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Genetically Engineered Crops for Biofuel Production: Regulatory Perspectives

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ABSTRACT

There are numerous challenges in realizing the potential of biofuels that many policy makers have envisioned. The technical challenges in making the production of biofuels economical and on a scale to replace a significant fraction of transportation fuel have been well described, along with the potential environmental concerns. The use of biotechnology can potentially address many of these technical challenges and environmental concerns, but brings significant regulatory hurdles that have not been discussed extensively in the scientific community. This review will give an overview of the approaches being developed to produce transgenic biofuel feedstocks, particularly cellulosic ethanol, and the regulatory process in the United States that oversees the development and commercialization of new transgenic plants. We hope to illustrate that the level of regulation for transgenic organisms is not proportional to their potential risk to human health or the environment, and that revisions to the regulatory system in the U.S. currently under consideration are necessary to streamline the process.

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Abbreviations:AFEX-Ammonia Fiber Expansion pretreatment; APHIS-Animal & Plant Health Inspection Service of USDA; Bt-Bacillus thuringiensis endotoxin; CBM-Cellulose Binding Motif; CMC-Carboxy-Methyl-Cellulose; E1-Endoglucanase I gene; EPA-U.S. Environmental Protection Agency; FDA-U.S. Food & Drug Administration; PMIP-Plant Manufactured Industrial Product; PMP-Plant Manufactured Pharmaceutical; USDA-United States Department of Agriculture.

INTRODUCTION

Renewable energy has gained renewed interest and funding recently due to high crude oil price, uncertainty in fossil fuel supply, and political and environmental issues associated with fossil fuels. In March 2008, the price of crude oil peaked above a record $100 per barrel, and putting greater pressure on creating a domestic and less expensive source of fuel. Biofuels are an attractive alternative as they have environmental benefits compared to fossil fuels, are renewable, and can be domestically produced. The two main categories of liquid biofuels for transportation are biodiesel and bioethanol. Biodiesel, which can be produced from any fat or oil through a relatively simple transesterification process, is easily incorporated into diesel engines with little or no modifications (www.biodiesel.org). Other advantages of biodiesel are that it yields 93% more energy than is invested in its production and produces less air pollutants than bioethanol and fossil fuels (Hill *et al*., 2006). However, one of the main disadvantages of biodiesel is that because the source is limited to seed crops, such as soybean and palm oil, it directly competes with food production. Food based fuel crops are a concern as it is predicted that in the next 50 years, food production will have to double to feed the growing human population (Hill *et al*., 2006). The other common biofuel is bioethanol, produced from sugars extracted from a plant source and fermented. Currently the ethanol produced in the United States is almost exclusively from the corn grain by the hydrolysis and fermentation of starch, making it also a food based fuel. In addition, corn grain ethanol only produces only about 25% more energy than is invested during production and will not be able to meet the energy demands of countries such as the United States (Hill *et al*., 2006). Another source of ethanol is cellulosic bioethanol, which utilizes the breakdown of cellulose to sugar. Cellulosic ethanol's main advantage is that is not a food-based fuel and can be developed using agricultural plant waste or plants such as switchgrass and sorghum that require low inputs of water, pesticides, and fertilizers. The challenge with cellulosic bioethanol is that hydrolysis of cellulose is currently a difficult process that requires acid, steam, or AFEX (Ammonia Fiber Explosion) pretreatment to destabilize lignin and hemicellulose and make cellulose accessible to cellulase enzymes. Utilizing transgenic plants with modified cell wall composition and engineered microbes for enhanced fermentation is expected to significantly increase the efficiency of cellulosic bioethanol production.

PLANT BIOTECHNOLOGY FOR BIOFUEL PRODUCTION

The use of biotechnology for biofuel production is very attractive. Theoretically biofuel yields can be increased without a comparable increase in the amount of energy needed for production or cultivated land. Significant progress has been made using molecular biology in the past decade to increase the activity of enzymes and the microbes used for biofuel production. However, by

many estimates, there is not enough arable land to produce sufficient biofuel feedstocks to replace more than 15% of transportation fuel using existing technologies (Gressel, 2008). The development of transgenic plants engineered for enhanced biofuel conversion is expected to be the most rapid and efficient solution, especially for the production of fuels from lignocellulosic biomass (Gressel, 2008).

Two main techniques exist for producing transgenic plants, differing in the method by which the DNA of interest is inserted into the plant cell. The first transformation technique was through the use of the soil bacterium Agrobacterium tumefaciens to successfully introduce the bacterial gene encoding neomycin phosphotransferase type II (nptII) into plant cells. The nptII gene provides resistance to aminoglycoside antibiotics and after treatment with agrobacteria tobacco cells exhibited tolerance to kanamycin (Fraley R, 1983). Agrobacteria naturally infect plants when the plant is wounded and insert a portion of their DNA into the plant cell, inducing the cell to proliferate and produce amino acid derivatives, opines, that are readily metabolized by the agrobacteria. Virulent agrobacteria contain a Ti plasmid on which a number of virulent (vir) genes are present. The proteins encoded by the vir genes shuttle a portion of the Ti plasmid defined by flanking border sequences (the T-DNA) from the agrobacterium cell into the plant nucleus (Tzfira *et al*., 2006). The T-DNA relies on a number of plant host cell proteins to transport the T-DNA from the cytoplasm into the nucleus and integrate it into the plant genome. The exact integration process is not entirely understood, but appears that the host cell machinery recognizes the T-DNA as broken DNA fragments and preferentially incorporates them into transcriptionally active regions of the plant genome (Alonso *et al*., 2003).

Researchers have modified agrobacteria for plant genetic engineering purposes by modifying the Ti plasmid to remove the genes directing opine biosynthesis and uncontrolled cell division from the T-DNA. These genes are instead replaced with the genes that are intended for integration into the plant, though there is still agrobacteria sequence in the T-DNA. The presence of agrobacteria DNA in the T-DNA incorporated into the transgenic plant has important regulatory consequences, as it triggers oversight by USDA and will be discussed later. The host range of agrobacteria was originally thought to just be dicot plants, but now have been successfully used to transform a wide range of important monocot crop plants too, including maize (Ishida, 1996), rice (Hiei *et al*., 1994), switchgrass (Somleva *et al*., 2002), sorghum (Zhao *et al*., 2000) and wheat (Cheng M, 1997).

The second technology developed to transform plant cells utilized particle bombardment to physically shoot exogenous DNA into the plant cell (Sanford, 2000). Any DNA can be coated onto tungsten microparticles in the 1 μm size range and bombarded into plant cells, where it appears to be integrated randomly into the plant genome by host factors. Biolistic transformation has successfully incorporated the chloramphenicol acetyltransferase (CAT) gene into maize cell cultures (Klein T, 1988), and shortly after generated stable transgenic tobacco plants expressing the β-glucuronidase gene (Klein *et al*., 1988). However, biolistic transformation often leads to integration of many (>

10) copies or fragments of the transgene in the plant genome (Wan Y, 1994), which can sometimes lead to gene silencing (Iyer LM, 2000).

With either using agrobacteria or biolistic transformation, the transformed plant cells must be regenerated into mature plants, a lengthy process that can take over six months depending on the plant species. Either transformation process can be used to generate the transgenic biofuel plants described below, though the choice of transformation technique can lead to different regulatory scrutiny.

