PROCEEDINGS OF A WORKSHOP ON:

CRITERIA FOR FIELD TESTING OF PLANTS WITH ENGINEERED REGULATORY, METABOLIC, AND SIGNALING PATHWAYS

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INTRODUCTION AND PURPOSE OF WORKSHOP

The rapidly growing number of field trials of transgenic plants reflects the rich diversity of types of genes and phenotypes becoming available for genetic engineering through plant molecular biology and genomics efforts. Increasingly, genes used in genetic engineering affect gene expression, metabolism, or signaling pathways and so may also have secondary effects on plant physiology due to pleiotropy or epistasis. These types of plant genes are being used to engineer a variety of phenotypic changes, including altered growth and development (e.g., altered flowering, fruit ripening, growth rates, yield), modified metabolism, increased tolerance to environmental stresses (e.g., frost, drought, salt), or novel disease resistances (e.g., viral, bacterial, or fungal resistance). The use of these "newer genes" contrasts with the first wave of commercialized transgenic crops, which predominantly utilized genes whose direct gene product (e.g., specific protein) conferred the desired trait of interest and in which the potential for pleiotropic or epistatic effects was more likely to be a result of position effects rather than gene function.

As useful genes emerge with more complex effects, identifying secondary effects and evaluating their consequences are integral components of biosafety assessments. Field testing of these products is the first regulatory challenge, as plants with engineered transcriptional control, metabolism, and signaling pathways are developed for commercial use.

This two-day workshop brought together regulators and industry and academic scientists working in various disciplines to discuss and evaluate current knowledge and research on secondary effects of transgenes that function as transcription factors, in signal transduction, or to modify metabolic pathways. The workshop focused on examples and commercially promising case studies to promote information exchange and discussion of data and experiments on secondary effects of these genes. We sought to evaluate what information is available and to identify areas that would benefit from additional research. In particular, secondary effects that could influence confinement, including gene flow to wild populations and adjacent, nontransgenic crops, were discussed. The collective knowledge and insight coming from this workshop should be valuable to those who develop these products for commercial purposes and to those who make regulatory decisions on field testing criteria of future transgenic plants.

There were four formal objectives for the Workshop:

(1) to promote a multidisciplinary discussion about field testing releases and management of newer, more complex genes emerging from plant genomics projects among geneticists, plant breeders, biotechnologists, physiologists, and ecologists from government, industry, and academia;

(2) to evaluate current standards for gene characterization and identification of secondary effects with respect to newer, more complex genes emerging from plant molecular genetic and genomics projects;

(3) to discuss whether emerging genes and the phenotypes they affect present any new environmental issues relevant to field testing releases and management; and

(4) if data or research gaps appear to exist, to discuss what additional data and experiments would identify secondary effects that may impact field testing releases of transgenes that affect metabolic or signaling pathways.

To accomplish these goals, the program consisted of a series of plenary talks followed by a day of discussion by small breakout groups.

OVERVIEW OF PLENARY TALKS

The first set of plenary talks focused on presenting an overview of field testing of engineered plants from several perspectives to provide participants with the context of how field testing is regulated, the approach industry uses to conduct field testing, and what biological factors may impact field testing.

Dr. Dave Heron (USDA-APHIS) provided information on how field testing is regulated by USDA. APHIS authorizes field testing through either a permit process or a notification process. Notifications use a simplified process for plants that are not noxious weeds, and six criteria must be met to be eligible for a notification procedure; whereas permits are used for any organism or trait but require more details, for example, on how biological containment will occur. The performance standards for field tests are intended to ensure biological containment so that the transgenic article will not persist in the environment. Notifications and permits occur with state concurrence and require field data reports within six months after the field test ends. Both may have site inspections. Any unusual occurrences (i.e., accidental release, plants destroyed by disease or other causes) must be reported to APHIS. Lack of regulatory compliance is subject to penalties of up to $500,000. Dr. Heron pointed out that more than 8,700 field tests have been authorized at approximately 30,000 sites since 1987, and no serious negative impacts on the environment have been reported. More than 36 species of crop plants, 10 species of grasses, 14 species of trees, and 9 species of ornamentals have been field tested since 1987.

