or how you regulate. There is difficulty, but we are motivated internally to make this system work, and it is possible for small companies to work with our system. Some successful applicants have been small companies.

Joachim Schiemann: The answer for Europe is clearly *no*. Requests to place a single event on the European market cost somewhere between 15 and 50 million euros. The regulatory burdens in Europe are so high, and it is so cost-intensive to provide the data, that small and medium-sized companies are completely excluded; they can do it only in cooperation with the big five or six. This is really a problem because Europe still has a lot of small and medium-sized breeding companies with varied genetic backgrounds. There is broad biological variability within these small and medium-sized companies, which are active and productive, but are excluded due to the high burden resulting from using the new technologies.

Neil Hoffman: In the United States, I would say *maybe*. Early on there was success with papaya, but there has been little success since then. However, if we are successful in revising the regulations the way we would like to, I think it would be possible again.

Alexa Schmitz (Boyce Thompson Institute): We've talked a lot about how we regulate products before they are released. Most of what I have heard is determining their safety prior to release. To what extent do we regulate how those products are used once they are released to farmers. Are companies responsible for making sure that protocols for proper use are put into action?

Schiemann: The European regulation provides consent for only 10 years. The company can apply for an extenion. During these 10 years, monitoring is mandatory. We have so-called *general surveillance* where you have to look for negative effects that cannot be predicted, which is scientifically quite demanding. In some cases we have so called *case-specific monitoring*. Case-specific monitoring is based on imagination that the new trait or the new event might have some negative effects. For example, in the case of *Bt*, it is the emergence of resistance in target organisms. In the case of herbicide-tolerance technology, it's the emergence of weediness.

Hoffman: In the United States, the USDA is not involved in oversight once the product has been deregulated. In the two examples mentioned, insect resistance to *Bt* and weed resistance to herbicide, the EPA is involved. Addressing weed resistance is a new development for the EPA. They have stepped up to the plate to provide oversight of the new 2,4-D product; a number of stewardship requirements are connected with the use of that product—monitoring for weeds that are becoming resistant and developing a rapid response to rectify that situation.

Shearer: In the best Canadian tradition, we do it just like Americans do. In addition, when we authorize a product, we have a condition that, if new information comes to light that may affect the specific use or safety of that product, then the farmer is required to notify us, possibly leading to reassessment or revoking of the authorization.

Robert Millman (MPM Capital): Heather, I have a question stimulated by your presentation on Canada about allergens. In any new event—any new engineering or editing event—where you are deleting a protein you could be creating new epitopes. How is that being addressed in Canada and the United States?

Shearer: In Canada, actually, that is already part of the assessment. Evaluators look for potential allergenic epitopes in the proteins, and, again, that is where we rely on that fallback. If new information comes to light, we need to be told. So, if the evaluators happen to miss something or something unprecedented causes an allergic reaction, that would be a perfect example of a situation where you would want to pull that authorization. That hasn't happened yet and hopefully it never does.

Maria Federova (DuPont Pioneer): Dr. Shearer, if a developer comes up with yet another glyphosate-tolerant corn, made by genome editing, how would you qualify that?

Shearer: Of course, we haven't seen that yet. A policy discussion would have to happen at the time we received such a product. I expect, at the very minimum, there would be a lot of bridging to past data generated for that particular protein. That's something we always encourage in our data submission—if a company has already developed a lot of data around the safety of a protein, they are certainly welcome to bridge to past data that they have submitted. It saves us time and saves them time.

Kay Simmons: Are the advances in DNA editing changing the number or the type of applications that you are now receiving?

Hoffman: I don't think so.

Shearer: Not yet. We have received one. I expect that we will see a lot more from what I've been hearing over the last couple of days.

Simmons: Since we have so many graduate students here, let me ask: how many scientists do you employ in your regulatory agencies?

Hoffman: We have staff of about eighty, and about two-thirds of them are scientists.

Shearer: I can probably count on my fingers how many we have. We are actually a pretty lean organization. Our risk assessors are not dedicated to novel risk assessments by any means. They assess a whole variety, not just genetically engineered foods.

Schiemann: In Europe the decision is made on the European level. For risk assessment, EFSA is responsible, the European Food Safety Authority. EFSA is supported by, I think, ten different scientific panels, one of which is on genetically modified organisms. Twenty-one experts are invited to work on that panel. For 6 years, I have been an expert on the GMO panel of EFSA. We have been meeting at least once a month in Parma in Italy, and the GMO unit of EFSA now consists of about fifteen scientists. That's Europe. You also have to break it down on the level of the member states, and in Germany we are absolutely overdoing it. We have five authorities, all of which are looking at new product placement on the market. All of these authorities have to provide opinions to the central authority, the Federal Office for Consumer Protection, which provides the German opinion to EFSA.

Michael Kahn (Washington State University): Recently, some organic producers have been bringing actions against people who are growing genetically modified alfalfa because they are claiming that cross-pollination is contaminating their crops and making them not organic. Does that constitute a weed under the definition that is being considered?

Hoffman: In terms of what a noxious weed is, we don't consider that as a weed. We consider that actually as a natural event, a cross-pollination, which happens. But in the discussions that can ensue, that is certainly a possibility. We never have considered that—cross pollination and the fact that a GE trait that may go into someone else's field—we have never considered that to be a weed harm, a plant pest or a noxious weed harm.

Shearer: In Canada, once something is approved it is considered to be the same as any other cultivar that's out there. So, if you were growing two cultivars of alfalfa in neighboring fields, no one would raise that as an issue; if it's a GM variety next to a non-GM variety, we stay right out of that. On the organic side, we encourage neighbors to cooperate, but that's about all we have to say about issues around GM. That's a socioeconomic issue. It's not CFA's mandate. But I want to mention one of my favorite topics and give a little plug for one of my favorite NGO organizations. They are called CBAN[1] and their website is at cban.ca where they have an excellent animation about gene flow in alfalfa. I really have to give them credit. They are a well-informed advocacy group. They ask intelligent questions and they are persuasive. If you watch this short—have a look at it while you are waiting for your plane today, for example—it's educational, it tells you what alfalfa does in agriculture and provides a lot of truthful information. And then it slips a little bit over into spin. But I think if you weren't well informed about the issue it's subtle enough that it sounds very real and very believable and makes a good case for why we should be worried about gene flow and corporations gaining control of agriculture.

Simmons: I'd like to thank the speakers for their services to the research community.

[1]Canadian Biotechnology Action Network.

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The State and Local Regulatory Landscape for Bioengineered Plants: September 2014[1]

Peter Whitfield
BakerHostetler
Washington, DC

pwhitfield@bakerlaw.com

Across the country, numerous state and local governments have enacted, or are considering, laws that impact the cultivation, use, and labeling of genetically engineered plants ("GMOs"). These laws are best described in three categories:

- Laws that ban the cultivation of GMOs;
- Laws that regulate the handling of GMOs; and
- Laws that impose disclosure requirements on the sale of GMOs, such as food labeling.

The landscape of GMO laws is constantly in flux. Although only a few laws are currently in effect, many more are being proposed. Additionally, some laws are subject to legal challenges and at least one has been overturned.

Laws Banning the Cultivation of GMOs

Laws that ban GMOs are straightforward prohibitions against any form of growing a bioengineered plant. Currently, there are versions of these laws in California (Marin, Mendocino, Santa Cruz, and Trinity counties, and the cities of Arcata and Point Arena), Hawaii (Hawaii County), Maine (Town of Montville), Oregon (Jackson and Josephine counties), and Washington (San Juan County). Generally, these laws state that it is unlawful for "any person or entity to propagate, cultivate, raise, or grow genetically modified organisms" in the specified county. Some versions of these laws are more stringent, making it unlawful to "sell, distribute, propagate, cultivate, raise or grow seeds or crops of genetically engineered organisms" (Arcata Ordinance 1350; Point Arena Code § 8.25). Other

[1]Text that reflects November 2014 election results and results of rulings and lawsuits in Hawaii were added in January 2015.

versions, such as that of Hawaii County, impose a general prohibition on the open-air cultivation, propagation, development, and testing of genetically engineered crops or plants, but exempt certain crops (*e.g.*, papaya) and allow the continued cultivation on land where GMOs were planted before the ordinance became effective (Hawaii County Code § 14-128).

A number of states have passed legislation prohibiting counties from adopting ordinances regulation GMOs or proclaiming such ordinances to be preempted by state law. Oregon, for example, passed bill 863 preempting counties from enacting GMO bans, although Jackson County was exempted from the ordinance.[2] Notwithstanding state preemption, Lane and Benton counties in Oregon are attempting to get similar bans on their ballots for the November 2014 election. More than a dozen other states have laws prohibiting county or local regulation of GMOs.[3] California and Hawaii are not among the states to pass such a law, which is significant because Humboldt County (Measure P) and Maui County have ballot initiatives that would prohibit cultivation of GMOs that are slated to be included as part of the November 2014 election.

While not a ban *per se*, Kauai County, Hawaii, passed Ordinance 960 that contained four operative provisions that imposed various notification requirements on commercial agricultural entities, created pesticide buffer zones, mandated a county environmental and public health impact study, and provided penalties for non-compliance. Specifically, the notification provision required commercial agricultural entities to make annual public reports to the county disclosing the growing of GMOs, including a general description of each, its geographic location, and the date on which it was planted.

Litigation

In Hawaii, Syngenta, Pioneer Hi-Bred, Agrigenetics, and BASF Plant Science challenged the Kauai County Ordinance 960 on numerous grounds including state and federal preemption. On August 23, 2014, the US District Court for Hawaii held that Ordinance 960 was preempted by state law. It also held that the law was not preempted by either the Federal Insecticide, Fungicide, and Rodenticide Act or the Federal Coordinated Framework for biotechnology. Nevertheless, the ordinance was struck down.

In June 2014, several associations, including the Biotechnology Industry Organization, filed suit challenging the Hawaii County ordinance alleging that it was preempted by both federal and state laws, violated the commerce clause of the US constitution, and was illegal under the Hawaii constitution. In July, the plaintiffs filed a motion for partial summary judgment on their preemption claims. The case went before the same judge that decided the Kauai County ordinance. After hearing oral arguments in October 2014, the court found that the Hawaii County ordinance preempted and enjoined its enforcement.

[2]Although Josephine County, Oregon has approved a GMO ban, it is expressly preempted by state law.

[3]These include Arizona (SB1282 Passed 4/22/05); Florida (HB1717 Passed 5/6/2005); Georgia (SB87 Passed 2/18/2005); Idaho (HB38 Passed 3/23/2005); Indiana (HB1302 Passed 3/25/2005); Iowa (HF642 Passed 4/6/2005); Kansas (HB2341 Passed 4/1/2005); North Dakota (SB2277 Passed 3/16/2005); Ohio (HB66 Passed 6/30/2005); Oklahoma (HB1471 Passed 4/18/2005); Pennsylvania (HB2387 Passed 11/29/2004); South Dakota (SB152 Passed 2/25/2005); Texas (HB2313 Passed 6/17/2005); West Virginia (SB580 Passed 4/16/2005).

On November 4, 2014, Maui County passed a ballot initiative that imposes a moratorium on the growth and cultivation of GMOs. A group of organizations and companies filed a suit challenging the Maui County moratorium in federal district court, raising the same arguments as against the Kauai and Hawaii County laws. Given the court's previous rulings on similar laws, the Maui County moratorium will likely be defeated as well.

Laws Regulating GMOs

A number of states have reserved the right to regulate GMOs by requiring permits prior to open-air cultivation. Idaho, for example, restricts the "shipment, introduction into or release within this state of any…genetically engineered plant…except under permit issued by the department, or as exempted by a rule" (Idaho Code § 22-2016). Minnesota (Minnesota Statutes § 18F-07), Nebraska (Nebraska Revised Statutes § 2-10, 113), Oklahoma (Oklahoma Statutes §2-11-36), Wisconsin (Wisconsin Statutes §146.60) and Washington (Washington Revised Code § 17.24.051) have similar laws.