AGRONOMIC BIOTECHNOLOGY TRAITS

The most likely near term transgenic plants that will be used for biofuel production are those already on the market or those for which the traits were already under development for other uses. For example, many of the major seed companies have recently begun marketing high starch corn varieties for biofuel use. Corn grain from these varieties would yield increased glucose levels compared to existing varieties and lead to higher levels of ethanol production from the grain. To be competitive with the existing varieties of corn grown in the United States, any new variety will likely need to incorporate traits such as insect and herbicide resistance or farmers will be reluctant to adopt them and suffer lower yields or higher input costs. The most common transgenic traits utilized for insect resistance are Bacillus thuringiensis (Bt) toxins. There are over 140 genes identified from Bacillus thuringiensis that encode endotoxins (Crickmore *et al*., 1998), and these proteins are specific to certain orders of insects (Lepidoptera, Coleoptera, etc) with limited cross reactivity. Each Bt protein acts by binding to a specific membrane glycoprotein receptor in the insect midgut. Once bound, the Bt protein inserts irreversibly into the cell membrane, forming pores that lead to epithelial cell death, and eventual starvation of the insect due to feeding inhibition Hilder, 1999 #45}. Bt proteins were first expressed in tobacco (Barton *et al*., 1987) and tomato (Fischhoff D, 1987)and currently over 35% of corn in the U.S. and over 50% of cotton are engineered to express at least one Bt gene (Fernandez-Cornejo J, 2006). However, only nine Bt genes have been utilized to develop transgenic crops (http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm), in part due to the rigorous regulatory process. The most widely used trait for herbicide resistance, glyphosate tolerance, has been engineered into over 85% of the U.S. soybean crop but only about 25% of the corn (Fernandez-Cornejo J, 2006). Glyphosate is a wide spectrum herbicide that acts to inhibit the shikimic acid pathway common to all plants. This pathway is responsible for the synthesis of the amino acids tryptophan, phenylalanine, and tyrosine (Steinrucken H, 1984). Glyphosate binds strongly to the enzyme 5-enolpyruvylshikimate–3-phosphate (EPSP) in plants, blocking its activity, but mutant versions of the gene were identified from bacteria such as Salmonella typhimurium in which a serine residue was substitute for the proline at amino acid 101, making the enzyme insensitive to glyphosate (Comai *et al*., 1983). When introduced

into tobacco, the mutated EPSP allowed the plants to survive exposure to glyphosate (Comai *et al*., 1985). This trait provides transgenic plants with a large selective advantage over conventional plants and allows farmers to spray their fields with glyphosate to kill off most weeds without harm to the transgenic crops.

In addition to high starch corn varieties, there are a number of potential crops for biofuel production that would benefit from insect or herbicide resistance. Varieties of soybean that have a higher oil content amendable to biodiesel conversion would likely need to be engineered to contain glyphosate tolerance before farmers will adopt them. Similarly, natural varieties of low lignin corn have been identified and are known as brown midrib lines. However to be commercially attractive to farmers such lines would need to express Bt endotoxins to provide resistance against corn rootworm and corn borers (Koziel *et al*., 1993; Moellenbeck *et al*., 2001).

The next category of transgenic traits that could be introduced for biofuel use includes plants engineered to have increased tolerance to abiotic stresses such as drought and salinity. These are the two leading causes of crop loss worldwide (Vinocur *et al*., 2005) and are projected to affect 50% of the global arable land by 2050. Researchers have been working for years to improve drought and salinity stress in crops, and these traits could become valuable for crops intended for biofuel production. As there is growing public concern with producing fuel instead of food on farmland, the most productive land should always be used to produce food crops. By increasing plants' abiotic stress tolerance, they will be able to be also grown in marginal cropland and increase the available area for biofuel production. These traits would be especially valuable for crops grown for biomass, as a likely decreased seed yield from suboptimal growing conditions would not necessarily affect the biomass production as severely.

There are two general mechanisms for increasing abiotic stress tolerance in plants, to enhance the environmental sensing and signal transduction pathways, or to enhance the stress response methods (Vinocur *et al*., 2005). The C-repeat/dehydration-responsive element binding factor (CBF) is an example of a family of transcription factors that transduce environmental signals into gene responses and recognize the cold and dehydration responsive element (CRT/DRE) (Yamaguchi-Shinozaki *et al*., 1994). The CRT/DRE elements have a conserved 5 base pair sequence that is found in the promoters of genes induced by cold and drought (Jaglo *et al*., 2001), and in the case of the DREB2A gene can be induced in as little as ten minutes after dehydration (Liu *et al*., 1998). Constitutive over-expression of OsDREB1A and OsDREB1B in rice (Ito *et al*., 2006) expression of Arabidopsis DREB1A in maize under the control of a stress inducible rd29A promoter (Al-Abed *et al*., 2007) improved stress tolerance under cold, drought and salt stress treatments. Additionally, constitutive overexpression of CBF1 in Brassica napus led to significantly increased drought stress in greenhouse conditions compared to wild type plants (Zhang *et al*., 2004). Another approach for enhancing the signal transduction pathway is through the control of the plants' stomatal pores on the surface of the leaves. Stomates open to allow the uptake of carbon

dioxide and release of oxygen, but they also allow the evaporation of water. Stomatal opening is controlled by the plant hormone abscisic acid (ABA), which triggers a signaling pathway in the guard cells that close the stomatal pore (Hetherington A, 1991). The signal transduction pathway is quite complex (Schroeder *et al*., 2001), but a protein farnesyltransferase ERA1 appears to play an important role in suppressing the ABA signal. When the gene is knocked out in Arabidopsis thaliana, era1 mutants show significant drought tolerance compared to wild type plants when not watered for two weeks (Pei *et al*., 1998). As era1 did not show any negative phenotypes (Pei *et al*., 1998), crop plants could be engineered with RNAi constructs to knockout expression of homologous farnesyltransferases and be better adapted to periods of water stress. Alternatively, LEA proteins accumulate in plant cells in response to drought stress and while their exact mechanism of action is not clear have been hypothesized to bind water molecules and sequester ions due to their hydrophilic nature (Swire-Clark *et al*., 1999). An LEA protein from barley, HVA1, provides increased drought tolerance when overexpressed in either rice (Chandra Babu *et al*., 2004) or wheat (Sivamani *et al*., 2000), and could be an attractive candidate gene to overexpress in biofuel crops to increase yields. There have been genes isolated that are comparable to HVA1 and DRE2B that provide tolerance to salinity stress (Vinocur *et al*., 2005).

BIOTECHNOLOGICAL APPROACHES FOR CELL WALL MODIFICATION

The production of biofuels from biomass holds great potential, as there is an order of magnitude more potential feedstock available for cellulosic ethanol production as there is for biodiesel from oilseed crops. The potential for biotechnology to modify the cell wall structure or lignocellulosic composition of the plant could yield considerable improvements in the ethanol yield per acre, or the energy balance and economics of cellulosic ethanol production. The key limitation of converting plant biomass to biofuel is the inability of appropriate hydrolytic enzymes to access and deconstruct the plant cell wall. This is thought to be due to the complex structure of the plant cell wall, which is a mixture of different polysaccharides (cellulose and hemicellulose) cross-linked with lignin, phenolic acids (ferulic and p-coumaric acid), pectins and structural proteins (Carpita, 1996; Grabber, 2005; Stone, 2006). The complex nature of plant cell walls is thought to be in part essential to provide mechanical strength, protect the plant from abiotic and biotic stresses and perform several essential functions in plants. However, the complex nature of the plant cell wall makes cellulosic biomass very difficult, or recalcitrant, to break down into simple sugars (Himmel *et al*., 2007). Thus, it is essential to understand plant cell walls at the chemical, biochemical and biological levels in order to develop plant feedstocks that are capable of maintaining essential plant functions, but are more amenable to efficient biomass deconstruction and conversion to biofuels.

