
Commercialization of a Protein Product from Transgenic Maize

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The concept of plants as factories was launched approximately 15 years ago as a method for bio-manufacturing pharmaceuticals, vaccines and industrial enzymes. The advantages of the plant system include low-cost manufacturing with limited capital investment for growing the biomass, easy scale-up with planting of increased acreage and a system without the threat of animal pathogens whether viral, bacterial or prion.

PROOF OF CONCEPT

The concept has been tested in a number of plant systems at several biotechnology companies, including: ProdiGene (corn; Hood *et al.*, 2003), SemBioSys (safflower; Moloney, 2002), Ventria (rice; Huang, 2004), Medicago (alfalfa; Vezina *et al.*, 2002), Biolex (*Lemna*; Gasdaska *et al.*, 2003), CropTech (tobacco; Cramer *et al.*, 2000), Large Scale Biology (tobacco; Grill *et al.*, 2002) and Planet Biotechnology (tobacco; Larrick *et al.*, 2001) as well as in university research laboratories. The proof of the concept is embodied in these research efforts. Plant systems can express proteins at high levels, assemble multimeric proteins such as antibodies, and correctly process them, *e.g.* cleave signal sequences and attach glycosylation sequences. Many types of proteins have been expressed in plant systems including those ranging in molecular weight from 5,000 daltons (Zhong *et al.*, 1999) to over 200,000 daltons (Lamphear *et al.*, 2004). These various examples show the versatility of the system. The subject of this paper—bovine trypsin produced in transgenic maize—illustrates the concept of gene to market, *i.e.*, steps in commercialization of a protein product.

This paper illustrates the steps in commercialization of a protein product.

Trypsin is a pancreatic serine protease involved in food digestion. Commercially, it is produced from bovine refuse from slaughterhouses for applications in cell culture and protein processing. Many of the cell cultures are for production of pharmaceutical or vaccine proteins, requiring pharmaceutical-grade manufacturing conditions. With the existing problems arising from hoof and mouth and mad cow (bovine spongiform encephalopathy) diseases, non-animal sources of trypsin are in high demand. Because bovine pancreatic trypsin is now available in maize lines, large-scale production for industrial and pharmaceutical applications can be achieved to meet this demand.

Molecular Biology

In order to express a protein at commercial levels (*i.e.*, as high as possible for the protein and plant host), several molecular and cellular parameters must be considered. The use of codons that are common to the host plant can enhance the translatability of the messenger RNA. From experience, the leading amino acids appear to be the most critical in this regard, presumably because they initiate efficient translation. In the case of bovine trypsin, the native *Bos taurus* gene was fused in frame with the maize-optimized barley alpha-amylase signal sequence (mo-BAASS; Rogers, 1985), providing twenty-five codon-optimized amino acids for translation (Woodard *et al.*, 2003), sufficient in this case to achieve good expression. Other parameters of interest for high expression of foreign genes for protein production include tissue specificity, subcellular localization, germplasm/breeding and protein-specific considerations. For bovine trypsin, tissue specificity was achieved through use of the maize globulin-1 promoter, which is embryo-preferred. Although several subcellular locations were tested, the best location for high protein accumulation was the apoplast (cell wall) (Hood and Woodard, 2002). By far, the greatest challenge with expression of bovine trypsin was regeneration of transgenic plants when the gene for the active enzyme was used. Therefore, the zymogen form of the protein, trypsinogen, was expressed from the holo-gene and transgenic plants, with high expression obtained (Woodard *et al.*, 2003). Thus, optimal expression of trypsin in maize was achieved by expressing the zymogen form of the gene from the embryo-preferred globulin-1 promoter, and targeting the protein to the apoplast using the maize-optimized barley alpha-amylase signal sequence. Using these conditions, the highest first generation seed showed 3.3% of total soluble protein (TSP) by enzyme activity (TRF, Figure 1).

