Controlling Traits in Transgenic Plants: Tools that Enhance Value and Reduce Environmental Release

Roger N. Beachy
Donald Danforth Plant Science Center
St. Louis, MO

Having just come from Montreal where I participated in the Meeting of the Parties of the Cartagena Biosafety Protocol and then later at BIO2005, it is clear that a number of issues face plant science and biotechnology. There is much to be said about the importance of public-sector scientists speaking their minds and stating the facts with regard to the issues of applications of agriculture biotechnology. Now is an absolutely appropriate time. Last year's conference—gleaned from leafing through the NABC-16 proceedings volume—reminded us how difficult it is for scientists in the public sector to develop a product via agricultural biotechnology. The costs required to take a product from the experimental stage to commercialization are overwhelming for scientists in the public sector. The regulatory oversight policies that have accompanied this industry have, in my opinion, gotten so far out of hand that we in the public sector can no longer effectively bring products to market.

How does one capture trait value and how does one prevent the escape of a trait?

Today I will discuss the rationale for controlling the expression of transgenes—including the use of gene-switching technologies—that will reduce the transfer of transgenic traits and may reduce regulatory concerns of agricultural biotechnology. A description of the technology of chemical control of gene expression will be followed by a brief discussion of some of the potential issues of concern about the use of gene-expression technology. Lastly, I'll put the technology in the context of the topic of this conference: How does one capture trait value and how does one prevent the escape of a trait where it is not wanted,

either through theft or through out-crossing, while reducing adventitious presence of a GM product in a non-GM crop.

It is important to find ways to continue to innovate while making the products of agricultural biotechnology profitable, if the technology is to reach its potential. My concern is that if we don't find ways to capitalize on the significant investments that have been made in the basic plant sciences by federal and non-federal funding sources by developing relevant knowledge and potential products, there will eventually be reductions in research funding in the public sector.

Controlling gene expression for commercially valuable traits may be necessary as a means of capturing value.

There are several benefits of controlling expression of transgenes in plants.

- For basic studies of gene function. Many gene functions are lethal if mis-expressed and it may be desirable to exert tight control over gene expression.
- To reduce the spread of a genetic trait to a weedy relative or to non-GM varieties of the crop plant. Control of trait expression can significantly reduce likelihood of adventitious presence of a controlled product and, of course, nonessential release to the environment. For example, when considering the use of plants to produce a novel food product, an industrial or pharmaceutical material, gene switching will limit expression of the materials to plants or plant tissues in which the gene is activated.
- To capture value of the new trait. Controlling gene expression for commercially valuable traits may be necessary as a means of capturing value, in particular in crops that are inbred, or where seeds can be saved.

Methods to Control Gene Expression and Applications in Biotechnology

The regulation of gene expression is a complex process that requires the coordinated activity of proteins and nucleic acids that ultimately determine whether a gene is or is not transcribed, and if transcribed, results in production of a protein that produces a phenotype. Most of the emphases of studies of gene expression have been on regulation of gene transcription, and a number of technical methods are used to affect the control of gene expression. First, one can use a promoter that has known regulatory characteristics; for example, a promoter that is expressed only in vascular tissues, in the leaf epidermis, seed endosperm or embryo, and so on. Or one can mix and match fragments of DNA and transcription factors to develop chimeric promoters that have the desired patterns and levels of gene expression.

In my laboratory we study a promoter that is expressed in plant vascular tissues [a promoter from rice tungro bacilliform badnavirus (RTBV)] and two transcription factors (Rf2a and RF2b). The factors, in conjunction with other co-factors and components of RNA polymerase II, are responsible for tissue-specific gene expression of the RTBV promoter. The RTBV promoter is expressed only in vascular tissues in transgenic rice, *Arabidopsis* and tobacco plants. However, when genes encoding RF2a or RF2b are constitutively expressed (using the constitutive 35S promoter) the RTBV promoter was likewise expressed constitutively (Petrucelli *et al*., 2001; Dai *et al*., 2004). As a consequence of this and other research, we identified the DNA-sequence element to which RF2a and other transcription regulators bind to govern expression of the promoter (Dai *et al*., 2006). We have used these and other elements to create a regulatable gene-transcription cascade that can be "put to work" to control the expression of transgenes in plants, including using a chemical gene switch.

The remainder of the discussion will be devoted to describing systems that can be used to control expression of genes at will. The challenge with all systems is that none is perfect for all applications, and it is important to define the intended application prior to making a choice. Some switches are more appropriate than others for experimental use and for field use. To date, most research on gene switching has been applied to laboratory studies. Nevertheless, the potential applications of gene switching are numerous and it is anticipated that a number of commercial applications will be developed.