Dr. Chuck Mihaliak (Dow AgroSciences) and Dr. James Astwood (Monsanto Co.) described the approach used by industry for preparing and conducting field testing. Dr. Mihaliak focused on a general framework of how industry develops products. Broadly, the stages progress from generating events, selecting events, characterizing events, and then launching the product. Each successive step involves screening products for desirable and undesirable characters. The safety of biotech products is established through evaluating gene, protein, and crop safety criteria that include ecological and human/animal health assessments. Dr. Astwood focused on safety assessments of metabolically altered plants and used two case studies, high carotenoid *Brassica napus* and amino acid enhancement in corn, to demonstrate his points. He illustrated how industry applied the concept of relative safety to evaluate food/feed safety, and in particular he focused on the approach to evaluate intended and unintended alterations of metabolites. Pre-existing natural variability is a key component for safety assessments because it provides the context for examining any intended or unintended alterations. Analyzing the targeted metabolic pathway can generate hypotheses that can be tested. He posed questions that could lead to insights when manipulating pathways: what, if altered, would be a concern? What is likely to be altered, and would it be a concern? What is actually altered? As in the case of high carotenoid *Brassica*, other species can serve as points for comparison.

Two speakers, Dr. Steve Strauss (Oregon State University) and Dr. Allison Snow (Ohio State University), outlined biological issues of importance for field testing. Dr. Strauss offered five contentions on the biological impacts of transgenes that affect regulatory, metabolic, and signaling pathways. First, he contended that although, as a class, these transgenes are less well known, they are far safer than first generation transgenes. Second, molecular biology would not provide general guidance on the potential for invasion, but rather phenotypes, fitness, and nutrition would be the most important criteria. Third, pleiotropic effects may alter development and are the rule in breeding, but these should not be equated with the potential for invasiveness. Fourth, the low frequency of transgenes present in small-scale field tests should minimize the spread of a transgene for most genes that alter existing regulatory, metabolic, or signaling pathways. And, lastly, genes that affect pathways will rarely improve fitness in wild populations. Dr. Snow described changes in plant development and morphology that could alter gene flow and plant fitness. In particular, she focused on how gene flow and plant persistence could be affected by changes in pollen (amount, longevity, dispersal distance,

and degree of outcrossing); in seeds (number, longevity, dispersal distance through attractiveness to pollinators or through aerodynamics); and from the extent and dispersal of vegetative propagation. She noted that uncertainties remained about isolation distances needed for containment due to the fact that pollen dispersal is highly variable and that a small fraction of pollen or seeds may travel very long distances.

The second set of plenary talks examined examples of plant genetic engineering involving metabolic traits, signal transduction factors, and transcription-associated factors. In each case, the speakers also explored what is known about secondary effects associated with expression of the genes in question.

Dr. John Ohlrogge (Michigan State University) described two examples of metabolic engineering of fatty acids: high oleic soybean and high laurate canola. High oleic soybean oil, which could provide direct health benefits by reducing the levels of saturated and polyunsaturated fats in our diet, was achieved via suppression of the 18:0 fatty acid desaturase, while production of laurate, which is used for soaps and surfactants, was achieved in canola via expression of a 12:0 acyl-ACP thioesterase derived from the California bay tree. Engineering for production of lauric acid showed that increasing levels of the 12:0 acyl-ACP thioesterase produced increasing laurate concentrations up to approximately 40% of the oil content, but had diminishing ability to increase laurate content above that point. Analysis of the inability to exceed the 40 – 60% plateau showed that, while making more thioesterase and laurate, the plants were not accumulating more laurate. High acyl-ACP thioesterase activity was associated with induction of at least two enzymes involved in fatty acid degradation. Several enzymes involved in fatty acid synthesis also were increased. Thus, accelerated fatty acid synthesis occurred to compensate for losses due to breakdown, resulting in a futile cycle of production and oxidation of lauric acid. Microarray analysis showed changes in gene expression, including some that encoded predicted fatty acid-associated enzymes, as well as other types of proteins, including putative transcription factors possibly involved in controlling expression of fatty acid synthesis enzymes. Overall, less than 1% of genes analyzed showed altered expression, indicating specific cellular response to altered fatty acid production rather than wholesale changes. The level of change can be contrasted with variations in gene expression as high as 30% at different stages of leaf development. The results indicated that it is possible to achieve metabolic changes, but such modification also can cause compensatory changes by the plant, including adjustments in both metabolic activity and gene expression.