Variation among transgenic events and among multiple plants from single events, *i.e.*, clones, is commonly observed in maize-derived transgenic plants (Hood *et al.*, 2003). Because of this, multiple individual lines from multiple events must be screened for the protein of interest, to choose the best lines to move forward. However, the maize varieties that perform best in tissue culture and transformation are not suitable for field growth. Therefore, lines selected for high first-generation expression are planted in field nurseries to be improved in agronomic characteristics through a breeding program. Breeding into elite inbred germplasm is conducted and resulting lines subsequently selected for high expression of the foreign gene of interest as well as for field performance. Trypsinogen was recovered in bulk seed lots at 58 mg trypsin per kg of seed, or 0.006% of dry weight from fifth-generation elite inbred material (Woodard *et al.*, 2003), or approximately double the first-generation high single seed.

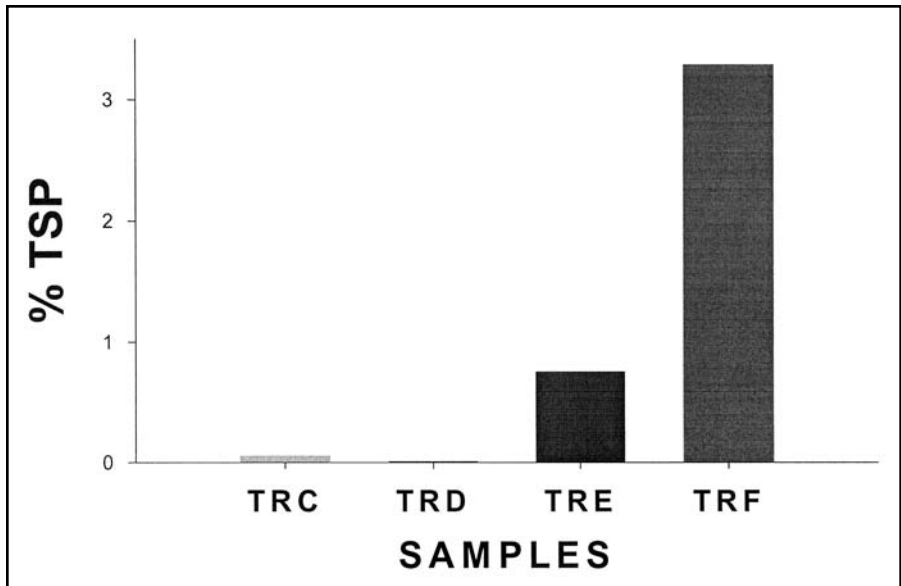


Figure1. Trypsin expression in various transgenic maize lines as a percent of total soluble protein (%TSP) from ground seed. TRC—zymogen form of trypsin expressed from a constitutive promoter and targeted to the cell wall; TRD—mature trypsin expressed from a constitutive promoter and targeted to the cell wall; TRE—zymogen form of trypsin expressed from an endosperm-preferred promoter and targeted to the amyloplast; TRF—zymogen form of trypsin expressed from an embryo-preferred promoter and targeted to the cell wall.

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Biochemical Characterization

For commercialization of a product from a new production platform, it is crucial to determine the biochemical qualities of the plant-derived protein, including its activity. Maize-derived trypsin was mostly active when extracted from ground transgenic seed, and exhibited several molecular-weight forms (Figure 2). The largest disappeared upon treatment with enterokinase, suggesting it was the trypsinogen form (Woodard *et al.*, 2003). When each of the remaining high-molecular-weight forms was subjected to N-terminal micro-sequencing, the sequences were the same and matched exactly the sequence of the native bovine trypsin, confirming that the N-terminal signal sequence was correctly cleaved (Figure 2). In addition, these forms were active (Woodard *et al.*, 2003).