These provisions require notification of, and approval by, the state before any GMO is grown within the state. Most of the laws contain exemptions where the Animal and Plant Health Inspection Service has permitted the release*, e.g.* Wash. Rev. Code § 17.24.051: "A special permit is not required for the introduction or release within the state of a genetically engineered plant or plant pest organism if the introduction or release has been approved under provisions of federal law and the department has been notified of the planned introduction or release." Some laws grant the state the authority to quarantine an area or take other action to prevent the spread of a GMO that is considered to be a noxious weed (Wash. Rev. Code § 17.24.041).

GMO Labeling Laws

There are two types of labeling laws: those that apply to food and those that apply to seed.

GMO Food-Labeling Laws: Adopted and Proposed Legislation

Only four states (to date) have passed GMO food-labeling laws: Alaska, Connecticut, Maine and Vermont. Of these, only the laws in Alaska and Vermont have immediate effect. The laws in Connecticut and Maine are not currently enforceable, but rather are contingent upon other states passing similar legislation. Connecticut's law requires four states to enact similar legislation, one of which must border Connecticut and the aggregate populations of the other states must total at least 20 million people. Maine's law becomes effective when five contiguous states enact similar legislation. Both of these laws seemingly require New York to pass a similar labeling law in order to become effective.[4]

Of the laws in effect, only Vermont's has real consequences because Alaska's pertains only to seafood, of which no GMO varieties are currently available. The Vermont law

[4]Maine also has a currently effective labeling law that establishes requirements for labeling a food as "as free of or made without recombinant deoxyribonucleic acid technology, genetic engineering or bioengineering." Maine Revised Statutes, Title 7, §530-A. This law allows "any food 1% or less of which consists of genetically engineered ingredients to be labeled as free of genetically engineered ingredients."

requires any food offered for retail sale that is entirely or partially produced with genetically engineered ingredients to be labeled (Vermont Statutes Annotated Title 9, § 3043). It also prohibits any GMO food from bearing the term "all natural" on its label. It defines genetic engineering as the process by which food is produced through "*in vitro* nucleic acid techniques" or the "fusion of cells" or "hybridization techniques that overcome natural barriers, physiological, reproductive, or recombination barriers, where the donor cells or protoplasts do not fall within the same taxonomic group, in a way that does not occur by natural multiplication or natural recombination" [Vermont Statutes Annotated Title 9. § 3042(4)].

The Vermont law lists a number of exemptions from the labeling requirements. Excluded commodities include food derived from a non-GM animal that was fed GMO feed or injected with a genetically engineered drug, non-GMO food processed with one or more genetically engineered processing aids or enzymes, alcoholic beverages, food containing 0.9% or less (by weight) of genetically engineered materials, food served at restaurants or intended for "immediate human consumption," medical food, and food certified by an independent organization to be non-GMO (Vermont Statutes Annotated Title 9 § 3044). The failure to label a food that is not exempt subjects a liable party to a penalty of "not more than $1,000.00 per day, per product" (Vermont Statutes Annotated Title 9 § 3048).

The Maine and Connecticut laws are nearly identical to Vermont's. They both use the same definition of "genetically engineered," require the same disclosure obligations, provide for the same exemptions, and impose the same penalties. Maine's law explicitly precludes citizen suits, however, and Connecticut's law contains an additional exemption for food sold at farmers' markets.

In addition to states with existing laws, a number of proposed laws will appear on state ballots in November 2014, competing with federal bills addressing GMO labeling.

Both Oregon and Colorado have certified ballot initiatives on the November ballot. These proposed laws are similar to Vermont's, with a few differences. Colorado's proposed law would have exempted chewing gum in addition to the listed exemptions that are the same as the Vermont law. Oregon's proposed law has fewer exemptions, as it contains no provision excepting food from animals fed or treated with genetically engineered food or drugs. Oregon's proposed law also differs from the others in that it contains a citizen-suit provision allowing a citizen acting in the public interest to bring an action to enjoin a violation of the law (and if the citizen prevails, (s)he is entitled to attorneys' fees and costs). Both of these ballot initiatives were defeated by voters in the November election, however.

California, New York and Hawaii have attempted to pass labeling legislation through the state legislative bodies. None of the bills introduced in the previous legislative sessions garnered enough support to make significant progress. In fact, one bill in New York was voted down before making it before the New York Senate. In Arizona, a group was able to put together a ballot initiative, but failed to obtain the requisite number of signatures. Thus, the initiative did not appear on the ballot.

At the federal level, there are two competing bills—one that would mandate labeling and one that would provide for voluntary labeling and preempt state labeling laws. Senator Boxer and Representative DeFazio introduced identical bills in the Senate (Bill 809) and House (Bill 1699) that would amend the Food Drug and Cosmetic Act (FDCA) to require labeling of GMOs, dubbed the Genetically Engineered Food Right-to-Know Act. These bills adopt the same definition of "genetically engineered" as the Vermont law and provide for similar exemptions. The bills are silent on the issue of whether food from animals fed or treated with genetically engineered food or medicine would need to be labeled.

Representative Mike Pompeo, along with four co-sponsors, introduced the Safe and Accurate Food Labeling Act (H.R. 4432) that would establish voluntary labeling standards regarding GMOs and require labeling only where a material difference in a food exists. The bill also amends the FDCA to create a mandatory premarket notification and review process that would obligate the developer of a GMO to notify the Food and Drug Administration (FDA) that a GMO is being proposed for food use. The FDA would review and evaluate the application and inform the applicant whether the agency agrees with the determination, objects to the determination, or concludes more information is required to make a determination. The bill would set national labeling standards regarding the presence or absence of GMOs and preempt all state GMO-labeling laws.

GMO Seed-Labeling Laws

Several states have enacted laws that require labeling of GMO seed. Under Vermont law, "[f]or all seed containing genetically engineered material, the manufacturer or processor shall cause the label or labeling to specify the identity and relevant traits or characteristics of such seed, plus any requirements for their safe handling, storage, transport, and use, the contact point for further information and, as appropriate, the name and address of the manufacturer, distributor, or supplier of such seed" (Vermont Statutes Annotated, Title 6 § 644). In Virginia, "[f]or transgenetic seed, in addition to any other requirements, the guarantor shall label all seed produced from transgenetic plant material pursuant to regulation" [Code of Virginia § 3.2-4008(K)]. In Maine , "the manufacturer or seed dealer of the genetically engineered plants, plant parts or seeds shall provide written instructions to all growers on how to plant the plant parts, seeds or plants and how to grow and harvest the crop to minimize potential cross contamination" [Maine Revised Statutes, Title 7, §1052 (2001)].

GMO Labeling-Law Litigation

In Vermont, a group of plaintiffs, led by the Grocery Manufacturers Association, is challenging the state's GMO food-labeling law alleging that the law violates:

- The First Amendment's protection against forced commercial speech in requiring a label;
- The First Amendment's protection against restricting commercial speech for preventing the use of the term "all natural" on food required to be labeled;
- The Fifth Amendment's due process clause for containing vague terms regarding the restriction of using terms "similar" to "all natural";

- The Commerce Clause for imposing unreasonable burdens on manufactures outside of Vermont; and
- The Supremacy Clause on account of the fact that the law conflicts with federal law.

The state filed a motion to dismiss the complaint for lack of standing and failure to state a claim. That motion has not been fully briefed.

PETER WHITFIELD'S practice focuses on environmental litigation with an emphasis on natural resources law. A former attorney with the US Department of Justice in the Environment and Natural Resources Division, he has served as lead counsel in numerous cases defending projects against legal challenges under various environmental statutes including the National Environmental Policy Act, the Federal Land Policy and Management Act, the Endangered Species Act and the National Forest Management Act. He defended the Animal and Plant Health Inspection Service in federal court against a challenge to permits allowing the cultivation of genetically engineered *Eucalyptus*.

He received his undergraduate degree from Duke University—where he double-majored in public policy and economics—and a juris doctorate and environmental law certificate from the University of Hawaii's William S. Richardson School of Law.

DR. WHITFIELD writes and presents on issues related to the labeling of genetically engineered food. His articles have appeared in *Law 360* and are featured on Mondaq, JD Supra, and Baker Hostetler's environmental law blogs.

Retailers, Trust and GMOs

Jeanne Colleluori
Wegmans Food Markets
Rochester, New York

jeanne.colleluori@wegmans.com

We have spent the last day or so talking basically about the scientific side of GMOs. As a food retailer, Wegmans has to face the emotional side of this issue because it's our job to understand our customers' perceptions because their perceptions are their reality, whether they are based on fact or not. Furthermore, customers worry about different things for different reasons.

Before we talk about customers and GMOs, we are going to talk about bird beaks. When my son was little, we developed a secret code in case someone else had to pick him up from school. I would tell that person, "When you see him, say 'bird beaks.'" When he heard "bird beaks," he knew that anything else that person said was okay with Mom. Also, if I was away and I needed to call the house and get a message to him and I had to have someone else make that call, that person would say "bird beaks" and he knew that anything else that person said was okay with me.

"Bird beaks": two simple words with amazing power. Two simple words that garnered complete trust from the recipient.

This group is accustomed to setting scientific angles to consumer stories. Recently I had an interesting conversation with someone who said, "I don't understand why consumers don't trust us, because the science is all there." On the other hand, as retailers, we have to listen to consumers and try to understand their concerns and their perceptions in order to gain their trust.

Trust and Transparency

Transparency without trust is useless, and trust doesn't happen overnight. You can't demand it. You have to earn it and you have to build it—block after block after block, day after day, week after week—and never stop. Trust and transparency are intricately intertwined. You can't wake up tomorrow and say, "Today's the day we're going to be transparent," because, if we haven't give consumers "bird beaks," chances are they are not going to believe us.

Wegmans is a chain of 84 grocery stores, based in Rochester, New York, and situated in six states. We have 44,000 employees and we work hard to ensure that our customers trust our transparency. Sometimes that entails discussing ugly topics. Sometimes it means admitting that we made mistakes, and sometimes it means saying that we are sincerely sorry. Those are all difficult conversations, but, because we are willing to have them, they make our company more human to our customers. This year, it's estimated that our consumer-affairs department in Rochester will handle approximately 100,000 customer comments, and we reply to all of them. They come in through emails and phone calls and tweets and blog responses and Facebook postings and letters, and sometimes it's someone who—believe it or not—just walks in the front door of our corporate office and says "I want to complain about something," and we go out and we talk to her/him. Some of the questions are easy, for example:

- At what time does your pharmacy open?
- Do you carry Grandma Brown's Baked Beans?
- Can I cash a payroll check at your store?

We can answer these quickly and the customer is on her/his way. But, sometimes, they have concerns that require a lot more of our attention and a lot more of our time.

Figure 1 provides a sampling of the 100,000 comments that come in each year. Some are based on moral issues such as animal welfare. Customers may say, "That's just wrong and I don't want anything to do with it." Other questions or concerns relate to safety issues, and I use product recalls as the example. What we hear from some customers is, "Oh my gosh, how do I know if my family is safe? Did I buy something that is really going to hurt my family?" Some other comments combine moral and safety concerns, and I'm using GMOs as that example. What we hear from those customers is the combination of "That's just wrong; I don't want anything to do with it" and "How do I know if my family is safe." Some customers simply seek reassurance, whereas others are more invested and want more information. We have found that the best way to deal with that is to offer answers to frequently asked questions (FAQs). We provide these answers to our store employees and to our call-center employees, and, for many customers, that suffices. But then, some customers are much more vested and want detail; they want to dig down into the "dirt." In such cases, we rely on third-party experts: "If you don't believe us, here's a couple of other organizations that may help you to better understand the situation." These customers are thirsty and what we try to do is give them a glass of water and let them decide how much they want to drink.