Plant cell walls are composed of several components including cellulose, hemicellulose, lignin, pectin, structural proteins and aromatic compounds (Carpita, 1996). They are broadly classified as primary or secondary cell walls, which are distinguished by the absence or presence of lignin. Primary cell walls can be further classified into two types, namely Type I, and Type II. Type I cell walls are the most predominant found in dicot plants and in non-commelinoid monocots. Type II cell walls are found only in commelinoid monocots, which includes all of the cereal crops and perennial grasses (Carpita and McCann, 2000). In Type I cell walls, cellulose microfibrils are interlocked by xyloglucan, the predominant hemicellulose. The cellulose-xyloglucan framework is then embedded in pectin and structural protein matrix. In Type II cell walls, the cellulose microfibrils are interlocked by a different hemicellulose, namely glucuronoarabinoxylan. An additional difference between these two types of cell walls is the presence of higher proportion of ferulic acid moieties on glucuronoarabinoxylan chains, which cross-link hemicellulose via diferulic acid diester bridges.

Secondary cell walls are characterized by the presence of lignin, which embeds the cellulose and hemicellulose matrix. Lignin in most of the dicots is composed of guaiacyl (G) and syringyl (S) units while in grasses lignin is composed primarily of p-hydroxyphenyl (H), guaiacyl and syringyl units (Dixon *et al*., 2001; Grabber, 2005). Lignin is cross-linked by carbon-carbon (C-C) bonds or by ether linkages to form a complex network. Lignin also cross-links cellulose and hemicellulose by ester and ether bridges and by diferulic acid diester-ether bridges in the case of grasses. The extensive cross-linking of cell walls by lignin is thought to be one of the key limitations for producing biofuels from biomass. Cell walls that are cross-linked are less accessible to hydrolytic enzymes, and necessitating physical or chemical pretreatment to allow hydrolysis to simple sugars.

CELL WALL BIOSYNTHESIS

The majority of the cell wall related mutants that have been isolated to date are the result of visual screens for an altered plant phenotype. For example, irregular xylem (irx), interfascicular fiberless (ifl), ectopic deposition of lignin in pith (elp) and reduced epidermal fluorescence (ref) mutants are all the result of visually examining plant populations for altered cell structure or absence of UV fluorescent secondary metabolites on the leaf surface (Turner and Somerville, 1997; Zhong *et al*., 1997; Zhong *et al*., 2000, Ruegger and Chapple, 2001). There are also a few cell wall mutants (mur) that were identified based on difference in cell wall composition (Reiter *et al*., 1997). Identification of such mutants has expanded our knowledge on cellulose, hemicellulose and lignin biosynthesis.

Cellulose synthase (Ces A) genes that are involved in cellulose biosynthesis were first identified from developing cotton fibers (Pear *et al*., 1996). However, a clear understanding of the role of CESA genes in cellulose biosynthesis came

from the characterization of irregular xylem (irx) mutants from Arabidopsis (Turner and Somerville, 1997; Taylor *et al*., 1999). There is sufficient evidence to suggest that specific CESA genes are involved in cellulose biosynthesis in primary and secondary cell walls (Gardiner *et al*., 2003). In addition to cellulose synthases, there are other proteins such as membrane anchored endo-1-4–β-glucanase (KORRIGAN; Nicol *et al*., 1998; Lane *et al*., 2001), a membrane associated sucrose synthase (Amor *et al*., 1995) and UDP-Glc:sterol glucosyltransferase (SGT; Peng *et al*., 2002) that are associated with cellulose biosynthesis.

Hemicellulosic polysaccharides that cross-link cellulose microfibrils are primarily synthesized in golgi by glycan synthases and glycosyltransferases. Because of the structural similarity of the back bone of hemicelluloses (β-1,4 linked glucose in xyloglucans; β-1,4 linked mannose in glucomannans and β-1,4 linked xylose in arabinoxylans) and cellulose (β-1,4 linked glucose) it is predicted that cellulose synthase like (CSL) genes may encode golgi localized glycan synthase (Richmond and Somerville, 2000). The evidence that CSL genes encode glycan synthases was first demonstrated by Dhugga *et al*., 2004 when β-1,4 -mannan synthase from guar seeds was identified as a member of the CSL A gene family. Subsequently, several CSL genes from different species were identified that are involved in mannan and glucomannan synthesis (Liepman *et al*., 2005); mixed linked glucan biosynthesis (Burton et al., 2006) and xyloglucan glucan synthase (Cocuron *et al*., 2007). In addition to glycan synthases, a few glycosyltransferases that add side chains to the hemicellulose backbone were also identified (Perrin *et al*., 1999; Madson *et al*., 2003; Zhong *et al*., 2005; Lee *et al*., 2007).

The traditional view of lignin biosynthesis in which p-coumaric acid, ferulic acid and sinapic acid acts as precursor to the formation of p-hydroxyphenyl, guaiacyl and syringyl lignin, respectively was challenged in the last decade (Humphreys and Chapple, 2002). Identification of caffeoyl CoA 3-O-methyltransferase (CCoAOMT) as a key enzyme that converts caffeoyl CoA to feruloyl CoA, and its role in lignin biosynthesis suggested an alternative to free acid pathway (Ye *et al*., 1994; Zhong *et al*., 1998). Subsequent characterization of ferulate 5 hydroxylase (F5H) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) enzyme activity in Arabidopsis and other species suggested that F5H and COMT displayed greater affinity for coniferaldehyde and 5-hydroxyconiferaldehyde, respectively than ferulic and 5-hydroxyferulic acid as originally hypothesized (Humphreys *et al*., 1999; Osakabe *et al*., 1999; Li *et al*., 2000; Parvathi *et al*., 2001). A key finding in our understanding of lignin biosynthesis is the characterization of the p-coumarate 3-hydroxylase activity that was believed to occur at the level of free acids through the conversion of p-coumaric acid to caffeic acid. CYP98A3, a candidate gene for C3H activity, converted p-coumaroyl shikimate and p-coumaroyl quinate into their corresponding caffeic acid conjugates and did not prefer p-coumaric as a substrate (Schoch *et al*., 2001). Further, characterization of Arabidopsis, alfalfa and poplar CYP98A3 by mutant or transgenic approaches showed that CYP98A3 is involved in lignification (Franke *et al*., 2002; Nair *et al*., 2002; Reddy *et al*., 2005, Coleman *et al*.,

2008). A gene that encodes for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) activity, which converts p-coumaryl CoA to p-coumaroyl shikimate or p-coumaroyl quinate was also identified and its role in lignin biosynthesis was unequivocally established (Hoffmann *et al*., 2003; 2004). These results suggested that the free acid pathway in which caffeic and sinapic acid act as precursors to lignin biosynthesis was incorrect. Additionally, Arabidopsis ref1 mutant characterization further suggested that ferulic acid and sinapic acid are partly derived from coniferaldehyde and sinapaldehyde respectively by the action of an aldehyde dehydrogenase (Nair *et al*., 2004). Thus our understanding of lignin biosynthesis has significantly changed over the last decade and each new finding helped us to establish a reliable roadmap to lignin biosynthesis.

BIOTECHNOLOGY APPROACHES TO IMPROVE BIOMASS YIELD AND COMPOSITION

The key factors that determine the cost of conversion of biomass to biofuels are the amount of biomass produced per unit area and the efficiency by which the polysaccharides present in biomass is hydrolyzed and converted to biofuels.