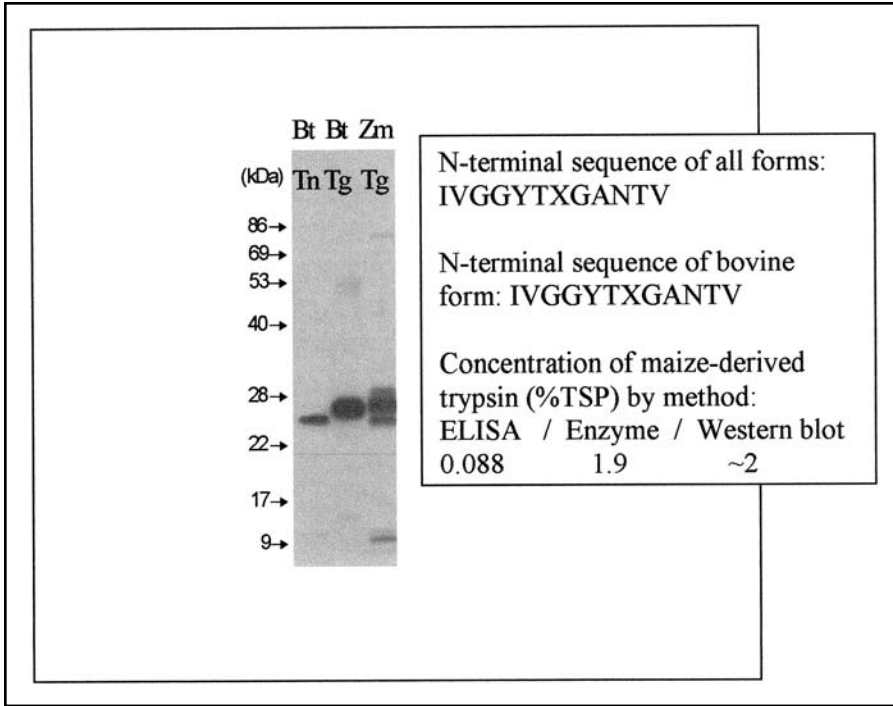


Figure 2. Trypsin characterization. Western blot of maize-derived trypsin. Bt = *Bos taurus*; Zm = *Zea mays*; Tn = trypsin; Tg = trypsinogen. Numbers to the left indicate migration of molecular-weight markers. Box to the right indicates N-terminal sequence of three active forms of maize-derived trypsin and values for trypsin concentration from several averaged samples using three assay methods.

Several methods of analysis were developed to determine the concentration of protein in transgenic maize seed. Enzyme-linked immunosorbent assays (ELISAs) provided disappointing results in that most transgenic lines exhibited very low concentrations of trypsin. However, Western blots suggested that the enzyme was present in substantially higher concentrations than the ELISAs had indicated (Figure 2). Subsequently, enzyme assays were developed to assess the activity of the maize-derived enzyme, and the results agreed closely with the Western blots, suggesting that the trypsin was digesting protein components in the ELISA.

Physical parameters of maize-derived trypsin matched the native protein in all instances with the exception of the molecular weight of extracted protein (Table 1; Figure 2). The differences in molecular weight were apparently due to O-linked glycosylation sequences on the maize-derived protein, and when removed by chemical means, the proteins had the same apparent molecular weight (Woodard *et al.*, 2003). Specific activity, pH optimum, Km and Vmax values were not significantly different between the two sources of enzyme (Table 1).

TABLE 1. BIOCHEMICAL CHARACTERIZATION OF BOVINE TRYPSIN FROM MAIZE AND NATIVE SOURCES.

Parameter	Maize-derived trypsin	Bovine trypsin
Molecular weight (MALDI TOF)	23,297 daltons	23,306 daltons
Specific activity	175 U/mg protein	166 U/mg protein
Glycosylation	O-linked	none
Km	2.7 mM	3.2 mM
Vmax	0.29	0.30
pH optimum	8.6	8.7

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PRODUCT DEVELOPMENT

A product is not just the protein of interest in a transgenic plant. It is the protein of interest utilized in a certain market application, each of which creates a different product. Thus, many products can be developed from one protein-expressing plant line. Development of crop-derived proteins into real-world industrial or pharmaceutical products comprises a number of activities including applications testing and market development, a freedom-to-operate assessment, patent protection, a safety assessment, breeding into elite material for best field performance, small-scale production and formulation requirements. All aspects must be developed in a way that meets cost targets.

Applications for trypsin include digestive aids, detergent additives, processing of commercial proteins, commercial cell culture, an active pharmaceutical product, eye-care products and leather processing. The major question for maize-derived trypsin is whether it can substitute for the bovine product in these applications. In several trials, the maize-derived enzyme functioned equally as well as the bovine counterpart (S.L. Woodard, unpublished).