Basic Components

The basic components of a good chemical gene-switching system include: (1) a suitable inducer; (2) a receptor-like protein that binds the ligand; (3) a promoter that is activated or repressed as a consequence of binding of ligand to the receptor. Over the last 15 years, a number of gene-switching technologies have been developed, including those induced by cations, phytohormones, steroid-related molecules, antibiotics, ethanol, herbicide safeners, and other organic molecules. Several recent reviews have described the state of the science in this topic area *(e.g.*, Padidam *et al*., 2003).

A suitable chemically regulated or inducible gene-expression system will have a number of characteristics, including: (1) high specificity of the ligand for the receptor to ensure that genes are tightly regulated to be "on" or "off" in the absence of the ligand; (2) the ligand should be readily taken up by the plant and move to all organs and tissues; (3) the ligand should elicit a rapid response; (4) the ligand should be non-toxic to the target and non-target organisms; (5) the ligand should be active at low concentrations and have a favorable environmental profile; (6) the ligand should be suitable for convenient application, either as a foliar spray, seed treatment, or root drench; (7) application should be low cost.

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The EcR-Methoxyfenozide System

In my laboratory, we have experimented with and adapted a system based on the ecdysone receptor (EcR). These receptors are found in lepidopteran insects that are activated by ecdysone, a compound that regulates insect growth. In this system an inactive EcR receptor remains in the cytoplasm until ecdysone, or a suitable agonist, binds, after which the ligand-receptor complex is transported to the nucleus where it binds to the responsive DNA-sequence element. Under appropriate conditions, binding of the complex causes a change in gene expression. In insects and other animal cells, the system is bi-partite, and requires an additional endogenous protein. Padidam *et al.* (2003) developed a mono-partite inducible expression system that is appropriate for use in plants and plant cells. The chimeric receptor comprises the VP16-activator domain from SV40, the *gal*-4 DNA binding domain, and EcR (the chimeric receptor is referred to as "VGE"). When the gene encoding the chimeric receptor is produced from a promoter that is either constitutive or tissue-specific, it remains inactive until ecdysone or a suitable agonist binds and causes the complex to activate gene expression by binding to the gal-4 cis element that is a component of the target promoter. Methoxyfenozide is a suitable agonist of ecdysone and a suitable ligand in this system. It is proposed that methoxyfenozide causes the formation of a dimer with the receptor, which binds to the DNA-binding site on a chimeric gene. The system is easy to use and is highly active in *Arabidopsis* and other plants. Methoxyfenozide is the active ingredient in the insecticide Mimic® (Dow AgroSciences LLC).

Methoxyfenozide has a suitable safety profile for use as a gene switch in laboratory and greenhouse conditions. Furthermore, it does not cause non-specific expression of a high number of genes in *Arabidopsis* (S. Dai, I. Ordiz and R.N. Beachy, unpublished data), an indication that the ligand has very little direct effect on the host plant. Furthermore, the level of expression of a target gene can be controlled by the concentration of the ligand. In other studies, we developed several hundred transgenic *Arabidopsis* plant lines that produce luciferase upon addition of methoxyfenozide. These studies confirmed that, like other transgenes, expression of the gene-switch system is controlled by position effects and different lines respond to different concentrations of the ligand and exhibit different rates of responsiveness (S. Dai, I. Ordiz and R.N. Beachy, unpublished data).

We used the methoxyfenozide gene-switch system to demonstrate that the ligand is taken up and systemically distributed in *Arabidopsis* plants and can induce the expression of the transgene in a variety of tissue types. These studies demonstrated that the ligand is taken up rapidly when applied to roots, is transmitted throughout the plant and causes expression in all cells. We then went on to show that the system can be used to induce expression of a gene encoding the coat protein of TMV-Cg tobamovirus, and induce coat-protein-mediated resistance against the virus following addition of methoxyfenozide (Koo *et al.*, 2004). In one plant line, the level of accumulation of coat protein exceeded the highest level produced by the enhanced 35S promoter. To date we have developed more than 750 plant lines using this system and have observed a variety of levels of gene induction, from 10-fold to more than 1,000-fold following addition of the ligand.

Applications of the Gene-Switch System

In an ongoing study, we are evaluating the methoxyfenozide gene-switch system to determine whether or not gene-switching technologies can be used to induce tissue-specific expression of genes. For these studies we selected promoters that are known to be expressed only in selected plant tissues, and constructed genes with the VGE-coding sequence. The gene was co-introduced with a *uidA (*encoding GUS) reporter gene that is under control of a minimal 35S promoter ligated with the DNA-binding site for recognition by the receptor. In these studies the reporter gene was silent in the absence of VGE and methoxyfenozide. Although the study is not yet completed, we are encouraged with the results and are confident that they will show that the gene-switch system can be used to restrict gene expression to specific tissues after addition of the ligand.

How might one use a gene-switching system? One of the experiments in progress is to develop a system to control multiple genes with application of the ligand. If successful, we will use the system to activate expression of genes that cause the repression/suppression of a gene in one or more metabolic pathways while activating other genes in the same or other pathways. If successful, this will make it possible to substantially alter primary and/or secondary metabolism in plants. We do not yet know the limits of the system, but are confident that it is sufficiently robust to make significant changes in the metabolism as well as growth and development of the target plant.