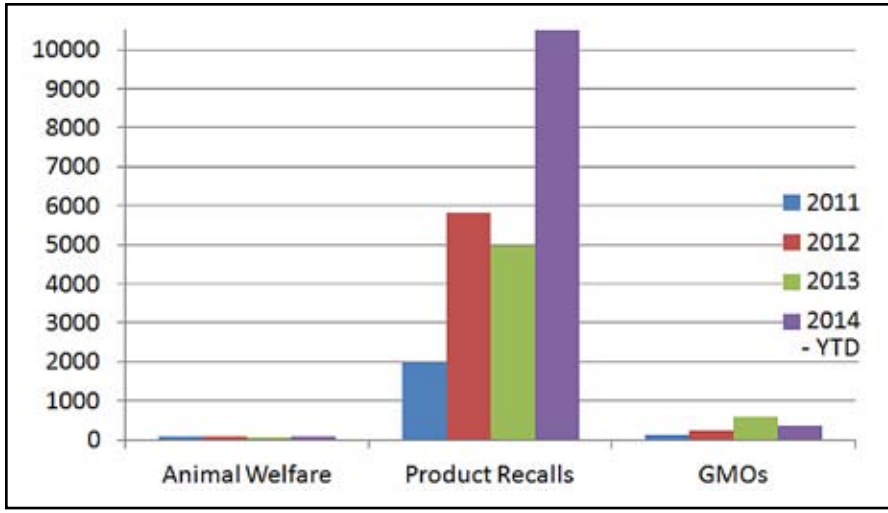

Figure 1. Incoming customer concerns.

In 2012, there was an intensely hot topic that the media affectionately termed "pink slime," and all who worked in the food industry were amazed, if not terrified, at how quickly the public demanded transparency. As the meat-retailing industry scrambled for answers, it was uncomfortable for our employees to say—and for our customers to hear—"We just don't know. We don't have answers yet for you." We weathered the storm, but we came out the other side questioning how to prevent being put in the same position again, to prevent our employees being faced with customers demanding information immediately.

Emerging Issues Taskforce

It is impossible to predict what the next surprise "pink slime" will be, but we formed what we call our Emerging Issues Taskforce, gathering, from around our company, experts in:

- Meat and seafood,
- Animal welfare,
- Organic foods and nutrition,
- Sustainability and procurement, and
- Communications.

The taskforce meets quarterly or more often if we have to, and at each meeting each of the experts brings a list of ideas that they think might be the next hot topic. We put these ideas out on the table, we swirl them around, we play with them, we talk about them and eventually a few of them bubble up to the top. We break into sub-groups to focus on particular topics:

If this happens, what are customers, going to ask? If they ask that, how will we answer? If we can't answer, which third parties can help us out?

We start creating the FAQ documents and contact third-party experts to seek expressions of willingness to speak on particular topics, and we document who they are and how we can reach them. We file that information away knowing that we will have to tweak it based on the details of the story that hits the media. But our preparatory work means that we may be better prepared to deal with the next food apocalypse.

Every Communication a "Bird Beak"

Every communication that comes from our company can be a "bird beak." It can build trust or not, including labels, brochures, press releases, tweets, Facebook postings, blogs, emails, phone calls and even personal conversations between our employees and our customers. Accordingly, we need to plan them, write them and deliver them carefully. Figure 2 provides an example from a few years ago. A man named Roger contacted Debbie at our call center with specific questions about GMOs. She did her research and got back to him and then he got back to her. The most important part of his comment was "Thanks for the honesty, it is becoming rarer and rarer." That comment makes me a little sad because it shows that he didn't expect us to be honest. He expected us to try to fool him. But we were honest and he recognized it and he appreciated it enough to get back to us. He then had a little more trust in Wegmans.

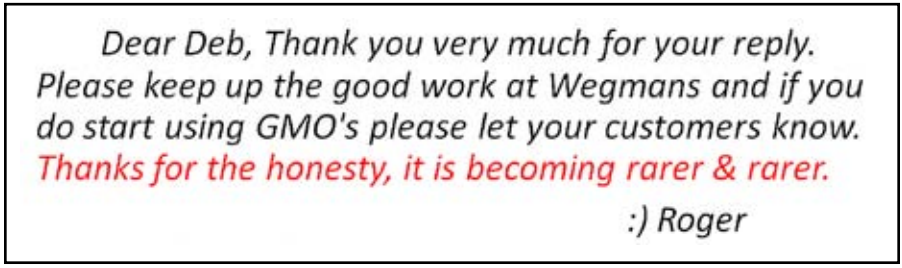

Figure 2. Every single communication can be a "bird beak."

Furthermore, every single employee, 44,000 of them, can be a "bird beak," and that's a little scary. We have to hire them, train them and support them so that they feel good about our company, not just when they are sitting in the office talking to a customer, but when they are sitting in their neighbors' backyards at picnics and someone says, "Hey, how's everything going at Wegmans?" Whatever they say about our company either builds trust in us or it doesn't. So we have to be cautious about whom we hire, how we train them, and how we support them.

A couple of years ago when Hurricane Sandy came roaring up the east coast, although our store in Ocean Township, New Jersey, was particular hard hit, we stayed open. A couple of weeks afterward, a customer got in touch (Figure 3).

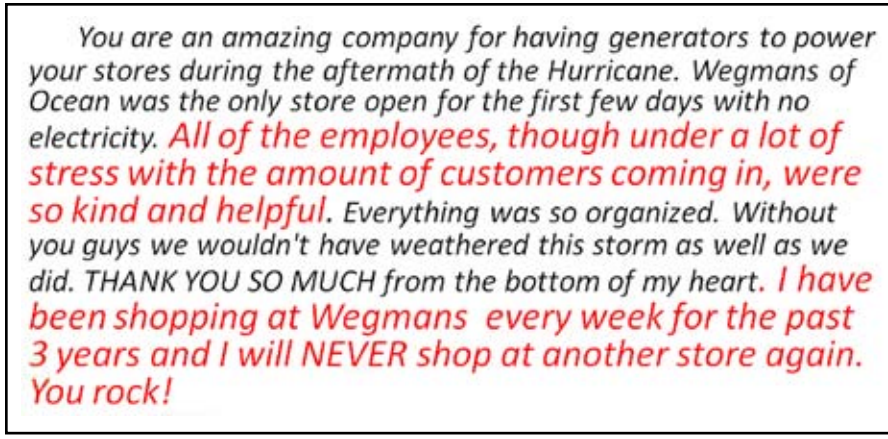

Figure 3. Every single communication can be a "bird beak"–2.

I think that the writer was saying that our company rocked, but, to me, our employees at the Ocean store, excelled. Those employees also had lost their homes. They also had no power. They didn't know where their next meals were coming from. They were living in the same circumstances as their customers, but they showed up at work every day, put on a smile, delivered service and built trust in our company. They were our "bird beaks."

As a retailer we have to identify our "bird beaks." We have to know what our employees need to build trust so that they, in turn, are loyal to the company. And we have to build that trust every single day. We have to tell our story whether it is good or whether it is bad. We have to admit when we are wrong. We have to admit when we don't know the answer. We have to promise to find answers and then we have to find those answers. "Bird beaks."

The GMO Debate

Turning to the GMO debate—customers on all sides of the table are passionate on this subject. They have strong feelings, as illustrated in Figure 4. No matter what one person says, an opposing point of view exists.

Pro-side	Con-side
GMOs can feed the world	GMOs are about profit
GMOs are better for the environment	We don't understand the GMO impact
GMOs enhance farmers' profitability	Corporate vs Family Farms
GMOs are safe to eat	Safety studies are biased

Figure 4. Customer debate on GMOs.

Because there is no standard for how GM foods should be labeled or identified, retailers are justifiably trying to satisfy their own customers. Whole Foods says that, by 2018, everything in their stores will labeled as to GMO content and Trader Joe's says that they won't allow GMO ingredients in foods bearing their private labels. These retailers are satisfying their own customers, but the consumer who goes from store to store to get the best deals is likely to be confused.

Then you have the state debate. Right now, Connecticut and Maine are close to mandating labeling, but there is some important trigger language, especially in Connecticut: they need four nearby states with a combined population of 20 million to similarly instigate GMO-labeling laws. Vermont doesn't have any trigger language, but they are being challenged by the Grocery Manufacturers Association. And then there are local debates; one of the Hawaiian Islands has pending legislation in two counties. Oregon has some legislation in place.

Quandary

How in the world are we supposed to label products when each state may have a different rule? As a retailer, we don't have warehouse space to store chocolate cookies to be sent to Maryland in a different location from those to be sent to Virginia or Pennsylvania. Furthermore, we don't want to have to print different labels for different states. Another problem lies in labeling a multi-ingredient product like chocolate cookies in terms of GM content. Figure 5 shows such a label; any one of the ingredients marked with an asterisk could potentially come from a GMO. We would have to figure out how to get that label with extra information into that space and we would have to provide a paper trail. Also, our suppliers would have to provide paper trails. And their ingredient suppliers would have to provide them, all the way back, to determine whether each of these ingredients comes from a GMO seed or not. Difficult stuff.

Customer Input

What do we hear from our customers? In early 2014 we were receiving about 40 comments per month concerning GM foods, which, within the grand scheme of things of 100,000 comments a year, is not very many. On the other hand, of the comments we receive about food, most concern GMOs. Figure 6 shows the types of questions being asked. Our Emerging Issues Task Force has decided that this is will be a focus topic. We have created an FAQ list, defined our position, and brought in experts from the opinion spectrum including the Center for Science in the Public Interest, the Non-GMO Verified Group and the Environmental Working Group for consultations. From these discussions, we developed a position statement (Figure 7).

For those wanting to avoid GMOs, we point to the growing section of certified organic products. We believe that it is now time for the FDA—through an act of Congress, if necessary—to mandate pre-market approval for all foods produced by GMO technology. And we believe that a national standard should be created for labeling non-GMO foods. The important thing right now is to remember that, as a retailer, at Wegmans we believe we are not a selling agent for manufacturers—we are not in the business of selling their foods. We are in the business of buying the foods that our customers want. This is on our website and people can click through to an FAQ and see the third-parties.

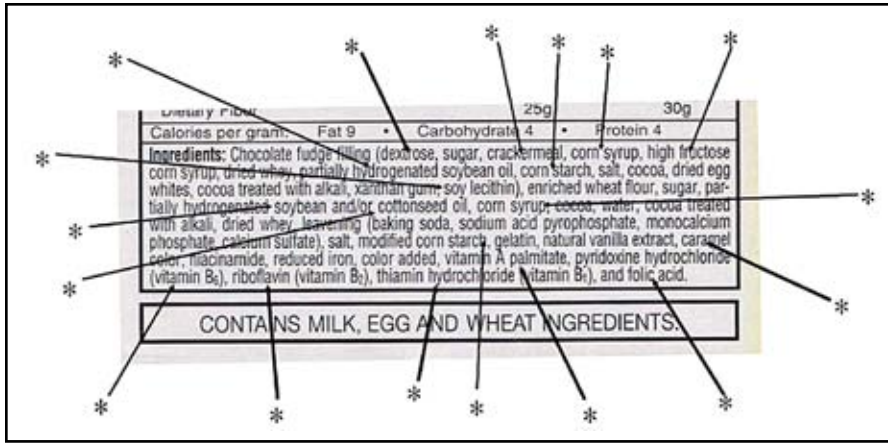

Figure 5. Could be from a GMO…

- In early 2014, receiving 40 comments/month
  - Does this product contain…
  - Can certified organic have GMOs?
  - What is Wegmans policy?
  - Why can't you label GMOs?

Figure 6. Customer input.

- Our job is to bring you a wide array of safe, delicious, healthy foods at consistent low prices. **For those wanting to avoid GMOs we point to a growing selection of certified organic products.**
- Here's what reassures us about the safety of GMO foods and ingredients currently available.
- All GMO seed companies have consulted with FDA on each potential product. According to FDA, the seed companies have satisfactorily completed every additional test that FDA has asked for, even though this consultation is not required by law.
- The testing protocols FDA uses are well established by a global collection of scientists from 168 nations.
- **We believe that it is now time for the FDA, through an act of Congress if needed, to mandate (officially formalize) pre-market approval for all foods produced by GMO technology.** This would assure us that all *future* GMO products will meet these well-recognized food safety standards no matter what country or company develops them. Furthermore, **we believe that a national standard should be created for labeling non-GMO items.** In that way consumers wanting to avoid GMOs can have confidence that consistent, non-misleading definitions are being followed no matter who distributes the product.

Figure 7. Position statement on GMOs (www.wegmans.com).

In addition, our senior VP of consumer affairs, Mary Ellen Burris, wrote a blog about this and our CEO, Danny Wegman, published a letter about GMOs in our *Menu* magazine, which comes out four times a year. Beyond those two communications, we also went on the road to train our employees—our store employees and our call-center employees. We even brought our top executives into the room and gave them the road show so that they could understand what GMOs are and how we are addressing the issue with our customers.