Market development for maize-derived replacement products for existing markets mainly comprises focus on the advantages of a plant-derived protein. First and foremost, the plant-derived enzyme can be utilized in markets that are sensitive to the source of the product and the associated need to avoid animal pathogens. This is particularly true for cell culture, pharmaceutical use, and commercial reagent proteins that are utilized to process pharmaceutical proteins. A second advantage is low cost for markets not sensitive to these cleanliness issues. A third advantage is the ability to scale-up for large market applications for which microbial production systems are less appropriate because of capital requirements.

Freedom to operate (FTO) is a complex issue for plant-derived products—“can I practice my processes and produce my product without running afoul of others’ patents?”

(Sweeney, 2002). Determining FTO is a process that requires constant diligence and many licenses, particularly in competitive areas of research and technology development. Whenever new materials are brought into an entity or the entity's materials are shared with others, FTO issues can be raised. And, obviously, not only materials are at stake. Often processes for creating products are patented; for example, transformation protocols and breeding strategies are equally subject to licensing requirements. Diligence in this area with company or university attorneys is critical to success.

Patent protection is essential to successful market entry, particularly if the technology or product can be manufactured by other firms, or the technology of interest makes a product possible when it previously was not available. Proteases in general are difficult to produce in xenogenic systems because of their detrimental effect on native protein content. In 2000, ProdiGene was issued a broad-based patent (USP # 6,087,558) describing the production of proteases in transgenic plants, claiming expression of any protease in any transgenic plant, where the protease is expressed in the zymogen form. This is a fundamental technology, because recovery of transgenic plants expressing high levels of active proteases is nearly impossible unless zymogens are expressed in seed.

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Maize transgenic events are generated in material that has poor agronomic characteristics. Thus, **breeding into elite germplasm** is essential for field performance that meets production requirements. This process takes from 2 to 4 years depending on the need to reach yield parity for profit margin and whether year-round nurseries are used for the acceleration of generations. If yield parity with commodity corn is required to meet production-cost requirements, then five to seven generations of crossing into elite inbred germplasm is required with two additional selfed generations before making a hybrid (D. Delaney, personal communication). The germplasm of choice depends upon the field production location, and if more than one zone will be required for crop production, more inbreds will be required for the breeding program. A minimum of two generations per year can be accomplished in midwestern US summer nurseries alternating with Puerto Rican (or other Caribbean island) winter nurseries. However, with year-round nurseries in Hawaii, 2.5 to 3 generations per year can be accomplished. If the cost of the product and immediacy of the market opportunity warrant, the higher cost of year-round nurseries can often be justified, cutting significantly the time involved in development of genetic production material.

One of the major goals of the breeding program, in addition to lines with adequate field performance, is generating lines with commercial levels of expression of the gene of interest. Minimum concentrations of protein in corn meal for different types of products—purified pharmaceuticals, orally delivered vaccines, industrial enzymes or cellulases—are shown in Table 2 (J. Howard, personal communication). For the TrypZean™ product, the minimum concentration of trypsin in dry seed material should be 0.01–0.1% to meet production-

TABLE 2. MINIMUM CONCENTRATIONS OF FOREIGN PROTEIN IN GENETICALLY MODIFIED PRODUCTION MATERIAL FOR COST-EFFECTIVE PRODUCTION.

Product	Application requirement	Minimum concentration
For oral delivery of vaccines	~1 mg required for dose	0.1% DW
For purified pharmaceuticals	Less expression-sensitive	0.01% DW
For industrial enzymes	<\$200/kg production cost	≥0.1% DW
For cellulase for ethanol	120 g/gallon ethanol	≥3% DW

cost targets because in this lyophilized powder formulation it is considered a cell-culture reagent protein, thus falling between the purified pharmaceutical and industrial enzyme application requirements. As stated above, the concentration of trypsin in fifth-generation breeding material is approximately 0.006% of dry weight, somewhat lower than what will be required for a profitable product. However, because breeding and selection can generate material that expresses the recombinant protein at ten to a hundred times higher than the initial transformant (Hood *et al.*, 2003), continued breeding and selection should yield lines with the required expression level.

During the breeding program, **small-scale production** can be accomplished from hybrid seed samples taken from the breeding material and subsequently grown in field plots. Two goals can be achieved with this material: assessment of the improvement in agronomic character of interim hybrids and small-scale extraction/purification of the protein of interest for applications trials. These two assessments are critical to timely identification of potential problems in the product-development timeline.