BIOMASS YIELD

Mutations in specific genes or expression of single genes by transgenic approaches increased overall biomass production in plants (Levy *et al*., 2002; Lefebvre *et al*., 2005; Sakamoto *et al*., 2006). One such approach is to express carbohydrate binding modules that bind to polysaccharide surfaces and affects the interaction of cellulose to hemicellulose in plants. Under in vitro conditions it has been shown that CBMs could interfere with attachment of hemicellulose to cellulose (Shpigel *et al*., 1998). Levy *et al*., 2002 showed that heterologous expression of a Clostridium cellulovorans cellulose binding domain in poplar enhanced plant growth. They speculated interaction of CBM during early stages of cellulose crystallization led to an increased rate of cellulose biosynthesis. Safra-Dassa *et al*., 2006 further showed that introduction of Clostridium cellulovorans CBM3 gene in potato plants enhanced plant growth during early stages of plant development. However, expression of CBMs from Piromyces equi and Cellulomonas fimi in transgenic tobacco resulted in plants with altered cell wall structure and delayed growth (Obembe *et al*., 2007). Thus the suggestion for use of CBMs for increasing plant biomass or plant cell wall modification needs further investigation.

An alternative approach to increase plant biomass is to delay flowering in plants so that energy needed during flowering and seed set could be diverted for continued plant growth. It has been shown that over-expression of Arabidopsis Flowering locus C (flc) gene in tobacco led to delayed flowering and increased

plant biomass (Salehi *et al*., 2005). One final approach to increasing biomass is a class of strategies by which the photosynthetic efficiency of plants can be increased for improved growth and yield. Transgenic tobacco over-expressing fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase showed increased dry weight (Lefebvre *et al*., 2005; Tamoi *et al*., 2006). Similarly, transgenic maize over-expressing sorghum phosphoenolpyruvate carboxylase (PEPC) showed improved carbon dioxide fixation rate and increased fresh and dry weight under drought conditions (Jeanneau, *et al*., 2002). Change in plant architecture to enhance photosynthetic capacity has also recently shown to be effective in improving biomass and grain yield in rice (Sakamoto *et al*., 2006).

BIOMASS COMPOSITION

The efficiency by which the plant biomass is utilized for biofuel production depends on the ability by which appropriate hydrolytic enzymes access and hydrolyze cell wall polysaccharides. Currently, biomass undergoes thermo-chemical pretreatment that may include high temperature, pressure or extreme pH to disrupt cell wall and to expose the sugar molecules to enzyme hydrolysis (Merino and Cherry, 2007). The key factor that necessitates this pretreatment is the extensive cross-linking of cell walls in both dicots and monocots by lignin. In the case of grasses, in addition to lignin, ferulic acid also cross-links cell walls (Grabber, 2005). Thus biotechnology approaches to modify lignin content and composition in plants is considered as an alternative to severe pretreatment to improve biomass conversion to ethanol.

Rapid progress has been made in understanding lignin biosynthesis in dicots as compared to monocots. Chen and Dixon (2007) showed that in transgenic alfalfa, lignin modification could improve fermentable sugars and bypass the need for acid pretreatment. They have analyzed transgenic alfalfa plants in which six lignin biosynthetic pathway genes (C4H, HCT, C3H, CCoAOMT, F5H and COMT) were independently down regulated. They showed that the recalcitrance of cell walls to enzyme digestion with or without pretreatment is directly proportional to the lignin content. In some of the transgenic lines, substantial reduction in lignin content resulted in reduction in biomass. However, the increase in sugar production in these transgenic plants offset the reduction in overall biomass yield.

Although substantial reductions in lignin content per se are likely to have deleterious effects on plant growth and development (Pedersen *et al*., 2005; Reddy *et al*., 2005; Franke *et al*., 2002), alterations of lignin composition may improve biomass properties without deleterious pleiotropic effects. Transgenic approaches have shown that plants tolerated a reduction in lignin content to a certain level without severe reduction in biomass (Reddy *et al*., 2005; Pichon *et al*., 2006). However, it needs to be shown whether this reduction in lignin content could reduce pretreatment needs of biomass for biofuel production. An alternative approach to lignin reduction is to alter lignin composition.

In angiosperms, lignin is composed of syringyl and guaiacyl lignin while in grasses lignin is composed of p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin (Dixon *et al*., 2001; Grabber, 2005). Because, H-lignin contains p-hydroxyphenyl alcohol subunits that have non-methoxylated C-3 and C-5 positions of the phenyl ring they allow for more C-C linkages during polymerization as compared to the coniferyl or sinapyl alcohols of G- and S-lignin. Thus, it would be attractive to redirect the flow of carbon from p-hydroxyphenyl alcohol and coniferyl to sinapyl alcohol and thereby increase the proportion of S-lignin to reduce C-C cross-linking. Ferulate 5-hydroxylase (F5H) that converts coniferaldehyde to 5-hydroxyconiferaldehyde is a key enzyme involved in S-lignin biosynthesis. In Arabidopsis, F5H mutations completely abolish S-lignin (Chapple *et al*., 1992; Meyer *et al*., 1996) and the over-expression of F5H in Arabidopsis, tobacco and poplar using a vascular tissue specific promoter altered the lignin composition to predominantly S-lignin (Meyer *et al*., 1998; Franke *et al*., 2000). The pulping efficiency of transgenic poplar plants with higher S-lignin content significantly improved as compared to control poplar plants due to reduced C-C linkages (Huntley *et al*., 2003). This promising approach needs to be explored in detail to determine whether changes in lignin composition without reduction in lignin content may improve biomass properties for biofuel production.

In grasses ferulic acid is another key factor that cross-links cell walls by forming linkages between glucuronoarabinoxylan (GAX), the predominant hemicellulose and GAX and lignin (Jung 2003; Grabber, 2005;). It has been perceived for a long time that any reduction in ferulic acid content may have adverse effects to plants since ferulic acid is considered as a precursor to lignin biosynthesis. In Arabidopsis hydroxycinnamic acids, ferulic and sinapic acids are primarily bi-products of lignin biosynthesis produced by the oxidation of coniferaldehyde and sinapaldehyde by an aldehyde dehydrogenase (Nair *et al*., 2004). Coniferaldehyde dehydrogenase activity that converts coniferaldehyde to ferulic acid is present in several plants including grasses such as maize (Nair *et al*., 2004). Homologs of Arabidopsis REF1 are present in maize and rice (Skibbe *et al*., 2002). Preliminary result in maize suggests down-regulation of REF1 homologues (RF2C and RF2D) led to a reduced level of ferulic acid and lower levels of GAX (Nair *et al*., unpublished results). Alternatively, identification of enzyme that transfers ferulic acid to the arabinosyl residue (feruloyl transferase) of GAX through an ester linkage will be a key target for ferulic aicd reduction. Recently, a bioinformatics approach was used to identify a candidate gene for feruloyl transferase (Mitchell *et al*., 2007). It needs to be shown either enzymatically or more importantly through mutant or transgenic approaches that the candidate gene encodes a feruloyl transferase.

There is also a strong interest in increasing the cellulose to hemicellulose concentration in biomass to improve ethanol yields. It has been suggested that increasing cellulose content in plants, especially grasses, may also improve plant standability (Dhugga *et al*., 2007). Over-expression of cellulose synthase genes in maize showed improved cellulose synthesis (Dhugga *et al*., 2005). Alternatively, reduction in hemicellulose content may improve the ratio of cellulose to hemicellulose content. As described earlier, our understanding of

hemicellulose biosynthesis is still in its infancy. While several enzymes that are involved in addition of side chains to hemicellulose backbone are identified (Zhong *et al*., 2000; 2005; Persson *et al*., 2007), it need to be shown whether transgenic approaches using these genes could lead to plants without severe developmental phenotype.