After Mary Ellen's blog went out, we received over 1,000 comments, 60% of which were negative including "I'm disappointed," "Whole Foods thanks you," and "Mary Ellen Burris should be fired." As we watched these 1,000 comments come in, very rarely did we have to join the conversation. We were able to watch the conversation amongst our customers; for a lot of the negative comments that came in, some positive comments came in as well, including "Did you really read the information they have there? A lot of the things you are worried about are addressed on the website." We learned that many customers don't trust the FDA or the biotech companies. They believe GMOs are harmful to their health and they want labeling for the presence of GMOs. That's where we differ, because we feel that, if there is going to be labeling, it should be for the *absence* of GMOs. No label states "This product is high in salt," whereas it may state "This product is low in sodium." We don't say "This is a high-fat potato chip," whereas we may say "This is a low-fat potato chip." Therefore, why would we say "This contains GMOs"? Why don't we just say, "This doesn't have GMOs"? It would be in keeping with the labeling that our consumers are accustomed to.

As said, in early 2014 we were receiving approximately 40 calls a month on GMOs. Since then we have put our information on our website. We have blogged about it and put it in a letter. We trained our employees so they can talk to our customers about it. Only 15 calls came in during the month of September, 2014. So the interest has dramatically dropped. We do feel this is probably temporary because, as elections come closer, it's probably going to be a topic of debate, a topic in the media. So, it will probably pick up again. But that's okay. We can talk about it. We can answer questions and do further research if we have to. In the meantime, we put the information on our website, Mary Ellen has blogged about it, Danny has written about it, and we have trained our employees so they can talk about it. These are our "bird beaks."

Retailers, Trust and GMOs

Q&A

Moderator: Margaret Smith
Cornell University
Ithaca, New York

Karen Kindle (Boyce Thompson Institute): Have you taken, or are you planning to take, your message about labeling products that have no GMOs to any of the political debates and conversations that are ongoing?

Jeanne Colleluori: We are members of the Food Marketing Institute here in the United States, which is a food-retailers' association. They were in the process of developing a position statement for the industry and we actually had gotten out in front of it. We were a little more prepared and so they came to us and asked *what did you do, whom did you talk to, and how did you go through the process?* We shared all of our information with them. They went out and polled their other members, and their position statement is actually very similar to ours. They are located in Washington, DC—on the hill all the time. So the message is getting out through FMI. That's where the grocery industry is heading, at least where members of FMI are looking.

Alexa Schmitz (Boyce Thompson Institute): Have you noticed any differential changes since you've made this announcement? I was here in Ithaca and I got all of the news and I heard how people felt about it here, but you are across state lines. Can you comment on how different states have reacted, either through feedback or through customer numbers?

Colleluori: We haven't looked on a state-by-state basis because, a lot of times, when customers comment to us—especially when they make negative comments—they are not always willing to tell us truthfully who they are or where they live. So, we don't really have a great idea regarding customer concerns on a per-state basis, but we are obviously keeping our eye on different types of pending legislation and proposed bills in the six states where we do business. We are keeping our eye on that.

Ralph Hardy (North American Agricultural Biotechnology Council): How have you dealt with the transparency issue on "no-GMO?" What's being classified as "organic" is certainly going to contain traces of GMO. The organic business is going to hurt itself until it establishes minimal thresholds. How are you dealing with that issue?

Colleluori: What we've been doing is saying to our customers is certified organic products don't come from GMO seed. That's the way we are addressing it, and we can go into more detail with customers if we need to. But that's exactly why we are saying that the labeling issue needs to be dealt with at a national level rather than state-by-state, county-by-county, or town-by-town. Until we have a standardized version of labeling, it just does not help the customer at all.

Adam Bogdanove (Cornell University): Like a lot of people in Ithaca, I love Wegmans and I trust them.

Colleluori: Thank you. Our bird beaks are working.

Bogdanove: I was bitten by the bird beaks. This is a follow-up on how representative are your customer comments. Eighty-four stores, six states, 100,000 comments a year—statistically speaking is that a good sampling of population to give you a good idea that the concerns expressed in that fashion are the most important concerns of your consumer base? The aphorism that the squeaky wheel gets the oil partly applies here, right? There are a lot of people who aren't sending in comments because they are happy.

Colleluori: Yes, you are exactly right. We know that, and, no matter what the topic is, if a customer is happy they are probably not going to call us and tell us about it, unfortunately. We hear from the people who have concerns, or just have questions. If you like a product or you like a service, I urge you to call and say so. But it's true, the squeaky wheel gets the attention; that's the only thing we have to base our work on.

Brian Iaffandano (Ohio State University): We've seen companies elect to put "non-GMO" labels on some of their products. Do you have any sense of how powerful that is in terms of monetary value or acceptance?

Colleluori: I can't speak for any other retailers, and we haven't started labeling like that whatsoever, so it's hard for me to address the power of applying such labels or symbols.

What we have done, so far, is say *if you are really concerned your best bet is going to be certified organic*, meaning that it did not come from a GMO source. We are seeing great growth in the area of organics. How much of that is related to GMOs, how much of it is related to sustainability, to local sourcing, to environmental concerns, is hard to tell. But we do see the area of organics as having continued growth potential.

JEANNE COLLELUORI joined Wegmans Food Markets in 1998 as a communications and media specialist for the Department of Consumer Affairs. Her responsibilities include research on, and communication of, ever-changing consumer concerns such as sustainable seafood, food safety and product recalls, genetically engineered foods, and animal welfare. Prior to joining Wegmans, she worked in the food brokerage industry for Daymon Worldwide, focusing on private label design for Wegmans. She also has held various advertising and marketing positions at Saatchi & Saatchi Advertising and Digital Equipment Corporation.

MS. COLLELUORI is a graduate of the College of Business at the Rochester Institute of Technology, Rochester, New York, is a member of the Food Marketing Institute's Seafood Sourcing Committee, and is a past founding member of the FMI Sustainability Executive Committee and FMI Consumer Affairs Steering Committee.

Tie-Up Session

Moderator: Karen Kindle
Boyce Thompson Institute
Ithaca, New York

Panelists:

Perry Hackett
University of Minnesota

Neil Hoffman
USDA-APHIS

Gregory Jaffe
Center for Science in the Public Interest

Drew Kershen
University of Oklahoma

Daniel Voytas
University of Minnesota

Donald Weeks
University of Nebraska-Lincoln

Karen Kindle: We will start with an overall question for the panel, then address some pre-submitted questions and then open it up to the audience for general discussion, because I think the conversation has been excellent during the audience Q&A. The thing I want to start with is—it's fun to go to a meeting and hear exciting talks and new ideas, but what is the impact when you go back to your day job? How do we progress from presentations to a call for action? In the areas of technology, regulation and public opinion, what should be the call to action for the participants of the meeting and for NABC? Who wants to start? Don?

Donald Weeks: This technology has potential to influence people's lives in a very positive way. It has the opportunity to impact how we feed the growing population we have across the earth. I see so many opportunities to make modifications to plants that are going to make real differences. I want to get back into the lab and help make that happen. What I'm not going to go back with, with great joy, is the realization that all my efforts up to this point—and maybe for the next few years—have come to naught because people who don't understand the technology, but who want to oppose it for whatever reason, are going to set up serious roadblocks to seeing the technology do good.

Daniel Voytas: We have focused a lot on how the technology can be used to create plants with new traits, and we have compared and contrasted it to state-of-the-art biotechnology. As a technology developer, I think that we can make it even more precise, more controlled, more predictable as to the outcomes of our genetic modifications, to minimize concerns about off-target effects. It's something we should continue to keep our focus on, so that when the public does ask questions we can say exactly what sort of modifications we are making.

Neil Hoffman: I prefaced my talk with some of what I had heard at this meeting. I have made additional contacts, including the kinds of information that Dan mentioned about additional reassurances from the risk point of view, which are always good to know. We have a long stair to climb and we need to go steady and keep pushing. I am glad to have met you people and any information that you have regarding safety may be useful for us in formulating our regulations.

Gregory Jaffe: I think that the take-homes for people in the audience are—and I would pick up on our luncheon speaker, Jeanne Colleluori[1]—transparency and providing information. Lots of great science is going on at universities and in industry, but don't stay in your ivory towers. Make sure that you are transparent about the information. There should be engagement and, especially, communication of science, not just to the public but, as opinion leaders, to the communities that you are in, as well as to the government. Government regulators are not what you think; they don't hear enough in comment periods from scientists—who can provide both technical and useful information—as well as from important stakeholders who need to be heard from.

Drew Kershen: I'm going to say, "I don't know." I don't know where we are headed. I really don't. I'm convinced that the science is sound. I am impressed—not just from this meeting but from other things I do—that the breadth and depth of the science is phenomenal. On the other hand, I am not convinced on my pessimistic days—and this is not necessarily a pessimistic day—but I am not convinced that this will go forward. The issues of public perception and how it is shaped are crucial. We had a very nice talk form Jeanne Colleluori about how Wegmans food stores are trying to navigate a very difficult field. But if the food retailers begin to say that we are not going to use these foods because we think that that is what our customers want, and if we get into additional international trade disputes, such as we are in with China right now, which has resulted in many expensive lawsuits against Syngenta—I'm not trying to favor one side of the other, I'm just trying to be a good reporter—I do not know which way it's going to tip. Someday, we may look back and say, "Why didn't we use this technology?" We've seen that already with Golden Rice, and we let thousands of children go blind and die each year as a result of regulatory obstacles that are basically unnecessary. You could release Golden Rice and let people try it. You may think I'm passionate, but I'm pretty good friends with Ingo Potrykos, one of the developers, and I'm not a quarter as passionate. Truthfully, Potrykos is in agony about

[1]Pages 223–230.

this because he spent his life trying to do something good for people, and he did. Now it's blocked and he's convinced he will die without ever seeing it help a single child. I'm not trying to be negative, I'm just saying I don't know which way it will go. I have my hopes and dreams and I have made a commitment to my own self and to other friends: I'm going to continue to speak and write and say what I think is the truth as best as I know it and stand up for it. If, at the end of the day I lose, I'll say that I tried my best.

Perry Hackett: I feel the same way, but I'm also taking back two messages to myself. One is that when I say you can't educate the public, I'm certain of it after today. I asked Greg Jaffe how he felt about GE foods, and he said, "Well I don't know." Come on! There aren't a hundred people in the world with your background and everything you've been exposed to, and you're not sure? You have patience. No one else has that patience. Not one aspect of GE foods has been brought up, that I know of, by anyone in any context, with animals at least, that offers any possibility—any possibility—of harm if it's not already harmful to the animal. I just don't know of any. And I don't know how you educate against proving negatives. That's what the public wants, but that's impossible. So that's one thing I take away. The second one is a simple question to the regulators: "Is it possible for a small company to get any GE product through?" And the answer is "no." Period. It's "no." It's the time that it takes. It's the expense of going through all the trials. I heard at this meeting that it costs $200 million—that's a maximum cost—to get deregulation of another event by a company that's already well established in the field. And Drew Kershen gave us the number $78 million so far for AquaBounty. Those are unrealistic numbers for people who want to bring some creativity into the world. So, where is it going? Well, you can be negative that it won't get out, or you can imagine that it's going to go underground. We can build essentially any genetically modified animal for about $100,000, and maybe about another $100,000 to show that it's actually healthy. This is leave-no-trace technology. There's nothing to show that if you do this in Brazil, Argentina or Nigeria and say, "Look, I have a new mutation; my cow doesn't have horns and I'll be sending 1,000 straws of semen to various places in order to get this genotype out," there is no way anyone can prove that that is not exactly what happened and the regulatory costs are essentially nil.

Kindle: Well, that is an interesting opinion and we hope that Drew will represent you and will be able to keep you out of prison. Let's go ahead to some maybe less controversial pre-submitted questions. First:

> *The specificity, the precision of gene editing depends on the target sequence and reagent, thus genome editing is not always precise. It's focus is on product. What are appropriate standards for determining whether or not there are off-target effects that affect the safety of the product?*

And second:

> *I agree that our regulatory system does not work well. However, should we be more forthcoming about off-target effects in our research and in these discussions, or does everyone really think they are so minimal we can ignore them without risk?*

Dan, why don't you start with this?