The final **formulation** of the protein product is entirely a requirement of the application. For maize-derived trypsin, the product TrypZean™ is a lyophilized powder bottled for laboratory use as a cell-culture-dissociation reagent. For other uses, the formulation might be 1) a stabilized liquid, 2) ground, unextracted corn meal, or 3) ground, defatted corn-germ meal. In all cases, the cost of formulation must be compatible with the sale price of the product.

A product **safety assessment** includes understanding the hazard of the specific protein of interest alone or within the plant material. Once the inherent hazard of the protein has been quantified, one can determine the risk associated with various levels of exposure to it (risk is proportional to hazard multiplied by exposure) (Howard and Donnelly, 2004). While this assessment will not satisfy all regulatory-compliance issues, it is a required component and will assist in establishing confinement measures for regulatory compliance. The field of product safety versus regulatory compliance is in flux and requires constant attention to remain apprised of status.

PRODUCTION

Production of crop-derived proteins requires scale-up of activities similar to those described in product development. Additionally, it requires a plan for compliance with US Department of Agriculture (USDA) and Food and Drug Administration (FDA) regulations, or

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those of other agencies depending upon the product. The steps involved in production, growing, harvesting and associated activities, processing, and extraction/purification are outlined in Table 3.

Corn containing bovine trypsin—or any transgene for protein production—should be grown in areas where crop development is efficient and cost-effective, *i.e.*, the midwestern corn-belt. Otherwise much of the advantage of using a commodity crop for low-cost production is lost (Howard and Hood, 2005). The total acreage required is a product of seed yield and recombinant protein concentration in the seed. For example, trypsin in the fifth generation was at a concentration of 0.006% of whole-seed dry weight. If the corn produced 150 bushels per acre, equal to 3,750 kg of grain per acre, the yield of trypsin per acre would be 225 g. If the desired production amount is 10 kg for market entry, approximately 45 acres would be required. Confinement to meet USDA regulations requires a buffer zone of 1 mile wide surrounding the field, plus other measures such as delayed planting compared to other corn-production fields in the area. Male sterility can also be utilized, but the trypsin material is not male sterile.

TABLE 3. PRODUCTION STEPS AND PARAMETERS TO BE CONSIDERED DURING PRODUCTION OF TRYPSIN FROM TRANSGENIC MAIZE.

Production step	Considerations for trypsin in corn
Growing	Biological requirements for corn, seasonal and geographic limitations, recombinant protein yield, confinement regulations
Harvesting/ Transportation/Storage	Mechanical issues, time, temperature sensitivity, cost, protein stability in tissue
Tissue processing	Stability in tissue, potential for enrichment, small batches, whole seed, large batches, dry mill for germ, extract oil
Extraction/Purification	Protein stability, biomass quantity versus yield, cGMP for pharma applications, formulations

Corn seed is harvested mechanically when the moisture content is below approximately 20%. Shelling is accomplished best when moisture content is below 15%. Field drying is the most cost-effective method, but harvest should occur before a severe frost, and if necessary the ears can be mechanically dried after collection. Current regulations require dedicated equipment for non-food products from genetically modified crops. Once the grain is harvested, dried and shelled, it can be stored for months to years. Most foreign proteins in genetically modified seed are quite stable in dried grain (Hood *et al.*, 1997; Hood *et al.*, 2003; Lamphear, 2004), and trypsin is no exception (S.L. Woodard, unpublished).

One of the advantages of grain crops for bioproducts is the ability to partition proteins into specific tissue sinks (Figure 3). Trypsin is expressed largely in the embryo of the kernel, with much less present in the endosperm. Because the embryo represents only 10% of the dry weight of the kernel, separating it from the endosperm effectively concentrates the protein ten-fold on a dry-weight basis. Tissue separation can be accomplished with either dry-milling or wet-milling operations. The recombinant protein must be compatible with the temperatures used in either process, and the solutions used in the wet-milling process. In the future, oil will be extracted and sold as a co-product when the germ is isolated,