CELL WALL HYDROLYZING ENZYMES

Numerous microbes have been isolated with the ability to completely degrade cellulosic biomass into simple sugars that are readily metabolized by the microbes. The best characterized is Trichoderma reesei (Sternberg, 1976), but most organisms share a common feature in that they produce a large variety of cell wall hydrolyzing enzymes that work in concert to deconstruct the hemicellulose surrounding the cell wall and then break down the cellulose microfibrils into individual glucose molecules (Lynd *et al*., 2002). Three classes of cellulase enzymes are needed to completely break down cellulose to glucose monomers that can then be fermented into ethanol (Sun and Cheng, 2002). Exoglucanases processively hydrolyze cellulose from the ends of the molecule, releasing glucose dimers (cellobiose), while endoglucanases cleave the cellulose molecule internally, producing additional reduced ends that can then be acted on by exoglucanases. High levels of cellobiose can reduce exoglucanase activity through feedback inhibition, but β-glucosidase enzymes cleave free cellobiose molecules to produce glucose (Sun *et al*., 2002). There has been extensive research and development on commercially producing enzyme mixes capable of hydrolyzing cellulose for food processing, and a number of genes encoding microbial cellulase and hemicellulase enzymes have been cloned (Warren, 1996). Large enzyme manufacturers have been working for the past five years to improve the activity of their cellulase cocktails on cellulose substrates with the goal of producing cellulosic ethanol. In 2001, the U.S. Department of Energy set a goal reducing the cost to manufacture cellulases through microbial fermentation from approximately $5 per gallon of ethanol produced to a cost of $0.05 - $0.10, but this has not yet been achieved. One alternative to the traditional method of producing enzymes in genetically engineered microbes is through transgenic plants. There can be considerable economic advantages of a transgenic plant expressing the microbial cellulase, as there are no large capital costs associated with constructing a large scale microbial fermentation facility and the level of enzyme production can easily be scaled up by planting more transgenic seeds.

One of the best characterized cellulase enzymes is the E1 endoglucanase from Acidothermus cellulolytics, a thermophillic bacteria originally isolated from decaying wood. E1 demonstrates a temperature optimum of 83 °C and a specific activity of 40 μmol glucose release from carboxymethylcellulose (CMC)/min/mg protein, and is described in detail in U.S. Pat. No. 5,275,944. It is synthesized as a precursor with a signal peptide that directs it to the export pathway in bacteria. The mature enzyme is 521 amino acids (aa) in length. The crystal structure of the catalytic domain of about 40 kD (358 aa) has been

described (Sakon *et al*., 1996). E1 was successfully expressed in transgenic potatoes (Dai *et al*., 2000) and tobacco (Dai *et al*., 2000; Ziegelhoffer *et al*., 2001)and observed to be highly active against the purified cellulose substrate CMC. Even though E1 was constitutively expressed throughout the whole, no visible phenotypes were observed compared to wild type plants and the transgenic plants had the same rate of growth and produced comparable biomass (Ziegelhoffer *et al*., 2001). This lack of phenotype is probably due to the fact that endoglucanases cleave cellulose internally and in the actual plant cell wall matrix the extensive cross-linkage between cellulose, hemicellulose, and lignin provides sufficient structural support to make up for the disruptions in the cellulose strands. Plant produced E1 is highly stable, as E1 tobacco seeds still showed high E1 activity after storage at room temperature for one year (Dai *et al*., 2005). This stability is an attractive feature of plant produced enzymes since it simplifies the storage and transportation of the enzyme. Additional cellulases from Thermomonospora fusca have also been successfully expressed in plants. E2 and E3 encode an endoglucanase and exoglucanase respectively that are active on crystalline cellulose (Irwin D, 1993), and both were singly transformed into tobacco, alfalfa, and potato (Zeigelhoffer *et al*., 1999). These lines produced E2 or E3 at levels up to 0.1% total soluble protein and plant produced E2 was active on CMC. Interestingly, there appeared to be a correlation between enzyme expression in alfalfa and increased digestibility during ensilement (Ziegelhoffer *et al*., 1999). As with transgenic E1 plants there did not appear to be a visible phenotype associated with E2 or E3 expression, which might also be from the structural support from hemicellulose and lignin. In addition to cellulases, other cell wall hydrolyzing enzymes have been expressed in plants. Endoxylanases act to hydrolyze xylan, the primary type of hemicellulose found in woody plants and monocot cell walls (McNeil *et al*., 1984), eventually releasing the pentose sugar xylose. The endoxylanase xynZ isolated from the thermophillic bacteria Clostridium thermocellum was expressed in tobacco (Herbers *et al*., 1995), and the plant extract demonstrated to hydrolyze purified birchwood xylan to release xylose and xylobiose.

The seeds of plants are natural storage units for the plant and have the capacity to accumulate large quantities of carbohydrates and protein. A number of groups have taken advantage of seeds' ability to accumulate up to 13% protein by weight (in corn, (Reussner *et al*., 1957) and targeted recombinant proteins to rice and corn grain using seed specific promoters such as glutein and globulin (Yang *et al*., 2003; Hood *et al*., 2007). These plants can be harvested as normal, and the grain collected and stored until the enzyme is ready to be extracted. Alternatively, the biomass can also be harvested with the grain, separated, and then processed at an ethanol processing plant using the plant produced enzyme to supplement the microbial cellulases used to hydrolyze the biomass. The pre-eminent biofuel crop in the United States is corn, and progress has been made with developing transgenic varieties expressing enzymes for this use. Both E1 and the exoglucanase CBHI have been expressed in separate lines of corn at high levels in the grain under the control of the seed specific glob1 promoter (Hood *et al*., 2007). The enzymes made up over 16% of the total soluble protein in the grain, and were readily

extracted and observed to retain their biological activities (Hood *et al*., 2007), suggesting that these transgenic lines could be grown as a reliable source of cellulase enzymes. However, these plant produced enzymes were not assayed on actual plant biomass substrates to confirm that they could be useful for lignocellulose hydrolysis.

The ultimate transgenic feedstock has been proposed to be a plant containing a high enough level of enzymes to perform autohydrolysis under the appropriate conditions to release fermentable sugars, or at least substitute for some of the pretreatment process or microbial enzymes needed for hydrolysis (Sticklen, 2006). The agbiotechnology company Syngenta is working towards this goal by developing a variety of transgenic corn expressing an amylase enzyme targeted to the grain, which hydrolyzes starch to release glucose (Ethanol Prod Mag http://www.ethanolproducer.com/article.jsp?article_id=2113). In standard dry mill processing of corn grain to produce ethanol, the corn grain is ground and liquefied, and then microbial amylase enzymes added to the slurry to break the starch polymers down to glucose. The amylase gene used by Syngenta was cloned from a thermophillic bacteria isolated from an undersea hot vent, allowing the enzyme to remain stable and active at the high temperatures (70-80°C) commonly used for starch hydrolysis. Syngenta planned to field test this variety of corn during the 2007 growing season, which is expected to greatly reduce the amount of external amylases needed for processing the grain.

While this variety of corn could reduce the cost of producing ethanol from corn grain, it does not address the problem of a limited amount of grain available, since even if the entire U.S. corn grain harvest were used to produce ethanol it would only replace 16% of the gasoline used annually (Gressel, 2008). Therefore, various groups, including Edenspace, are working towards the goal of incorporating cell wall hydrolyzing enzymes into the plant biomass, so that a portion of the remaining 80% of the corn biomass left behind after the grain harvest can be converted into ethanol or other alcohol fuels (Sticklen, 2006). Sufficient crop residue will need to remain on the soil to maintain soil quality. The E1 endoglucanase was the first enzyme engineered to be expressed in all tissues of corn (Ransom *et al*., 2007) and rice (Oraby *et al*., 2007) plants. Similar to the E1 tobacco, there was no visible phenotype in these transgenic plants compared to wild type lines yet the E1 was active on CMC. The E1 rice biomass was pretreated using AFEX to examine the impact that E1 had on the hydrolysis of the rice biomass. Even though AFEX is considered to be one of the mildest pretreatment conditions available, the E1 activity in the plant biomass was reduced 65% after the pretreatment (Teymouri *et al*., 2004). This illustrates one of the main challenges with using in planta enzyme for cellulosic ethanol production. The pretreatments necessary to strip away lignin and hemicellulose from cellulose, making the cellulose accessible to the cellulases, are likely to inactivate the enzymes. Higher expression of the enzymes may overcome this challenge, though may also produce detrimental phenotypes on the transgenic plants. However, the E1 could be extracted from the biomass using a simple protein extraction procedure and added back to the biomass after the AFEX pretreatment along with a β-glucosidase to

complete the hydrolysis of cellulose to glucose. This approach yielded a 22% conversion of cellulose to glucose (Oraby *et al*., 2007), which is promising as no exoglucanase was added to the reaction.