Voytas: As I have said, the technology is powerful and, in fact, off-target effects are rather infrequent. However, I think that we could set very reasonable standards for the types of products that are released. We have pretty good genome sequences for most of the plants and animals we work on. For a fairly modest amount of money we could determine the genome sequence in the plant or animal we are about to release to show that, in fact, the only mutation is the one we actually wanted.

Weeks: I'll second what Dan said. Especially with the cost of sequencing going down very quickly, it's not going to be a major burden to take the event that you want to bring to market, do a complete genome sequencing and show that there are no new mutations in any gene of any significance that you can recognize in the whole genome, and use that as good evidence that there are, likely, no off-target effects. One of the frustrations in science is that you won't get a scientist to say that that something is never going to happen. You can't be honest and say that. You can say that you have looked the best you can and the chances of something negative coming out of what you have done have very little probability. That you can do, and I think we will be able to say that in terms of looking at off-target effects.

Kindle: Thank you. The next question relates to the precision of the process and, in my mind, how much it can be scaled up:

> *Enzymes and transcription factors from different organisms have different efficiencies. Do these technologies allow for closely related enzymes and transcription factors, one or two different amino acids in the active site to be precisely modified, effectively mutagenizing a poor-efficiency enzyme or transcription factor into a more effective one found in other organisms?*

That's the first part, and I have a related question:

> *What is the potential throughput of this technology, especially for enzymes that can be tested only within their native context, and how much optimization of proteins should we anticipate?*

Voytas: With respect to the first question, we can go in and make pretty much any modification we want in any gene we want whether it has a transcription factor or not. That capacity is currently there. As we look towards the future, screening *in vivo* for variations of interest is certainly a possibility. Plant-pathogen interaction is often a receptor-ligand interaction that triggers a response, and so we have that continued war between plant and pathogen. You could imagine screening large numbers of variants *in vivo* with resistance genes that would recognize new ligands produced by various pathogen—a whole new approach jumping into the evolutionary process yourself and engaging in the war between plant and pathogen in a useful way. Those kinds of opportunities will be there as the technology improves and becomes more efficient.

Kindle: Let's move to the area of communicating with the public and public acceptance:

> *How should scientists address the public on the subject of gene-edited crops and livestock?*

And a second, related question:

> *It's clear that many people in this room can be thought leaders within their communities. Are there non-technical thought leaders who might be receptive to technical arguments?*

Could we go to Kim Kardashian or Justin Bieber? We don't want to waste our time talking to people who are never going to be open to the arguments, but having someone who has changed her or his mind or learned something and is willing to be a proponent seems to me an opportunity. I think Greg will be the person to address that.

Jaffe: A lot of people are open to scientific arguments. I didn't mean to leave the impression that they aren't. But, you can't assume that all consumers are going to act rationally or be convinced by scientific arguments. There are lots of opinion leaders and there are lots of nonscientists who will listen and be persuaded by scientific arguments. I think we need to do both. We need to both talk to people about the science and about the evidence that's out there—not dumbing it down but realizing that you may be talking to people of various backgrounds. You also have to understand, and not be frustrated by, people who do not agree with you even if you think that the science is overwhelming; they may base their thinking on nonscientific factors that are important to them, rendering the science irrelevant to them. There's a false sense that, if you keep saying louder and stronger that GMOs are safe, it is going to convince some people. There are lots of rational people and lots of organizations and government people and those who work for NGOs and others who are convinced by scientific argument. I'm often in scientific audiences who think, "If they only understood the science then, obviously, they would be with us." I want people to understand that that's not always the case.

Kindle: Heather Shearer[2] suggested a website that she has found useful. Are there are gaps we should fill, or is there plenty of available information that people can refer to?

Jaffe: There is definitely a lot of consumers or a lot of the public out there with perspectives about information, for example from corporations that may be right or wrong, but they do have that. And they may have specific views about specific corporations. The biotechnology industry has added a new "GMO answers" website thinking it would be the solution for this, that it would provide opportunities to interact more with consumers and answer their questions. That may persuade some people, however a lot of people will see it as a biased source of information and are more likely to be swayed by academics at Cornell and elsewhere. Although Wegmans is a corporation, it's a trusted source for many

[2]Pages 193–199.

consumers. Their responses to GMO-related frequently asked questions are scientifically accurate, understandable to their customer base and written in a folksy way that fits their corporate personality. That's probably not persuasive to 100% of their customers, but it is persuasive to a lot of their customers who visit their website and their stores. I often tell people to look at that because we need more similar communication from credible sources. And academics and universities can be included in those sources.

Voytas: The notion of consumer traits was brought up several times. We don't really have the capacity to market our technology, but with products like acrylamide-reduced potato and allergy-free peanuts—benefits that consumers can directly see—it will bring a link between the consumer and the science.

Kindle: I'll make this the last of the pre-submitted questions and then open the discussion to the audience. This one talks about the harmonization of GM regulations in order to maximize international trade, but while this works for large corporations it may not help small farmers. I think the questioner wants to explore plusses and minuses of having a more harmonized international approach to regulation.

Hoffman: I don't know the answer to that. It's hard enough to harmonize now and, if we change things, I can see that as a real key issue for us. I'm sorry I'm not the right person to answer that.

Jaffe: I caution people on harmonization. In the international arena, a lot of time when you have harmonized regulations, it ends up being a lowest common denominator. While we may all think that that is fine for GM, it might not be for *Salmonella* or for *E. coli* or Ebola or something else that we, as Americans, really want to trust our FDA on and may want them to have stronger standards than some other countries in the world. With that in mind what I do support, and strongly urge, is harmonization of requirements of scientific data and risk-analysis frameworks. To me, that is very important and I think that is something that can be achieved so that the scientific community and governments and others can come together and say, "These are where the potential risks are in a GMO for food safety, and these are the tests that are state-of-the-art and this is the way you do those to cut down on the cost factor." If all countries would harmonize that, then they may still make different decisions and they may have different timelines and different public-participation processes in the formulation of regulations, but they all will be looking at similar data, which for developers and scientists will result in savings in cost and time.

Kershen: Some other ideas on international harmonization—it would be helpful if countries would be willing to allow recognition of decisions of other nations. It would be helpful if you didn't have to go country by country because you have to repeat the data. Part of it is just what Greg said: the data requirements from country to country vary, but even if you had the same data requirements, the question is, "Do you require that they be redone for each country?" Let's say we're talking about growing. Well, our environ-

ment is different from that of Canada and you can carry that into, "Well, our county is different from the next county." How far do you carry that? And so the issue becomes, "How often do you have to repeat these?" It would be very helpful at the international level if we could just simply agree to give some level of recognition to the data in other dossiers done by other nations so that we don't have to constantly do it again and again. And there are, allegedly, mechanisms to do that through the World Trade Organization, but the WTO hasn't really addressed these issues significantly. A lot of the issues that I see in terms of barriers to international trade are actually violations of WTO agreements and obligations. However, that is a very difficult thing one, to prove, and two to enforce, and so international relations are still very much an area in which there are lots of treaties and regulations and laws, but the enforcement side in getting cooperation is still lacking.

Kindle: I would welcome anyone wishing to share what they will take home from NABC 26 and what they will do as a result of what they've heard. Also welcome are questions for our panelists. So, let's open it up now.

Reuben Tayenga (Washington State University): For those of us who are considering using these technologies to modify genes of interest, what are the chances of success at the first try? Secondly, what are the chances of modifying genes closely related to the gene of interest?

Voytas: A lot of people have used the technology successfully. Whether at first try, that's hard to say. Even in my lab, my graduate students and postdocs sometimes don't get it on their first try. Particularly CRISPRs are pretty transparent and easy to understand; protocols are well established and can be followed. With regard to your question about affecting closely related genes, it comes down often to the design of your nuclease. You can get a nuclease that will bind to one particular gene but not to another in the same gene family because of sequence variation and the DNA-binding recognition domains. So that is definitely doable to target one member of the gene family and leave other members unscathed. But it takes a little bit of effort and thought in terms of your nuclease design.

Weeks: On the other hand, you can knock out a complete gene family if they are very homologous, so there is a world of variation there. It's powerful.

Gary Rudgers (Dow AgroSciences): One thought I had on off-target effects and sequencing the entire genome—unfortunately, not only does the public, but also regulatory agencies in some countries, think that one corn-plant genome is identical to the next corn plant and the next corn plant, that there is no variability whatsoever. That's not true in all countries, but I know that it is true in some. So, when we look at off-target effects trying to sequence the genome to show that there is none would be an impossible task because you would then be asked to confirm and justify every single mutation that you identify, which could be in the thousands or even tens of thousands. What I would hope to see is comparison of the off-target effects with these technologies to what occurs naturally and

also to compare them to chemical and radiation mutagenesis. There have been conversations that the off-target effects could be significantly less using these technologies. That is something that does need to be addressed, and I think we need to put it in some sort of context there sooner rather than later, to help clarify that to regulatory agencies. I know that, in Japan, they are very interested in this. The regulatory agencies in Japan are concerned about off-target effects, even though they have been told that the off-target effects would be less than with chemical and radiation mutagenesis in which Japan is probably number 1 or number 2 of the countries that use products developed through radiation and chemical mutagenesis. So, I don't know if there are any thoughts or comments on that, but I know that reports on this topic would be extraordinarily helpful to clarify the off-target effects of these various technologies.

Weeks: I agree with that. I should clarify that I think that anyone who is setting out to do genetic modification with these technologies—who does want to compare genomes—that they start with "a" genome of "a" plant that the sequence is already known so that they can compare to that one plant not every plant out there.

Voytas: Robin Buell and Nathan Butler are here from Michigan State and we have a joint project to make a handful of modified plants from the same mutation in a herbicide-resistance gene using TALENs, CRISPR/Cas, EMS mutagenesis and transgenesis, introducing the gene, and then sequencing the genomes of those handful with individual events to get an assessment of the amount of variation you see. We and others are trying to gather that sort of data, which will be useful.

Patricia Polowick (National Research Council of Canada): Two things—one comment and one question, totally unrelated. The first comment is, I do think it is possible to move through the regulatory system more cheaply than what has been mentioned, with a small company. We've heard a lot about the Arctic apple and I know that's a very small company because two technicians have been embedded in my lab for a number of years. I know of only five employees in the company, including the owner, who is supported by other orchard growers around him and they've gone through the whole procedure one step at a time, outsourcing, for a lot less money than has been suggested. My question: I've caught the tail end of a series of commentaries—I don't remember the website—suggesting that some of the bigger companies, like Monsanto, offer money to anybody who wants to do research to prove that there are dangers to GMOs. Would there be any merit in that or would it attract only scientists already accepting of the science?

Kimble: Who would like to take that?

Weeks: I think that's a brand new idea to most of us. I've thought a lot about this. If I were one of those evil Saturday-morning-cartoon scientists, how would I modify a plant—and I guess this would pertain also to animals, but plants are my business—how would I modify that to do harm? And, boy! That's a tough nut to crack. I couldn't come up with what I

thought would be a significant approach to that. That realization tells me that this effort would probably not succeed. I can't say it wouldn't, but it's hard to prove a negative.

Voytas: Scientists are already contributing to that thought—Gilles-Éric Séralini, for example. You are saying that a company with deep pockets is offering money to a scientific group to show that GMOs are dangerous. We consider Séralini's experiment as bad science, so I'm wondering about the feasibility of this. Would the company be paying for bad science? In a Greenpeace-supported study, some Russian woman found that GM diets created harm in an animal-feeding study. There are several examples of that. How would you separate the bad scientists, the people who have an agenda and are receiving money? Now would they be getting it from industry to do the same sort of studies?

Polowick: I look at it more as "put up or shut up."

Voytas: There's a fairly large literature of poorly done studies that don't stand up to the weight of evidence, and now you're asking industry to feed those kinds of efforts. I think you would find takers, is what I'm saying.

Kindle: Let's move on to the next question.