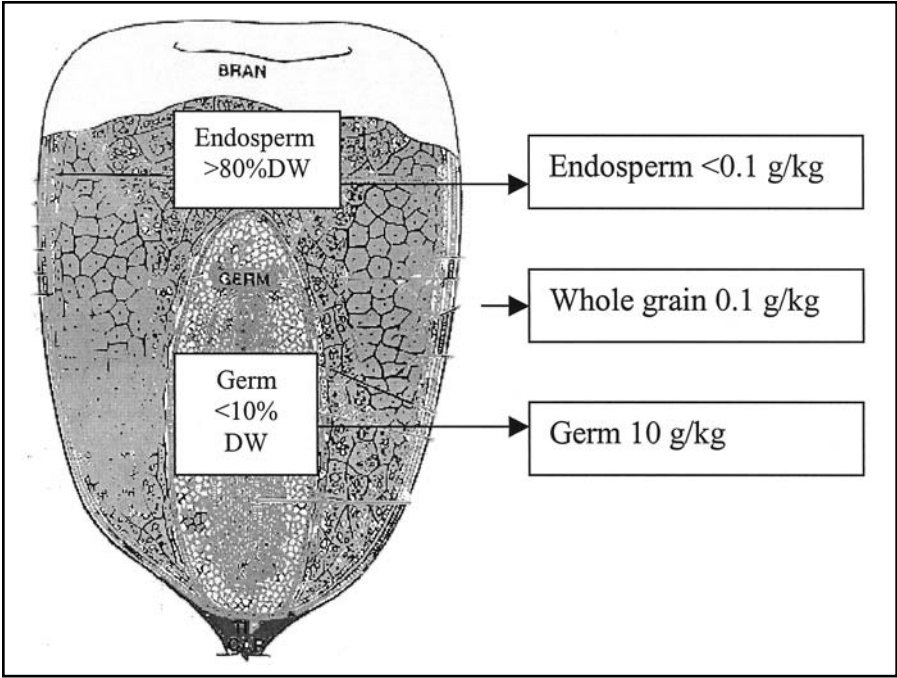


Figure 3. Relative contribution of maize kernel tissues to total seed weight. Numbers to the right compare the concentration of recombinant protein in each tissue based on a whole-seed expression level of 0.01% dry weight.

particularly if the transgenic product is deregulated. Tissue processing is not usually cost effective at less than a million bushels. Therefore, at 7,500 bushels from 45 acres of corn, whole-seed grinding and subsequent extraction will be a more cost-effective production method than seed fractionation for trypsin.

Currently, trypsin is a purified, lyophilized product, TrypZean™ (Sigma Chemical Co., St. Louis, MO). Thus, extraction and purification are necessary for its production and sale. Extraction buffers have been developed to maximize trypsin recovery and minimize extraction of native corn proteins. Subsequent purification protocols maximize recovery and stability balanced with purity. In many cases, 50% or less of the protein is recovered from the starting material, indicating that twice the acreage estimated above would be needed for production.

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PUBLIC ACCEPTANCE AND SALE

A lack of public acceptance is the major barrier today to producing biopharmaceutical or bioindustrial products in plants. Response to this public distrust has driven current regulations to be quite restrictive. The scientific community and the regulatory agencies are striving to gather substantive safety data to support regulations that are based on scientific principles and will protect the public as well as allow this new industry to develop. This topic has been discussed in detail in recent reviews on bioproduction and product safety (Howard and Donnelly, 2004; Howard and Hood, 2005). The critical asset for general public acceptance is whether the consumer sees benefits and whether these perceived benefits outweigh costs and risks. When products with obvious benefits are available to the consumer, public acceptance, science-based regulations and sales will fall into place.

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Hood has over seventy publications and patents to her credit and she has been an advisor for graduate programs at several universities. Currently she holds adjunct positions at Texas A&M University in the Department of Biochemistry and Biophysics and in the Molecular and Environmental Plant Sciences program. Her previous positions were with Utah State University, the Swedish University of Agricultural Sciences, Washington University in St. Louis and Oklahoma State University. She earned her PhD in plant biology from Washington University and an MS in botany from Oklahoma State. Her research interests are in the areas of renewable resources, foreign-gene expression in transgenic plants, plant cell-wall structure and function, and plant cell biology and protein targeting.