Drs. Harry Klee from the University of Florida and Peter McCourt from the University of Toronto examined manipulation of hormone and signal transduction pathways. Harry Klee discussed alterations in ethylene synthesis and perception. Controlled ability to induce ethylene production can be of value for increased postharvest fruit quality in which ripening is ultimately desired, whereas inhibited perception can be of value where indefinite delay of senescence (e.g., floral senescence) is desired. Induction of ethylene perception was associated with an array of undesirable secondary ethylene-related effects, including increased disease susceptibility to specific pathogens, reduced adventitious root formation, reduced ability of roots to penetrate soil, reduced ability to develop mature seeds, and reduced ability of stems to elongate in response to low light conditions. These phenotypes are consistent with the broad range of functions associated with ethylene action. Thus, although it is possible to make ethylene insensitive plants, negative consequences may severely impair performance and competitiveness. Ethylene responses can show clear cell autonomy, indicating that tissue- or developmental-specific promoters may assist in targeting appropriate specificity for desirable ethylene insensitive phenotypes.

Peter McCourt examined the effect of plant hormones on coordination of development and interactions among signaling pathways. A screen for mutants involved in water use efficiency was performed by identifying individuals with increased abscisic acid (ABA) sensitivity. These mutants were then used to identify second mutations affecting ABA response. The resultant genes were not only involved in ABA processes, but also were related to ethylene, gibberellins, and sugar sensing. These results indicate a com-

plex interplay among different signaling pathways and may explain why many hormones have overlapping functions. Dr. McCourt emphasized that the analysis of the genome is only the first level of understanding. The resultant proteome is much more dynamic, as protein expression changes during development and in response to environmental stimuli. The expressed proteins, in turn, form complex interactions with other proteins and other cellular components, as has been demonstrated by profiles of networks of yeast-interacting proteins. Ultimately, it is the total network that is responsible for phenotype.

Two speakers discussed transcription-related factors, Dr. Xinnian Dong from Duke University and Dr. Mike Thomashow from Michigan State University. Dr. Dong examined host-pathogen interactions, with emphasis on two types of genes involved in the systemic acquired resistance (SAR) pathways, *cpr* and *npr*. Selective forces operate on both the pathogen and the host to achieve a balance between virulence and resistance and the associated costs of each. Resistance mechanisms involve many genes and a diversion of resources, so that in many cases resistance responses, such as SAR, are inducible, rather than constitutive. Microarray analysis indicates that SAR induction results in induction of hundreds of genes. Mutant *Arabidopsis cpr* lines constitutively expressing SAR have increased disease resistance but reduced growth, indicating metabolic costs of constitutive SAR expression. Overexpression of the *NPR* gene, which encodes a master regulator of transcription of SAR-related genes, does not cause constitutive SAR expression. Thus, overexpression of *NPR* leads to enhanced resistance without negative effects on growth. Experiments measuring fitness in the growth chamber and field also showed negative effects with constitutive expression of *cpr*, but not *npr*. However, only *cpr* overexpressors, but not *npr* overexpressors, reduced disease severity rating. Neither *cpr* or *npr* overexpression gave increased seed yield relative to controls, even in the presence of the pathogen. It was concluded that constitutive activation of SAR has substantial fitness costs that outweigh benefits of enhanced resistance.