REGULATORY ISSUES REGARDING BIOFUEL BIOTECHNOLOGIES

The issue of genetically engineered organisms has always been a contentious issue, so while the laws and implementing regulations regarding oversight of transgenic products require that regulation be science-based, risk analysis always requires some judgment on the part of decision-makers. This may be particularly the case when laws are applied to the regulation of transgenic products because the United States does not have an overarching biosafety law and instead uses existing laws to regulate transgenic organisms.

The general U.S. policy on regulation of transgenic products was laid out in 1986 in the Coordinated Framework for Regulation of Biotechnology. This document, which was composed by the Reagan administration's Office of Science and Technology Policy, highlights that regulation should be science-based and product-based, rather than viewing products created through a particular process (i.e., genetic engineering) as inherently different. Consistent with this approach, the Coordinated Framework outlines how the U.S. Department of Agriculture (USDA), Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) are expected to regulate transgenic products using existing laws, which continues to be the U.S. policy. However, to implement the Coordinated Framework and apply the relevant laws to transgenic products, the agencies established separate regulatory staffs and USDA and EPA also found it necessary to write regulations that are in essence, if not in letter, specific to transgenic products. Despite the oft-repeated objective of U.S. policy that regulation of transgenic organisms be product-, not process-based, regulators only examine transgenic products even though plants with similar traits that were developed through conventional means are not regulated. In fact, the agencies evaluate each plant transformation event separately. Even if a plant expressing a certain transgene (for example corn expressing EPSP) determined not to present a risk and was approved for release, another corn plant expressing EPSP using the same transformation vector but generated on a different day would have to be assessed independently and could not be released until it had gone through the regulatory process. Thus a process-based system has been established, since it has been judged to be politically unfeasible to regulate products of conventional breeding in the same way as products of genetic engineering. This is despite the finding of several National Academy of Science committees that conventionally bred and transgenic crops pose the same types of risks and thus there is no scientific justification for not regulating conventional plants (NRC, 2002). For example, plants that are genetically engineered to be herbicide tolerant

fall under the scrutiny of the USDA; whereas, conventionally bred herbicide tolerant varieties do not, even if the conventionally bred lines are resistant to the same herbicide as the transgenic ones.

The Animal Plant Health Inspection Service of USDA (USDA-APHIS) uses the authority of the Plant Protection Act to regulate transgenic organisms. Among other things, this law gives USDA the authority to restrict the introduction into the environment of plant pests, which are defined as living organisms that cause disease in or damage to plants not including humans and non-parasitic plants (U.S. Congress, 2000). USDA-APHIS has interpreted this "plant pest authority" to regulate transgenic organisms based on the potential plant pest risk caused by the use of plant pest (e.g., viral) sequences or vectors (e.g., Agrobacterium) in the creation of many transgenic plants (USDA-APHIS, 1997). For plants created through biolistic transformation that do not have plant pest sequences (such as corn expressing the Arabidopsis DREB1A under the control of the Arabidopsis rd29A promoter), the regulations can be imposed on articles that the APHIS administrator has "reason to believe" pose a plant pest risk (USDA-APHIS, 1997). Technically, it may be possible that the use of plant pest components in the creation of a transgenic plant could create a new plant pest or increase the transgenic plant's susceptibility to a disease. However, the rarity of these effects in transgenic plants and the dependence on the reason to believe clause point out weaknesses in APHIS' justification for regulatory authority over transgenic plants (Huttner S, 1992).

In part to address this issue, in 2004 USDA-APHIS initiated a process to revise its regulations based on consolidated authorities in the Plant Protection Act, including the so-called "noxious-weed authority" (USDA-APHIS, 2004). This authority gives USDA-APHIS the ability to restrict introduction into the environment of noxious weeds, which are defined as "any plant or plant product that can directly or indirectly injure or cause damage to crops, …other interests of agriculture, …natural resources of the United States, public health, or the environment" (U.S. Congress, 2000). This definition is remarkably broad. Parallel to the reasoning used in regulating based on potential plant pest risk, the revised regulations would regulate based on potential noxious weed risk of transgenic plants, greatly expanding the reasons for USDA-APHIS to assert its authority. On the other hand, using the noxious weed authority to regulate transgenic plants could further undercut the goal of product-based regulation, as one could argue that conventionally developed varieties are just as likely to have the characteristics of a noxious weed (USDA-APHIS, 2004).

EPA solely regulates transgenic plants that contain pesticidal elements. To use this law to regulate transgenic plants, EPA defined a new pesticide type, the plant-incorporated protectant (PIP), as "a pesticidal substance produced by the plants and the genetic material necessary for them to produce the substance." (EPA, 2001) Some have criticized EPA for too restrictively regulating PIPs by establishing requirements that are not commensurate with the risks posed by transgenic plants and are unnecessarily burdensome for applicants, including refuge requirements that are not obligatory for chemical pesticides. Nonetheless, EPA's efforts may improve acceptability of the technology, and, in the case of Bt crops, extend the lifetime of their benefits to agriculture.

FDA regulates foods derived from transgenic products under the Federal Food Drug and Cosmetic Act (FFDCA). As published in 1992, FDA policy is that it will regulate foods derived from transgenic products in the same way as those derived from conventionally developed products (FDA, 1992). FDA's regulation is based on whether the product has altered nutritional properties or contains a food additive, which is defined as a substance introduced into food that is not a pesticide and is not generally recognized as safe (GRAS) (FDA, 1992). As for foods conventionally developed, FFDCA makes it the responsibility of the developer to determine that transgenic-derived foods are safe and any substances new to the variety are GRAS, but FDA provides a voluntary consultation process to help developers determine this. The consultation process, through which developers submit data to FDA scientists until FDA has no more questions regarding safety, is available for both conventional and transgenic products. FDA records show that all transgenic products currently approved in the United States have completed the consultation process.

Given the complexities of the U.S. regulatory system for transgenic crops, how does it actually work? The USDA, EPA, and FDA use a science-based risk assessment approach to evaluate the safety of transgenic crops before approval for food, feed, or planting use in the United States. For this endeavor, the agencies consider the potential impact of a large number of environmental and health related effects of the transgenic crops. Notably, the list of concerns that the agencies evaluate includes all of the potential environmental hazards that transgenic plants could cause, as identified by a recent National Academy panel, which are the following (NRC, 2002):

- The transgenic plant itself could become weedy or invasive.
- The transgenic trait could be passed to a wild or weedy relative and increasing its weediness or invasiveness.
- The transgenic trait could negatively impact non-target organisms in the environment.
- Organisms that the transgenic trait is intended to harm could develop resistance to the trait.
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USDA-APHIS considers agricultural and environmental effects of transgenic plants as it reviews all transgenic plants that applicants want to release into the environment in an unconfined manner (e.g., for commercialization). FDA reviews transgenic plants for food and feed uses through a voluntary consultation process with applicants. If the product expresses a pesticidal protein then EPA also evaluates the transgenic plant for health and environmental effects before it can be sold commercially. Currently, agency scientists and risk managers evaluate each submission on a case-by-case basis and determine the specific data the applicant should submit depending on the product. However, there are a number of general concerns that the agencies typically evaluate for every product they assess, though for EPA those are only plants producing PIPs.