Adam Bogdanove (Cornell University): I want to share some comments related to a couple of questions back about off-targeting. I advocate caution in generalizing about the precision of the technologies, because inherent to the technologies is selection of the target. And, depending on the target, your off-targeting will vary, either as a function of the sequence itself and how commonly related sequences occur in the genome or as a function of the reagent you are using, particularly in the case of TALENS in which a TALEN designed for that particular target may have greater or lesser specificity as a function of its RVD composition. The other part of that relates to Reuben Tayenga's question. Gene redundancy is a big challenge for gene-functional analysis. If you want to knock out many members of a family, I think you can do this, in some cases, with a single reagent. If, for one thing, as Dan Voytas says, you can find a conserved sequence that that reagent will target, but also—I just wanted to pitch this idea again—that with this sort of tunable specificity of TAL effectors where they can be engineered, we have examples from nature in which they have more or less stringent specificity, we can capture that and exploit that for engineering to get sort of a specificity profile with just the right degree of lax specificity to capture a series of paralogs that may have polymorphisms across. This off-targeting is something we seek to avoid in genome editing. But I think it's something we might be able to exploit for cases like this.

Hackett: I'd like to make a comment about off-targeting. I breezed over it in my presentation, but we can use human beings as an example, at least for mammals, in terms of genomic variation, because close to 10,000 human beings have been done now. There have been several triads—mom, dad and afflicted child—and there have even been some

quads done, which means close siblings or even twins, to try and narrow down the genetic basis of disease. Right from the get-go, we know that we each have about 6 million single-nucleotide polymorphisms. There are a hundred active retrotransposable elements in each of our cells. Most of them aren't actually activated and hopping around, but many of them do and the more we look the more we find. Generally, we just disregard those changes. We have any number of larger changes. Some of us may be afflicted with triplet nucleotide diseases and the like. We know that over 200 genes are inactivated in each one of us on average and 20 to 50 of those are, indeed, disease-related genes. So there is a context for off-targeting, but we have to appreciate that nature can take a lot of variability in genomes and I think that, in this room, all of us are doing just fine.

Abel Ponce de León (University of Minnesota): In the last 36 hours I have heard many, many interesting opinions, and I thank all the speakers for what they have presented and the audience for what they have contributed. Depending on where we stand, each one of us is trying to look for a response, a yes-or-no kind of situation. And I think this is not the case because there are very many positions and variations in between, and many very different opinions some of which we are witnessing right now. This conference is an attempt to assemble the available information in one place to try to see where we go from here into the future. I guess everybody wants the best outcome, never mind which position you have. The public, the consumers, the industry, the researchers—everyone—the goal is the same. But the art of negotiation to get to that point is what we need to master. I wonder what should be the objective of this conference. Should we start defining a path to take—because we are all here in some way representing different sectors—that will make this a successful outcome. And if it is possible to start working on definitions of what to do where we, the different sectors, become more comfortable and at least allow us to go 10 or 20 steps forward, without reaching the ultimate goal but at least moving in that direction. Can any of you address that?

Weeks: Certainly, I agree that this conference has been every informative. We have seen different sides of the issues from basic science to regulatory aspects to public acceptance or non-acceptance of this technology. Finding a way forward is terrifically complex though, because there are many aspects. Many people in science, many people in the regulatory agencies, and certainly many different voices out there in the public exist, and how you address all of those is a major challenge. But, I couldn't agree more with Jeff Wolt's comment that at least we in the science community have to be available to speak about these issues and tell things as they are, what the capabilities of the technologies are, and what these technological capabilities are not. If a pathway can be laid out that people can agree to, many will rally behind it.

Voytas: Many of us have gone to the USDA and asked, "Is our product regulated or not?" That is a great first step because it gives some definitive clarity to the modifications we have made and whether or not we can start to deliver products. I worry about coming back to colleagues again and again and saying the same things, but at least that is something tangible we can do and move forward.

Audience Member: Recently it was suggested that genome-editing technologies facilitate the gene-drive process which could lead to species extinction—elimination of mosquitoes, for example. In a letter to *Science*, an Israeli scientist suggested that gene-editing technology should not be made public because it could be useful to terrorists. He even equated it to the atomic bomb, which it certainly is not. What are your opinions on how to eliminate potential gene-drive issues.

Hackett: I made a prediction a month ago that, two years from now, that's all anybody will be talking about—how scientists will use gene drives to propel certain species to extinction, and that everything we are talking about today will be old-hat history and totally dead. In other words, I think it will terrify people what the power of homing nucleases can be when used for purposes of driving species to extinction. It's one step to say that you are going to have a gene drive in a mosquito that might bite a cow that is going to be eaten by a person for somebody to claim that it will drive human beings to extinction.

Ralph Hardy (North American Agricultural Biotechnology Council): I want to follow up on Abel's comment. We have here a strong representation of the scientific community. A contribution that could be made from this meeting is a definition of DNA editing. We need to define what we are talking about before we can start to sequence what the steps are down the road. So, I would appreciate it if we can have comments on what you think the definition should be. We at NABC will take your comments and synthesize an overall comment, and then send everyone a copy of that. If you cannot live with it, and I use that proviso, if you cannot live with the way it is, we want to hear back from you so that we can find something that you can support. If we can develop a statement like that from this meeting I think that it will be useful to a number of entities as we go forward. I would appreciate inputs on a definition for DNA editing.

Voytas: One reason for a definition is to "anchor" regulation and policy decisions. If you break or lose a DNA sequence—that form of editing—we have some regulatory guidance from the USDA that this is not a regulated article. But when you talk about replacement, it becomes more tricky. How many nucleotides over what span of DNA is it an edit versus a transgene? Is it 30 nucleotides over how many base pairs? It seems like you have to make some sort of arbitrary decision to guide you in then evaluating the plants and the modifications in them. I can edit a soybean into a corn plant. That's a significant amount of change that I am going to make to that soybean genome. I hate setting arbitrary decisions because I can also make 30 nucleotide changes in a kilobase, and I could create a very immunogenic protein that is going to cause allergies. You still have to have a case-by-case basis, but you need, I think, some framework for guidelines. I'm not comfortable saying what number of nucleotides for what span of DNA it would be, but I think maybe something like that is needed.

Hoffman: From a regulatory point of view, we are moving to a place where we are not concerned with what techniques are being used and whether one base is changed or a

million. We are interested in what the phenotype is going to be. Maybe I didn't emphasize this. If we change our regulations, we are going to be changing the regulatory trigger and this notion of what we are saying now *vis-à-vis* "Am I regulated?" may not hold true with a revised regulation. I'm not sure that I emphasized that enough. We are really interested in what the phenotypes of these organisms are, as opposed to what processes are being used. And that's the message I was hearing from many of you in the audience, that that is what's important.

Jaffe: Ralph, I can't give you specifics of the definition. I think it would be good to have a definition, but I also think that it's not the be all and end all, because, from a policy or regulatory or legal point of view, as we saw from Peter Whitfield[3], there's a bunch of definitions out there that may have all kinds of unintended consequences as technology changes. To me, the more important thing is flexibility. Flexibility to learn and then to adjust as needs be and as new technologies come along. It's important to put down what things are today, but you have to have the flexibility to put things in and take things out and move things around as we learn more and technologies change. Otherwise, we may waste a lot of effort if we are so set on one definition that it takes a long time to come up with a new definition. The better thing in the policy context is to have flexibility.

Hardy: This dialog is great. Let me put the definition off to the side. I am beginning to get a synthesis from you of what is important in the long term. The more comments we get to provide a good synthesis, the more productive this conference will be.

Weeks: If I may make a comment as a scientist involved in this technology—I don't want to put words in your mouth at all, Neil (Hoffman)—but if there was a paradigm shift in going from process to product, what a terrific event that would be!

William Haun (Cellectis Plant Sciences): When Jeanne Colleluori[4] was talking about labeling, the comparison she made was GMO to high-fat and high-salt food. She didn't compare non-GM to high fat and high salt, and. when telling us about her proactive task force, she compared GMOs to pink slime—not directly, but that was her train of thought. Even for the person we brought in to speak to a group that is very "pro-everything you've been talking about today," it was natural for her to make the comparisons of GM to high salt and high fat and GM to pink slime. I think that is interesting.

Kindle: I'd like also to return to consideration of semantics with another presubmitted question:

> *Should we help smooth the way with the public by choosing our terminology for gene editing to be more consumer friendly?*

[3]Pages 217–222.
[4]Pages 223–233.

You could expand that to other forms of GM-type technologies. There's an opportunity for scientists to get training in talking to lay audiences because it's difficult to take jargon out of our vocabulary. There are some excellent books about how to put together sticky messages and cursive knowledge.

Jaffe: It's important to make things accessible to the public; what you choose to call something can have significant impact. When I got out of law school, I considered myself an environmental lawyer and worked for eleven years with the government on pollution mitigation. I worked against corporate lawyers and people who were defending corporations who also considered themselves environmental lawyers. I didn't consider them environmental lawyers, but they tried to capture that phrase and call it their own. The language you use and what language gets captured in the debate is vital. In the United States I use the term "genetically engineered crops," which I think is more scientifically accurate. When I go to Vietnam or Kenya, for example, I use "GMO" because that is what they know and that is all that they know. But, in my view, that is not technically correct and has negative connotations and the people who initiated that term in some ways have won. They've started the discussion at a level that I think is loaded in some ways. I am forced to use "GMO" because if I say the other thing they don't know what I am talking about. When we move forward with these gene-editing techniques, what we call them and the language we use around them will be critically important.

Michael Kahn (Washington State University): There's another aspect to the problem that you are talking about, something I call "entanglement." When you start talking about GMOs and then you put that together with high-fructose corn syrup, for example, people will say, "Well, GMO high-fructose corn syrup is bad for you, therefore GMO is bad for you." And the science of GMO came from Monsanto, and that is a bad word. Perry did a very nice job of breaking apart a number of different arguments that are used in this area. What you find when you move into the public domain is that people slip from one of those topics to another very, very quickly. So that, if you are defending GMOs, you may find yourself defending high-fructose corn syrup when that wasn't your intent, or defending Monsanto's right to screw farmers when that wasn't your intent. One of the things that we have to learn to do is to break out some of these arguments and try to deal with them as unconnected, unentangled arguments. In most cases they can be dealt with one by one, but, when you are talking to the general public, they don't see these segments—it's all one picture, and I don't know how to deal with that. How does one keep control of the topic?

Jaffe: Your example could be another one of language. The high-fructose corn-syrup industry is now petitioning to call it "corn sugar" specifically for the reason that people are beginning to think that high-fructose corn syrup sounds artificial. I come back to the idea that things need to be put into context, and the public's lack of knowledge of agriculture and of science around agriculture is a real problem. We are always going to have trouble communicating about genetic engineered crops, for example, when people just don't

understand the breadth of what goes on in agriculture and what it takes to bring food to the table. To me, the most important thing is context. I don't have any good answers, but in CSPI's newsletter we deal with highly scientific topics and summarize the latest research on diet, cancer, *et cetera*. The people who write in my office do a really good job of taking highly scientific, technical peer-reviewed articles and put them in language that people can understand without dumbing it down. Our subscribers can understand the information and apply it to their diets. We need to have communicators who have those gifts to work with people like you and me.

Kindle: In addition to not understanding agriculture, many people do not understand how science works. And I'm not talking about the scientific method, I'm talking about the fact that if a scientist expresses doubt, it means something very different to a lay person than to someone who understands how the system works. Recently I read an interview with a plant molecular biologist in Florida and almost the entire exchange was about how science works. As scientists, we assume that everybody understands that, and yet they don't. They don't know what peer review is and how you prove or disprove a hypothesis. Things like that. So, there is an opportunity for that kind of communication as well.

Margaret Smith (Cornell University): A couple of comments about language, I would remind us all that "GMO"—also my least favorite term for genetic engineering—was coined by those who were trying to think of a term that sounded more friendly than "genetic engineering." It was actually coined by the people who supported that technology in order to make it sound softer and less like "nasty scientist in the lab" than genetic engineering. It's important to be thoughtful about your terminology, and you have to realize that it may be co-opted. The other thing I thought was interesting is the way we talk about this. One of the questioners talked about us being an audience who are pro-GM. What we should be is an audience who are interested in the pros and cons of particular phenotypes rather than being pro a tool. So, we also need to be careful how we think about ourselves. And, further to one of Greg's comments, I think we need a jingle and one possibility is "apply it to your diet."