Fitness effects of constitutive expression of normally inducible responses were observed with freezing tolerance responses as described by Dr. Mike Thomashow. Environmental stresses severely limit crop productivity, both in terms of where crops can grow and the yield potential at those locations. Adaptations to these stresses involved complex physiological responses including action of multiple genes. Microarray analysis showed cold temperature induction of ca. 200 *Arabidopsis* genes and down-regulation of approximately 100 more genes. One approach to increase resistance is regulon engineering, allowing for coordinated induction of a suite of relevant genes by expression of appropriate transcription factors. A promising transcription factor is CBF, which is rapidly induced by cold, and in turn, induces a subset of ca. 40 of the cold-induced genes, including the *COR* (cold-regulated) genes. Overexpression of *CBF* causes constitutive expression of *COR* genes, a higher level of *COR* gene expression following cold induction, and increased freezing tolerance for both pre-acclimated and non-acclimated plants. Other stress-related responses such as increased proline and sugar accumulation also are observed with overexpression of *CBF*. CBF-induced genes are also associated with other dehydration-related stresses such as drought and salt stress, indicating similar underlying mechanisms of resistance to the different stresses. In another cited example (Park *et al.,* 2001), stress and pathogen resistance was correlated, suggesting cross-talk among responses. Despite increased resistance, there were negative effects on growth, indicating that inducible rather than constitutive activation of complex systems may provide fitness benefits. Once again, targeted expression, e.g., via the use of stress-responsive promoters, may be of value in engineering desired phenotypes.

Collectively the speakers gave insight into the types of genes being used and the types of phenotypes being regulated, and touched on several recurring themes demonstrating interconnectedness of genetic, signaling, and metabolic pathways. Plants, like all living organisms, have evolved a complex web of cellular activities that produce and receive feedback from the internal and external environment. Manipulation of one aspect often results in alteration of several others, including compensatory changes. While this can cause secondary effects, the majority of those effects have negative impacts on plant growth

and fitness. This range of phenotypes and their effects on fitness provides a backdrop for evaluation of possible implications for field testing of these classes of genes.

An additional point made by several speakers was that many of the traits we might manipulate today have been (or can be) altered by conventional methods and often using similar types of genes. For example, Dr. McCourt related that although the specific gene product was not identified at the time, the short stature wheat and rice varieties critical to the Green Revolution were achieved through the use of naturally occurring gibberellin-insensitive mutants. Dr. Klee also discussed the wide range of phenotypes available in natural populations (such as in the cultivated and wild tomato species) and emphasized the importance of examining effects of genetic engineering efforts within the context of the natural range of genetic variation available by crossing.

SYNTHESIS OF THE GROUP REPORTS

The groups were asked to respond to a list of questions developed prior to the workshop by its organizers. The discussion had two phases. The first phase of questions was intended to have groups address general issues associated with field testing of plants with engineered regulatory, metabolic, and signaling pathways. The second phase asked the group to answer questions specific to a particular case study. The six case studies focused the questions on the use of transcription factors (cold tolerance, disease resistance), on alterations in signal transduction (altered ripening, altered flowering), and on modifications to metabolic pathways (lignin/wood modification, oil modification).

General issues discussed

The general questions posed to the groups included the following:

1. Given the regulatory criteria of field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?

2. Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring these changes?

3. What are the strengths of the industry approach to characterize genes from plant genomics projects? Are there areas where the approach should be improved?

4. Do any new environmental issues relevant to field testing releases and management arise when considering emerging genes and the phenotypes they affect?

Most groups highlighted gross morphological changes as most likely to impact confinement of transgenic plants. Although biochemical changes or changes in gene expression may have predictive value, their use will depend on how much we know about traits that could impact field testing confinement. Most groups pointed out that biochemical changes or changes in gene expression would be less critical to monitor at this stage of product development, primarily because correlations among biochemical changes or changes in gene expression and traits that would significantly impact field testing are not well established yet. The key issue for detecting and observing changes that could impact field testing became "what is a significant change?" Understanding how an alteration fits within or outside the range of natural variation was a recurring theme for answering the question “what changes would be important to detect?”