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The Next Generation

The next generation of chemical gene switches will likely be substantially different and better than current systems. There will be improved receptors that eliminate proteins of animal origin, and receptors that provide active repression as well as activation of gene expression. We also anticipate that a variety of ligand:receptor pairs will be developed, and that future developments will create plants that will respond to multiple gene switches.

RheoGene Co. (Philadelphia, PA) has developed several different receptor:ligand pairs that function in animal cells. It is anticipated that some of these will function in plants either in a two-protein or one-protein gene-switch system. This may make it possible to use one ligand to turn on a target gene and a second ligand to turn the gene off. Such flexibility in the system would have other uses: for example, company A might want to use a unique receptor:ligand pair while company B will want another receptor ligand pair, and so forth.

It is likely that there will be additional opportunities for ligand-receptor development with a variety of different biological characteristics. The challenge is, of course, to identify gene-switching systems that are safe for the environment, the plant, and for the final product. And, if a chemical ligand is to be released to the environment, it must pass

standard EPA toxicology tests. For this reason, scientists anticipate that early adoption of chemical gene-switching systems will involve ligands that have been approved as safe or that can be thus approved with modest investment.

Regulatory Considerations

The development of safe and reliable gene switches that are used commercially will rely on new applications of existing chemistry or on new chemistry; regulatory approvals will be required either for new use or for new chemicals that will be used. Furthermore, one must ensure that there is strict on/off control of gene expression; leakiness of gene expression will not be acceptable. This is essential if one expects the public to agree to selected types of agricultural biotechnology.

In order for chemical gene switching to be widely used, it will be necessary for regulatory agencies to adapt and undergo certain types of change. At the present time, the agencies regulate transgenic organisms on the basis of the presence or absence of transgene DNA and/or the presence of the gene product. Many scientists agree that the most important criterion for phenotype is not the presence or absence of a transgene *per se*, but whether or not the product of gene expression (*i.e.*, the RNA or a protein product) and the resulting phenotype are produced. Thus, the manner in which products are subjected to regulatory control will need to be established.

There is concern amongst some parties that limitations on trait expression will limit access of some technologies to those farmer/producers that can afford to pay high technology fees.

Public Discussion

As with any new technology, it is important to engage the public, both academic and non-academic, in discussions related to issues that may have impact on regulatory structures, on environmental safety, and with regard to possible ethical issues that may arise. It's perhaps more important now than it was in following the first breakthroughs in agricultural biotechnology in the 1980s. In January, 2005, we held a workshop at the Danforth to discuss chemical gene switching with ethicists and environmentalists to help us better understand the challenges that might be faced in bringing forward a viable gene-control system. I think that engaging the public and non-scientists in such discussions is important for all of us.

There is concern amongst some parties that limitations on trait expression will limit access of some technologies to those farmer/producers that can afford to pay high technology fees. It is considered likely that gene-switching systems will be first used on crops that will produce high-value materials; uses on field crops or other food crops are much less likely, except to restrict trait flow to non-GM crops. It is highly unlikely that gene switching will be used in the foreseeable future in commodity crops or crops that

are produced by small-scale, economically disadvantaged farmers. Unlike the so-called sterile seed technologies, gene switching as outlined here will not be applied to restrict seed germination *per se*.

I am convinced that we have an opportunity to make outstanding strides forward in biotechnology, but am increasingly concerned that much of the potential will not be realized unless we learn to deal with some of the issues that can be addressed by gene switching. Whether or not gene-switching technologies emerge as a tool to bring new agricultural biotechnologies to the public marketplace depends on many factors. Challenges notwithstanding, the potential of the technology is high and I am confident that it will be an important component of agricultural biotechnology.

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Roger Beachy is president and director of the Donald Danforth Plant Science Center in St. Louis, MO. He is recognized for his work in molecular virology and gene expression, and for development of transgenic plants that are resistant to virus infection.

Dr. Beachy received a PhD in botany and plant pathology from Michigan State University in 1972. After postdoctoral work at the University of Arizona and Cornell University, in 1978 he was appointed to the faculty at Washington University, St. Louis. In 1991 he joined The Scripps Research Institute in La Jolla, CA, as Head of the Division of Plant Biology holding the Scripps Family Chair in the Department of Cell Biology. In 1999 he accepted the position as founding president of the Danforth Center.

Beachy was elected to the National Academy of Sciences in 1997 and received the Wolf Prize in Agriculture in 2001. He is a Fellow of the American Society for Microbiology and of the American Association for the Advancement of Science. He is a strong proponent for training of, and cooperative research with, scientists in developing countries and is an advocate for implementation of policies of technology management that encourage sharing of intellectual property, and research for the public good.