EPA, FDA, and USDA-APHIS all evaluate a detailed molecular and genetic characterization of the product, including demonstrating stable inheritance

through Southern blotting and quantification of the transgene product in various plant tissues with western blots or ELISA. The molecular characterization provides information about the identity of the transgenic plant and helps confirm that the inserted gene is functioning as intended. In addition, FDA and USDA-APHIS examine plant composition, also to gauge unintended, pleiotropic changes due to transgenesis. FDA and EPA evaluate allergenicity potential of the expressed protein(s) by in vitro digestibility, heat stability, and sequences analyses of the protein and when applicable, any changes in expression of known endogenous allergens.

Both EPA and USDA-APHIS are concerned with the potential of the transgenic plant to become an agricultural weed and for gene flow to occur from the transgenic plant to wild relatives. Consequently, EPA has set isolation distances for commercially grown Bt-cotton in tropical areas of the United States where cotton relatives grow that may be able to hybridize with commercial cotton in order to minimize the potential for pollen-mediated gene flow. In order to satisfy National Environmental Policy Act and Threatened and Endangered Species Act requirements, both agencies determine whether there will be an impact on endangered species by the planting of the transgenic crop. In the case of plants engineered to produce a Plant Incorporated Protectant (PIP), a pesticidal substance produced by the plants and the genetic material necessary for them to produce the substance, USDA-APHIS and EPA evaluate whether there will be toxicity to non-target organisms (soil organisms, insects, birds, and fish) that might come into contact with the crop or its residues.

Lastly, APHIS determines the potential of a transgenic plant to cause damage to agriculture through the introduction of a novel plant pathogen produced by the transgenic plant or a change in plant susceptibility to pests. APHIS also assesses whether the modification will alter the farming practices associated with the crop.

As described above, transgenic plants are highly regulated compared to plants with identical phenotypes developed without the use of molecular biology, which undergo none of those assessments (Bradford *et al*., 2005; McHughen, 2007). The time necessary to bring a transgenic plant to the market ranges from six to twelve years from the isolation of a gene of interest and it has been reported that the time necessary to obtain regulatory approval has increased in recent years. In addition, it is estimated that the cost to bring a transgenic plant through the regulatory process is between $20-$30 million (McElroy, 2003). A significant fraction of this cost is from the need to also obtain regulatory approval in key export markets, as regulatory approval in one country does not transfer to others. These factors, along with intellectual property and political issues, have led to the small number of transgenic plants that have been approved for commercial use. Crops engineered with traits for biofuel production are expected to have greater public support than crops containing agronomic traits such as herbicide tolerance because consumers will see more direct benefits in lower fuel costs and reduced environmental impacts from oil use. However, to realize these benefits, transgenic biofuel crops will need to navigate the regulatory system in a timely manner, otherwise it is likely that industry will focus on other technologies that do not offer the

same benefits.

The regulation of the first category of transgenic biofuel crops described above, those expressing existing Bt or EPSP genes, would be straightforward. Both of these traits have been very well characterized in corn and these varieties of transgenic corn have been grown for over a decade (Fernandez-Cornejo J, 2006). While there are no significant human health or environmental concerns with these traits, any new lines of transgenic glyphosate resistant plants would need to formally assessed by APHIS and Bt plants formally assessed by both APHIS and EPA. This assessment would add at least a year to the commercialization timeline, as applicants would need to prepare data packets describing a thorough molecular, genetic, and compositional analysis of the transgenic plant along with an analysis of the allergenicity potential of the recombinant protein. The process would be extended for Bt plants since they would also need to submit additional data to EPA such as the results of field trials studying the impact on non-target organisms.

The next type of biofuel crop, those modified for increased abiotic stress tolerance, will encounter a more rigorous regulatory process. No transgenic plants with drought or salinity tolerance have been deregulated by APHIS, so the assessment process will take longer. Since these traits could lead to increased weediness and APHIS is not familiar with them, it will likely determine the necessary data requirements during the assessment process. Researchers have performed field studies with transgenic sunflower expressing Bt genes (Snow *et al*., 2003), which appeared to increase fitness and fecundity, and it is possible that APHIS might require applicants to perform similar field trials to test transgenic plants with increased abiotic stress tolerance. The applicant will provide data characterizing the transgenic plant as described for an herbicide resistant plant, but for these novel traits APHIS will review the data submitted and then request additional data and trials until it is satisfied that it can issue a Finding of No Significant Impact for the release of the transgenic plant. This process could obviously take years to assess the potential environmental impact, since field trials to evaluate the fitness impact of drought or salinity tolerant plants would need to last multiple years. This uncertainty makes planning the development of the transgenic product difficult for the applicant.

Plants engineered to contain altered cell wall structure or increased biomass, would not raise the same environmental concerns that the second category of transgenic plants would, since those traits would not be expected to provide a fitness advantage in the environment. Varieties of switchgrass, bromegrass, and alfalfa, were identified from natural populations that contained between 13% and 8% less lignin content respectively than the natural populations (Casler *et al*., 2002). After four growing seasons increased mortality was observed in the switchgrass and alfalfa, but not the bromegrass (Casler *et al*., 2002). However, since no traits from this category have previously been deregulated by APHIS the agency would still need to determine the necessary data requirements during the assessment process, making the deregulation process almost as difficult for the applicant as for products in the second category.

Lastly, transgenic plants expressing enzymes to be extracted or engineered

to produce high quality fuel oil (Kinney *et al*., 2005) could have a very challenging path through the regulatory system. This is because of APHIS's regulation of plants engineered to produce industrial compounds. Transgenic plants expressing a Plant Manufactured Industrial Product (PMIP) are defined as plants that meet the following three criteria:

1. The plants are engineered to produce compounds that are new to the plant;
2. the new compound has not been commonly used in food or feed; and
3. the new compound is being expressed for non-food, non-feed industrial uses. (68 FR 46434-46436. http://www.aphis.usda.gov/brs/fedregister/BRS_20030806a.pdf)

This is a broad definition that can include most compounds, including recombinant proteins, which have not been previously expressed in plants. These plants will undergo significantly greater scrutiny during the assessment process than other transgenic plants. For example, field trials involving PMIP crops will be inspected an average of four to six times, while an average field trial would only be inspected once or twice. Additionally, the time required to obtain an environmental release permit necessary for a field trial of a PMIP crop will be at least 120 days, as compared to 60 days for a conventional transgenic plant. In addition to the standard data requirements described for the other categories of transgenic plants, the industrial compound will be scrutinized for potential human and wildlife health impacts, necessitating numerous animal feeding studies.

CHANGES TO THE REGULATORY SYSTEM

As described earlier, APHIS has the broadest overview over transgenic plants for biofuel use and has been working to revise its regulatory process for transgenic plants since 2004 to address concerns with emerging transgenic technologies. Recently APHIS released proposed changes to their regulatory authority that could significantly change the way transgenic plants are regulated, including those intended for biofuels. Most of these changes are expected to benefit the regulation of transgenic plants by streamlining the regulatory process and developing a more science based assessment system, and are highlighted below.

SCOPE OF REGULATORY OVERSIGHT

APHIS currently regulates transgenic plants based on the potential to become a plant based from the use of agrobacteria to transform plants. Emerging technologies may lead to transgenic products produced via methods that will

not trigger review under APHIS's current regulations, so APHIS has proposed evaluating transgenic plants as potential noxious weeds. This broad definition should reassure the public that all transgenic products will be regulated, but could be considered excessive or unbalanced, as non-transgenic plants with comparable traits would present the same noxious weed risk but are not regulated. APHIS is also considering trait-based rather than event-based regulation, so that an approval of a plant (i.e. maize) expressing a specific transgene would allow other maize plants expressing the transgene to be released. Trait-by-species regulation would be more consistent with the report by the National Research Council (NRC, 2002) that stated the focus of regulatory assessment of transgenic organisms should be on the phenotype (i.e. trait) of the product, and not the method by which it was produced. This would allow applicants to more readily evaluate multiple lines containing the same transgene, while conserving APHIS's resources for greater scrutiny of higher risk products.