The groups agreed that standards and methods for gene characterization and identification of secondary effects were largely adequate but there may be cases in which more information is needed. The altered flowering group in particular suggested that APHIS include a question to trigger an investigator to think about possible secondary effects (i.e., are you working on a trait that could alter confinement?) so that investigators begin to think about how their manipulation might affect confinement.

Groups also agreed that as field trials progress to larger scales, the need to test a much broader array of traits is created.

Groups noted the strengths of the large biotech organizations: strong bioinformatics and databases to draw upon; multidisciplinary research groups; a conservative approach due to costs, liability, and product stewardship; and an awareness of consumer safety issues. Within the groups, industry representatives stated that industry should have the responsibility to make their scientists aware of the potential for secondary effects and should help academics or smaller companies with procedures or experimental designs that would facilitate the identification of secondary effects. One concern noted was the need for more transparency, although it was also noted that the balance between transparency and protecting intellectual property is a challenge for industry. Data generated are not always of interest to journals (i.e., crop variety development trials) and the current corporate culture may make it logistically difficult to publish.

Each group stated that these newer genes do not change current criteria for field testing, but that specific protocols (e.g., isolation distances) could be affected. Again the key is the effect on changes in pollen, seeds, flowering, and plant persistence and how these changes may compare to the range of phenotypic variability and affect fitness. For small field releases, the spread of genes may be more likely to occur when fitness is increased; however, it was noted that decreased fitness also may have unintended effects, and these may be a concern at larger scales of release.

Questions directed to the particular case study included the following:

1. Does the case study gene/trait differ from currently commercialized genes/traits in ways that are relevant to regulatory criteria for field testing?

2. Is there evidence to indicate that engineering the pathway under consideration may produce effects (either directly or secondarily) that impact confinement of field trials?

Altered flowering

A complex genetic network regulates the transition to flowering and involves some 80 genes in multiple pathways. Interplay among pathways activates key genes. The flowering regulatory system includes features such as quantitative regulation of gene expression, redundancy, suppression and promotion of floral transition, having related genes with opposite effects, and operation of transcriptional, post-transcriptional, and epigenetic regulation mechanisms. Current knowledge of genes that regulate floral transition comes from work with *Arabidopsis*. Data from a limited number of genes cloned from other species suggest that the function of some of these genes is conserved among divergent species. However, the extent to which function is conserved remains unknown.

Based on work in *Arabidopsis*, the altered flowering group noted that engineering with regulatory genes that control flowering could potentially produce unintended changes, including dwarfism, increased branching, altered growth, sex-altered flowers, and changes in nectary formation. These alterations could impact the movement of pollen and affect confinement, but only if these changes were unnoticed and if protocols for confinement were not already adequate.

However, although these changes were possible, the current regulatory criteria for producing altered flowering is no different than for commercialized transgenic products. Protocols to meet these criteria may need to be altered.

Oil modification

The oil modification group focused on two case studies that have already been deregulated, high oleic acid soybeans and high laurate canola, and also discussed in general future oil modifications to plants for producing better food or animal feed or for industrial use.

Given that most modifications to plants will not increase total oil levels dramatically, this group emphasized that plants engineered with changes in oil metabolism were unlikely to have altered fitness characteristics of significance to the regulatory criteria for field testing. They also pointed out that the use of tissue specific promoters, such as seed specific promoters, for the case studies they considered helps to focus the risk evaluation on categories that involve that particular site.

Cold tolerance

Engineering cold tolerance has been accomplished through a wide variety of means, including overexpression of enzymes (sorbitol synthase, superoxide dismutase) or of genes that regulate stress response pathways (*CBF1*). The group indicated that the production of stress tolerance traits through engineering pathways or the use of transcription factors raised no new issues for field testing, but also noted that the range of possible phenotypes, and therefore unpredictability, might be increased. Similarly, the group pointed out that the cold tolerance phenotype might be more likely to affect life history traits than phenotypes engineered with Bt endotoxins or current herbicide tolerance phenotypes.