Dana Carroll (University of Utah): Perry, I think it's okay if I tell people what Scott Fahrenkrug calls introgressing a natural trait into a different breed. He refers to it as molecular breeding, and I don't know if "molecular" will kick it out of camp, but it's another attempt to modify the terminology to separate what he has been trying to do at Recombinetics. I have a couple of comments, trying to make useful suggestions. People talk about Frankenfoods and Peter Whitfield[5] showed a picture of a banana with a fish head; so, I think that, in promoting what the technology can do, we need more pictures. It's a vocabulary that the public in general can understand better than arguments that are based on producing obligate heterodimer modifications with a cleavage domain with zinc-finger nucleases (which actually is a really good thing to do; it improves safety). So,

[5]Pages 217–222

one idea is to get more images that can be used in the argument. The other idea is to enlist local outlets to make the case in a way that is more easily digested. One example would be—I don't know whether this sort of thing has happened—where a small company has sprung up, perhaps a newspaper or TV or radio station could be enlisted to do a feature on the company from the perspective that it is locally advantageous economically. It's doing something that is positive, like an apple that won't brown. It would direct the conversation away from the international level and down to a local level and influence neighbors and friends with simple, locally understandable conversations, and lots of positive images may help.

Greg Gocal (Cibus): I'd like to extend that comment regarding influence locally and broadly. At Cibus we've had an internship program for forever. Over years we've had 30 interns who have worked through me, and even if they decide not to stay in science ultimately, I think it's a good way—just like talking to your neighbor—for them to acquire experience and knowledge about the sorts of things that we work on on a daily basis and the advantages of them. The more interns and the more academics talking to their classes about variation and how we can move that around and the new technologies that are beneficial, the more likely we are going to have broader influence. I can't remember where I heard this, but in our lifetime we probably meet 1,000 people or 10,000 people—in that range—and if we deliver the same message to each of those 1,000 or 10,000 people, we have the opportunity to share the message around the whole globe. And so we, individually, have to take responsibility for choosing the right words to deliver our message, and I agree that that is very important, and we have to tell people clearly why we are trying to do this in terms of food and the local population and the potential advantages. I will leave it there. This has been a great meeting and we have a collective challenge that we have to address.

Student Voice[1] *Report*

Patrick Di Bello[2]
U. Arkansas

Christine Kraus[2]
Boyce Thompson Inst.

William Serson[2]
U. Kentucky

Fu-Chyun Chu
NC State U.

Will Cody
Texas A&M U.

Brian Iaffaldano
Ohio State U.

Bolormaa Jamiyansuren
U. Minnesota

Ben Kilian
Washington State U.

Bastian Minkenberg
Penn State U.

Andrew Read
Cornell U.

Alexa Schmitz
Boyce Thompson Inst.

Poonam Singha
S. Dakota State U.

Caleb Squires
Washington State U.

Reuben Tayengwa
Washington State U.

Li Tian
U. Kentucky

Sarah Turner
U. Winconsin/

Marshall Tyler
Boyce Thompson Inst.

Indu Upadhyaya
Iowa State U.

The twenty-sixth NABC conference, held in 2014, *New DNA-Editing Approaches: Methods, Applications and Policy for Agriculture*, included presentations on developments, applications, and regulatory concerns for DNA-editing technologies, particularly targeted mutagenesis using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs).

The *Student Voice* discussions were focused mainly on the following areas:

- Scientific advocacy,
- Consumer benefits, and
- Regulation.

[1]The Student Voice program was inaugurated at NABC 19 in 2007 to promote graduate-student participation in the annual conference. Student representatives from NABC-member institutions attend the keynote and plenary sessions and meet separately to develop what they see as the most significant points of interest that emerged.

[2]Co-presenter of the *Student Voice* report at the conference.

Scientific Advocacy

Western civilization has a long history of scientific mistrust and speculation, perhaps most famously the conflict which existed between Galileo and the Roman Catholic Church during the Renaissance. Today, there is still a large part of the public which remains skeptical and adverse to Darwin's theory of evolution. While public distrust of good science is always troubling, in the two examples mentioned above, it mainly threatens the scientists' well-being, and does not directly affect the general public that the science of today so often tries to help. Major issues that will affect the planet and the human population at large in the twenty-first century include feeding nine billion people by 2050 and reducing the harmful effects of climate change by reducing greenhouse-gas production. One avenue that may help the hunger crisis while reducing inputs to agriculture, thus reducing carbon dioxide and other greenhouse-gas emissions, is the use of genetically engineered organisms, more commonly known as genetically modified organisms (GMOs).

In the late 1980s it became possible to deliver foreign genes into an organism, and have that gene up taken by the organism with expression leading to a more desirable phenotype. Many in the plant biology community, especially from the industrial sector, quickly noted the merits of such technology and the potential to help farmers and the public. Shortly after that, in 1996, Roundup Ready soybean varieties were released to growers, who benefitted by simplifying their herbicide-application regimens to only requiring the use of glyphosate, the active ingredient in Roundup herbicide. Other traits which were released include *Bt* corn and cotton, which conferred resistance to insect predation. After release of these traits and learning about the technology used to generate them, the public began to grow restless and wanted to know about the risks related to consuming such foods and the effects on livestock that ate the affected seeds. Since then, hundreds of studies have been released which indicate that GMO consumption does no harm to livestock or humans. However, non-government organizations (NGOs) such as Greenpeace have raised the alarm about the "dangers" of genetic modification and the harm that would befall the environment. In the true spirit of the precautionary principle and due to public pressure, government regulators such as the USDA and FDA quickly issued stringent regulations controlling GMO releases, virtually shutting the technology down for use in modern agriculture and stifling real life-saving innovations such as Golden Rice.

The resulting outcry from the scientific community, especially the agricultural biotechnology sector, quickly followed, sparking a public showdown between scientists, regulators, the public, and NGOs. Scientists responded quickly with a flurry of studies that demonstrated that there are no discernible health effects from consuming GMOs, and demonstrating that environmental concerns, while an issue in some circumstances, were largely overblown. In spite of this, regulation continues to be excessive and the consumer response has been lackluster, and many are still quite wary of GMOs in spite of overwhelming evidence that they are safe. This combination has left the scientific community quite frustrated, and many will not live to see their discoveries reach a hungry public. It is quite evident that if we want to see things change, we will need to change tactics, while being aware of those who have fought the battle before us, learning from their mistakes, and capitalizing on their successes. The timing could not be more critical; we may have the

opportunity in the near future to re-cast the narrative, as the public begins to understand the applications of the new DNA-editing techniques to plant and animal improvement, some of which will soon be up for review and will undoubtedly gain attention.

One of the issues we can examine is that of climate change. Evidence continues to stack up supporting the theory of global warming, and that the rise in temperature is tightly associated with levels of carbon dioxide and other greenhouse gases in the atmosphere. In spite of these facts, it is a politically polarizing subject in the United States, resulting in delayed action on climate-change legislation and the regulation of greenhouse-gas production. Rarely, though, does one hear about climate change from climate experts, and most information is disseminated through media outlets and politicians, who may suffer from greater levels of mistrust even than scientists! As greenhouse-gas emissions continue to rise, little is being done due to partisan gridlock while other countries have long ago transitioned to green energy solutions such as solar, wind and nuclear sources. As we cast alliances with public figures such as celebrities and politicians, we need to be wary that we do not alienate half of the country and create a partisan rift.

The pharmaceutical industry has been much more successful at pushing its products to the public and they now benefit a vast majority of American consumers, in spite of recalls and settlements when some users exhibit extreme side-effects. Though the industry is (rightfully) tightly regulated, many new products are released every year, in stark contrast to the virtual moratorium placed on GM crops. While few consumers are enthused about paying for and taking prescription drugs in the face of side-effects, millions do. This begs the question, why do people trust big pharma enough to risk side-effects and mistrust ag-biotech advocates the same way they mistrust climate-change advocates? How can ag-biotech avoid the polarity that climate change has engendered?

The key difference is advocacy. Pharmaceutical industries have thousands of advocates scattered throughout the country. These advocates are physicians and other medical practitioners who possess the ability to not only understand how drugs work, but also to make sure their patients also understand. While we may never be able to emulate for ag-biotech exactly what physicians do, we certainly can learn a good deal about the way they operate. First, they are actively engaged with the public on a daily basis. Though some of them could have been promising researchers, they chose instead to work with the public. In the same spirit, some up-and-coming leaders in ag-biotech will have to turn down careers in research and pursue avenues where they can engage the public, including legislation, policy, scientific communication and teaching at the secondary and post-secondary level. However, this does not release researchers completely from responsibility, and they can play a special role being on the forefront of innovation and having the land-grant system in place. Land-grant universities often include extension agents who go out and speak to farmers and the public in general about crops. It would not be challenging to set aside one day a year for a lab, or at least one of the graduate students, to organize a talk about GMOs and explain it from the perspective of someone doing the research. Other avenues could include public high-school science demonstrations and botanical gardens. Together we could form a network of advocates by sacrificing as little as one day per year and become a positive voice for ag-biotech.

Consumer Benefits

A frustrating issue that was raised in our discussion was the question, "How can we appeal directly to consumers?" Clearly, we need to hear more of genetically engineered products that directly benefit members of the public at large. Currently, the benefits of genetic engineering are clear to growers, but are largely hidden from consumers. A good example of a consumer benefit from agricultural biotechnology is the potato—a specialty crop—that produces significantly less toxic acrylamide during frying.

Products that lead consumers to realize that the quality of their lives is being improved by genetic engineering will open doors to improved communication, which, in turn will open doors to acceptance of other products of genetic engineering beneficial not only to farmers but also to consumers.

Regulation

A large portion of the NABC 26 meeting focused on regulatory concerns, both within the United States and internationally. It is clear that existing regulatory terminology fosters confusion about gene-editing approaches, even among leading scientists. During our conversation, it was unanimously agreed that a universal, standardized set of definitions should be developed and utilized to mitigate confusion about the regulation, adoption, and legislation surrounding gene-editing technologies and their resulting products.

As mentioned by many speakers during the meeting, there is a tendency to regulate the process in *lieu* of the product. This method not only adds many years to the regulatory process, but reduces the amount of resources devoted to testing the safety and quality of the actual product. Additionally, the extensive regulatory process perpetuates consumer distrust of genetic engineering, *i.e.* "if the product is safe, why does it require so much testing and paperwork to release?" Although we recognize there is no easy solution to this issue, we propose reduction or elimination of regulation for gene-editing technologies in favor of increased regulation and testing of the final organism.

As graduate students, we are especially apprehensive about the impact of regulation on funding opportunities for recent graduates. Our greatest concern is the high barrier of entry created by the costs and restrictions imposed by existing regulations. The current system favors large corporations and narrows research and funding opportunities for entrepreneurs and public-sector scientists. This circumstance stifles innovation and limits the ability of scientists to address more crucial issues, such as climate change and food security.

PART VII–POSTER ABSTRACTS

Development of a Germline Transformation System for the Western Corn Rootworm, Diabrotica virgifera virgifera

Fu-Chyun Chu, Stephanie Gorski, Yasmin J. Cardoza and Marcé D. Lorenzen
North Carolina State University
Raleigh, North Carolina

fchu@ncsu.edu

The western corn rootworm (WCR) is a major pest of maize and is notorious for rapidly adapting biochemically, behaviorally and developmentally to a variety of control methods. Despite much effort, the genetic basis of WCR adaptation remains a mystery. Transformation-based applications such as transposon tagging and enhancer trapping have facilitated the genetic dissection of model species such as *Drosophila melanogaster*. Following this model, we are developing a genome-wide mutagenesis system for use in WCR. However, we must first establish a germline transformation system for use in this beetle. Here we report results from our first sets of experiments. These include: 1) testing marker genes (EGFP and DsRed); 2) testing heterologous promoters (*Tc-alpha-Tubulin* and *Dm-heat-shock-70*); and 3) testing transposable elements (*piggyBac* and *Minos*). Since our overall goal is to create a jumpstarter mutagenesis system that is analogous to that used in another coleopteran, the red flour beetle, *Tribolium castaneum*, we are also using the CRISPR/Cas9 system to: 1) create a white-eyed mutant strain; and 2) specifically target transposon insertions to precise genomic locations (*i.e.* locations analogous to those known to enable efficient *piggyBac* remobilization in *Tribolium*). Establishing transgenic technologies in WCR is the first step towards bringing a wide-range of transformation-based tools to bear on understanding WCR biology, which can then be extended to other rootworm species.