RISK-BASED CATEGORIES (EXPANDED TIERED PERMITTING SYSTEM)

APHIS has two broad categories for regulating transgenic plants. The notification system is streamlined and applicable for a limited number of plant species expressing well understood traits, while the permitting system requires a significantly higher level of scrutiny. Most plant being developed for biofuel use will fall under the permitting system solely because they are novel traits, not because they are expected to have an increased risk to human health or the environment. There is significant uncertainty for developers in knowing how much scrutiny their transgenic products will undergo during the permitting process, making it difficult to anticipate how much time and resources will be needed to gain regulatory approval. Fortunately, APHIS is considering defining different classes for transgenic products based on the potential risk of the transgene and the plant. A science-based approach to assessing transgenic products should lead to more assessment categories with clearer guidelines for developers, and can also streamline the regulatory process for low risk products. Importantly, familiarity with a crop or trait can be incorporated into the assessment categories so that new products will have a defined process to move into lower risk categories (or in some cases higher risk categories) as APHIS gains experience assessing similar transgenic plants.

There are concerns with certain types of transgenic plants under development that have been classified based on the function of the engineered compound as a plant manufactured industrial product (PMIP) or plant manufactured pharmaceutical (PMP), and APHIS has proposed that these types of products automatically be assigned to a high risk assessment tier. While these classes of compounds could have higher biological activity than compounds in other transgenic plants, it is not good policy to regulate based on the presumed

market function of the product because this could cause inconsistent regulation. For example, a plant engineered to express a modified lignin more readily gasified to produce fuel (Yoshida *et al*., 2004) might be considered an PMIP plant, while the same plant used to increase the feed value of a crop by increasing digestibility would be considered an agronomic product. The PMIP plant would automatically be assigned to a higher risk tier than the plant modified for agronomic use. The assessment tiers should be based on trait-plant combination (e.g. weediness potential, food or feed crop, wild relatives) including the transgene product (e.g. toxicity, potential allergenicity, source of the transgene). The intended use of the plant is certainly a factor to consider in a risk-based evaluation, but often will be a rather unimportant factor. Historically hundreds, if not thousands of plants or plant products have been used for medicinal purposes, including some that are still currently consumed by humans such as cocoa beans (Theobroma cacao) and turmeric (Curcuma longa). The fact that a pharmaceutical or industrial use protein is produced in a food or feed crop does not necessarily mean there is increased risk compared to food or feed crops expressing other types of traits.

Many new varieties of transgenic plants are expected to be developed in the near future, and assessing each one at the existing level of detail will likely produce a significant burden on APHIS's staff and unnecessarily delay the introduction of products that benefit public health and the environment. The establishment of clear, risk-based tiers will simplify the process of identifying products that necessitate increased scrutiny and regulation and help best utilize APHIS's limited resources by streamlining the assessment process for most low risk products.

LOW-LEVEL PRESENCE OF REGULATED BIOTECHNOLOGY MATERIALS

There have been no significant negative impacts from biotechnology products that have gone through regulatory evaluation by APHIS and commercialized. On the other hand, there have been a number of incidents where regulated material has been detected at low levels in the environment or food supply. In none of these cases of adventitious presence were any harmful impacts on the environment or on human health detected or foreseeable, nor would harmful effects be expected for low-level presence of the vast majority of biotechnology products. Nonetheless, these incidents required regulatory action that was clearly disproportionate to the actual science-based risk of the product. The best known case in the United States is of Aventis' StarLink corn containing the Bt gene Cry9C. StarLink was approved by the EPA for animal feed but not for human consumption due to concerns that the stability of the protein under low pH might lead to allergic reactions (Bucchini, 2002). When traces of Cry9C were identified during 2000 in food products made from corn, there were rapid recalls of almost three hundred corn based products and Aventis spent approximately $100 million buying back corn that might have commingled with StarLink at a premium to try and remove StarLink from the market (Bucchini, 2002).

While a few individuals claimed to have experienced allergic reactions, none of them tested positive for IgE antibodies against Cry9C (Sutton *et al*., 2003). This suggests that there was no human health risk associated with StarLink corn, supporting EPA's own assessment that Cry9C had a low probability of allergenicity (Bucchini, 2002). In contrast, there have been numerous examples during the 20th century of foods imported into countries that have led to life-threatening allergic responses, such as the case with kiwi fruit (Lucas *et al*., 2003). No conventionally bred plants are screened for allergenicity, raising the question of whether the risk of the high scrutiny for transgenic plants is justified, when just the presence of Cry9C DNA in 1% of the corn based products was enough to trigger the StarLink recall (Dorey, 2000). In comparison, actual hazards such as rodent feces, pesticide residues, mycotoxins, and heavy metals are allowed at low levels in foods.

The need to ensure complete containment of transgenic plants under development adds significant cost to developers often without providing any benefits to the environment or human health, as the risk from most transgenic plants is negligible. In part to address this issue, APHIS is working to develop criteria under which occurrence of regulated articles would be non-actionable. The product would still be a regulated item, but an accidental low-level presence in the environment would not necessarily require action to be taken unless justified by actual risk. Applicants would still be obligated and should strive to contain regulated material, but it seems reasonable that biotechnology products presenting no known or foreseeable hazard should also be allowed reasonable tolerances. Plants engineered for altered cellulose or lignin content, or to produce increased biomass are examples in which a low-level presence would not likely present a risk to human health or the environment, so would be good examples of plants that might not require regulatory action if a low-level presence was detected.

CONCLUSIONS

Through the oversight provided by USDA, EPA, and FDA, transgenic plants have been more heavily scrutinized than any other comparable agricultural product. This high level of regulatory scrutiny is due to political rather than scientific consideration, since plants developed through traditional breeding or chemical mutagenesis with the same trait as a transgenic product (i.e., herbicide tolerance) undergo no formal review. Consequently, the development time and cost for transgenic plants is greatly increased compared to conventionally developed crops. For the benefits of transgenic crops to be fully realized in a timely manner in both the United States and developing countries, both the regulatory challenges and general public mistrust of transgenic plants need to be reduced and scientists need to continue to educate the public on the actual risks and benefits of transgenic plant technologies. The proposed changes to USDA-APHIS's regulations are expected to streamline the assessment process for a number of transgenic crops while still retaining a thorough evaluation

of the plants. These changes will be essential in the near future, given the rapidly growing interest in developing new crops for biofuels, nutrition, and novel agronomic traits will likely lead to a boom in the number of transgenic plant products needing to go through the regulatory process. If each new transgenic plant will need to undergo the same thorough level of assessment as is performed today then APHIS will quickly be overwhelmed and the time it takes to obtain regulatory approval will continue to increase.

While it is not possible to conclusively demonstrate that an agricultural product will have zero adverse environmental or human health impact, the agencies have determined to the best of their abilities that the transgenic plants on the market are safe and this conclusion has been supported by field observations. After a decade of growing transgenic plants commercially and over fifteen thousand field trials involving transgenic plants, there has been no documented case of a negative impact on human health or the environment. As we enter the second decade of plant biotechnology, serving new markets for energy and health care as well as growing demand for food and feed, it will be essential to be sensitive to the public concerns with transgenic organisms while not smothering the development of new and useful technologies.

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