Unraveling the Unique Mechanism of Ethylene-Induced Abscission in Non-Climacteric Sweet Cherry, Prunus avium

Benjamin Kilian
Washington State University
Pullman, Washington

kilianbe@gmail.com

Sweet cherry, *Prunus avium*, is a unique non-climacteric member of Rosaceae that comprises some of the classical climacteric species such as apple, pear and peach. Previous phenotypic data established that exogenous ethylene treatment has a genotype-dependent effect on pedicel retention as the fruit nears maturity. This is an intriguing result because sweet cherry is considered non-climacteric and, therefore, does not display positive reinforcement in ethylene production and elevated respiration. This observation prompted our inquiry of what could initiate the response to ethylene of certain genotypes of a non-climacteric fruit in the formation of an abscission zone? Part of the answer at least can be derived from gene-expression studies. I have addressed this question by measuring abscission-zone formation indirectly by pedicel fruit retention force (PFRF) and gathering contemporaneous RNA-Seq profiles to correlate the physiological data. For the experiment, three sweet cherry genotypes were selected which best represented the diversity of responses to ethylene. 'Chelan' does not respond to ethylene application. 'Skeena' also does not respond to ethylene; however, it forms an abscission zone naturally. 'Bing' is the unique genotype that forms an abscission zone in response to ethylene, lowering stem-retention force enough to enable mechanical harvesting. The experiments assessed relative genotypic responses to ethylene in the final phase of fruit maturation prior to harvest. Surgically defined abscission zone tissues from each genotype were collected and used to generate quantitative gene-expression data (RNA-Seq). The sequence data have been *de novo* assembled into genotype-specific transcriptomes and relative expression for each gene showed the differences among genotypes. With these studies we have identified specific genetic elements specifically in 'Bing' that are differentially expressed in response to ethylene. These include transcription factors rapidly responding to the ethylene signal and their potential target sequences, more delayed cell wall-modifying enzymes, and others connected to programmed cell death processes. We are currently validating expression

of these genes over multiple seasons and plan on performing subsequent experiments to demonstrate ethylene response. Agricultural labor availability and safety is currently a large problem in the United States in general, and the Pacific Northwest in particular. One solution is the facilitation of mechanical harvest, which would lower cost of production and increase worker safety. Identifying or transferring the required element(s) via classical breeding or through genetic modification into target species has the potential to facilitate more efficient harvesting. My PhD work revolves around the approach that we can predict and/or induce the formation of a pedicel-fruit abscission zone in sweet cherry, a crop with particularly high labor demand and cost. While aimed primarily at sweet cherry, the technology could be applied to mechanically harvesting a wide range of crops.

Early Flowering Salvia hispanica *(Chia) Composition*

William Serson[1], Paul Armstrong[2], Timothy Phillips[1] and David Hildebrand[1]
[1]*University of Kentucky*
Lexington, Kentucky

[2]*USDA-ARS Grain Marketing and Production Research Center*
Manhattan, Kansas

william.serson@uky.edu

A sustainable, affordable source of omega-3 fatty acids has been of high interest to those interested in human and animal nutrition due to known heart-health benefits. Chia is an ancient crop that has experienced an agricultural resurgence in recent decades, though it can only be grown in central latitudes due to its need for short-day flowering. Using EMS and gamma radiation, researchers at the University of Kentucky have developed a long-day flowering mutant which can be grown in the heartland of the United States. While the agronomics continue to be hashed out, we are interested in observing seed composition. If EMS and gamma radiation did not mutagenize genes relevant to seed composition, then we would anticipate no significant changes in seed composition. Five lines with agronomic potential were evaluated for fiber, oil, protein, and fatty-acid content and compared to the parent line, 'Pinta'. A Dunnett's test confirmed that there were 3 varieties with significantly higher fiber, one with higher protein, and one with higher 16:0 levels. Some of these changes could be due to mutations of genes that are responsible for helping determine seed composition. However, further trials will be needed to investigate genotype by environment interactions.

Effect of Different Gums on the Functional Properties of Soy-Based Nile Tilapia Feed

P. Singha and K. Muthukumarappan
South Dakota State University,
Brookings, South Dakota

p.s.technologist@gmail.com

Plant-origin polymeric binding agents, cellulosic gum, and starch are often used to improve the cohesiveness of extruded aquafeed. Exopolysaccharide gum such as pullulan and xanthan secreted by microorganisms has unique properties that have been used in food and pharmaceutical products for decades. Isocaloric ingredient blends were formulated for Nile tilapia using a constant level of defatted soybean meal (DFSBM) as a fishmeal replacer and graded levels of five different binders (guar, wheat gluten, xanthan, CMC, and pullulan). Extrusion trials were performed using a single-screw extruder at two levels of screw speed (100 and 150 rpm). To acquire more distinct results for the effect of utilized binders, the extrusion-barrel temperatures, blend-moisture content, die diameter, and screw-compression ratio were kept constant at 100–120–140°C, 20% db, 3 mm, and 3:1 respectively. A fishmeal-based diet was used as the control diet. Physical properties of the extruded products including expansion ratio, durability, water stability, water solubility, and densities were quantified. It is hypothesized that exopolysaccharide binder can provide similar binding properties in extruded aquafeed formula when compared to conventional binders.

Evaluation of Carrot (Daucus carota *L.*) *for Traits Related to Early Seedling Establishment and Canopy Growth at Different Planting Densities*

Sarah Turner and Philipp W. Simon
USDA-ARS, University of Wisconsin
Madison, Wisconsin

sdturner2@wisc.edu

Carrot production is limited by erratic germination, poor seedling growth, and delayed canopy establishment, all of which make weed control a major challenge. Plants with early germination, quick seedling growth, and competitive growth response are one viable option for improving weed management. Preliminary field trials have demonstrated that carrot genotypes have variable germination rates and responses to planting density, ranging from no response to an increase in canopy growth as planting density increases. This project aims to elucidate competitive growth response in diverse carrot-breeding stocks. Four genotypes with small canopy size and four with large canopy size were planted at different densities (30, 60, and 90 plants per meter) using a randomized complete-block design with three replications. Emergence, canopy height, and canopy width were monitored throughout the growing season and postharvest measurements of fresh leaf weight, root weight, and dry leaf weight were taken. Plant height was significantly affected by planting density, genotype, location, and genotype by location interaction. In general, increasing planting density promoted top growth at the expense of root weight, but this response varied by genotype. Current progress will be reported.

Targeted Mutation and Precise Genome Editing in Plants with CRISPR/Cas9 System

Kabin Xie, Bastian Minkenberg, Xiangling Shen, Qin Wang and Yinong Yang
The Pennsylvania State University
University Park, Pennsylvania

yuy3@psu.edu

The bacterial cluster regularly interspaced short palindromic repeats (CRISPR)-associated nuclease (Cas) system has recently emerged as an efficient and versatile tool for genome editing. In this study, we have demonstrated targeted mutation and precise genome editing with CRISPR-Cas9 system in both monocot and dicot plants. The engineered gRNAs were shown to direct the Cas9 nuclease for precise cleavage at the desired genomic sites and introduce specific mutations (insertion or deletion) at a high efficiency by error-prone non-homologous end-joining repairing. In addition, new strategies and tools are being developed for simultaneous mutation of multiple genes, site-directed mutagenesis, site-specific gene integration and precise deletion of chromosomal fragment in plants. To assess potential off-target effects and increase the specificity of CRISPR-Cas9 system, we have performed genome-wide prediction of highly specific gRNA spacer sequences and targetable transcription units in eight model plants and major crops. A bioinformatic database and web tool have been developed to help design highly specific gRNAs and assess their off-target potential. With improved bioinformatic prediction and new experimental strategies, CRISPR-Cas9-mediated genome editing is rapidly becoming a powerful tool for plant functional genomics and genetic improvement of agricultural crops.

Apurva Bhargava
Bayer CropScience

Le Ann Blomberg
USDA-Agricultural Research Service

AdamBogdanove
Cornell University

Katherine Boor
Cornell University

Ewa Borejsza-Wysocka
Cornell University

Robin Buell
Michigan State University

Nathan Butler
Michigan State University

Janet Carpenter
SugarCane Refined Genetics

Dana Carroll
University of Utah

Suma Chakravarthy
Cornell University

Shiyan Chen
Cornell University

Fu-Chyun Chu
North Carolina State University

Archie Clutter
University of Nebraska-Lincoln

Will Cody
Texas A&M University

Jeanne Colleluori
Wegmans Food Markets

Alan Collmer
Cornell University

Meridith Cook
Cargill

Paul Debbie
Boyce Thompson Institute

Patrick Di Bello
University of Arkansas

Hui Duan
J. R. Simplot Company

Allan Eaglesham
NABC

Sarah Evanega
Cornell University

Maria Fedorova
DuPont Pioneer

Robert Gaeta
Monsanto

Greg Gocal
Cibus US LLC

Dalia Rosin Grunewald
Evogene

Brian Gwin
The Ohio State University/OARDC

Perry Hackett
University of Minnesota

Ralph W.F. Hardy
NABC

William Haun
Cellectis Plant Sciences

Neil Hoffman
USDA-APHIS

Brian Iaffaldano
The Ohio State University

Michael Irey
Southern Gardens Citrus

Thomas Jacobs
Boyce Thompson Institute

Gregory Jaffe
CSPI

Bolormaa Jamiyansuren
University of Minnesota

Michael Kahn
Washington State University

Drew Kershen
University of Oklahoma

Ben Kilian
Washington State University

Karen Kindle
Boyce Thompson Institute

Christine Kraus
Boyce Thompson Institute

Rick Kress
Southern Gardens Citrus

Brian Larkins
University of Nebraska-Lincoln

Susanne Lipari
NABC

Lyza Maron
Cornell University

Greg Martin
Boyce Thompson Institute

Robert Millman
MPM CAPITAL

Bastian Minkenberg
Penn State

Jack Okamuro
USDA-ARS

Jongjin Park
Samuel Roberts Noble Foundation

Patricia Polowick
National Research Council Canada

F. Abel Ponce de León
University of Minnesota

Steven G. Pueppke
Michigan State University

F. Ann Ran
The Broad Institute

Andrew Read
Cornell University

Gary Rudgers
Dow AgroSciences

Joachim Schiemann
Julius Kühn Institute

Alexa Schmitz
Boyce Thompson Institute

William Serson
University of Kentucky

Heather Shearer
Canadian Food Inspection Agency

Kay Walker Simmons
USDA-ARS

Poonam Singha
South Dakota State University

Margaret E. Smith
Cornell University

Martin Spalding
Iowa State University

Caleb Squires
Washington State Univeristy

Vibha Srivastava
University of Arkansas

Reuben Tayengwa
Washington State University

Gary A. Thompson
Pennsylvania State University

Li Tian
University of Kentucky

Tien Tran
Cornell University

Sarah Turner
UW-Madison/USDA-ARS

Tom Turpen
CRDF

Marshall Tyler
Boyce Thompson Institute

Indu Upadhyaya
University of Connecticut

Joyce Van Eck
The Boyce Thompson Institute

Vijay Vijayaraghavan
Sathguru

Dan Voytas
University of Minnesota

Xiaohong Wang
Cornell University

Donald P. Weeks
University of Nebraska

Troy Weeks
J.R. Simplot Company

Hailei Wei
Cornell University

Peter Whitfield
Baker Hostetler

Jeff Wolt
Iowa State University

Brent Woodward
Cooperative Resources International

Yinong Yang
Penn State

Bing Yang
Iowa State University

Wenzheng Zhang
The Samuel Roberts Noble Foundation

Yingxiao Zhang
The Ohio State University

North American Agricultural Biotechnology Council*
Boyce Thompson Institute, B15
533 Tower Road
Ithaca, NY 14853-1801
USA

607-254-4856 fax-254-8680
nabc@cornell.edu
http://nabc.cals.cornell.edu

Ralph W.F. Hardy, President

Allan Eaglesham, Executive Director

Susanne Lipari, Executive Coordinator

*Member institutions are listed on page v.