While recognizing the potential for increased unpredictability and effects on life history traits, the group saw no need to alter regulatory criteria, but noted that procedures used to comply with the criteria may need a change of emphasis, depending on familiarity with the phenotype and the crop. The group recommended drawing upon knowledge from traditional breeding and information about common molecular processes to provide evidence on what correlated changes might be likely and in need of monitoring during field testing.

Disease resistance

For both current products with disease resistance genes and those under development with signal transduction modifiers, the potential for enhanced persistence in the environment due to release from pathogen pressures will be a concern if gene flow occurs between disease resistant transgenic plants and their wild relatives. Alterations to pathways that produce broad disease resistance may provide a selective advantage to wild relatives, but evaluating this effect will depend on the biology of host:pathogen interaction of wild relatives. The group indicated that pleiotropic or epistatic effects associated with manipulating disease resistance pathways would be more likely to be detrimental, although they noted that changes that could impact confinement were not without possibility.

Lignin modification

Overall, the group agreed that field testing criteria for low-lignin transgenic plants would be similar to criteria for currently commercialized transgenic plants. The lignin biosynthesis pathway is of great interest given the importance of lignin for digestibility of forage crops, for conversion of lignocellulose for bioenergy products, and for wood quality and paper-making. Some lignin group members felt that metabolic or phenotypic changes in low-lignin transgenic plants would fall within the range of natural variability for that species, and, therefore, at the field testing stage, would not be of any greater concern than changes resulting from conventionally bred low-lignin plants. However, other members noted that lignin is ubiquitous in the plant body and therefore, by modifying its content, unexpected metabolic or structural effects, which impact confinement or non-target species, could occur.

Altered ripening

The plant hormone ethylene plays a critical role in a number of processes, including fruit ripening, seed germination, abscission, senescence, root formation, and disease resistance. Given the interconnectedness of ethylene action, secondary effects would be expected as a consequence of modifications to ethylene synthesis or response. The group highlighted, as an example, transgenic petunias expressing a mutated version of the ethylene receptor gene *Etr*1, which clearly exhibited altered patterns of ripening as expected. In addition, these plants revealed a number of secondary effects that reduced plant fitness, including reduced rooting of cuttings, increased incidence of disease, brittle stems, and prostrate growth habit.

RESEARCH NEEDED

Groups were also asked to discuss whether areas exist that would benefit from additional research and, if so, to suggest what data or experiments would address these areas. Each group included lists within their reports, but several recurring themes emerged from these lists. Several groups stressed the continued, basic study of these genes, their control, and interactions as necessary for understanding secondary effects and for minimizing negative, unintended effects. Multiple groups suggested that a database of information detailing natural variation of characteristics related to gene flow could provide background against which to evaluate any observed changes. Lastly, groups indicated that research on mini-

mizing gene flow and on understanding the consequences of gene flow was also needed. Suggestions included studies to validate current isolation distances and to investigate the use of gene excision technologies and their effects on confining pollen, as well as others.

OVERALL CONCLUSIONS

A recurring theme from all breakout groups was that phenotypes and not specific genes are ultimately the relevant criteria for field testing considerations. Although alterations in metabolism, signaling, or transcription may in turn bring about additional changes in gene expression or metabolic profiles, specific information about those changes is less important than the translation of those changes into relevant phenotypes such as those influencing flowering, pollen biology, or persistence properties. Thus, the use of these new genes, per se, does not appear to provide novel concerns for confinement. However, their potential for more broad-reaching effects should stimulate researchers to look beyond the primary expected phenotype when establishing field trials and the regulatory system. It was noted that at larger, pre-commercial stages of field testing, monitoring is already required for these and other traits. Several groups also indicated the importance of phenotypic context (i.e., is the observed phenotype within the range of naturally occurring variability for that trait in the domesticated species and wild relatives?), and establishing phenotypic ranges may be an area where additional information/research is needed for some crops.

WORKING GROUP REPORTS

INTRODUCTION: PURPOSE OF THE WORKSHOP

Rebecca Grumet
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INTRODUCTION

The purpose of this workshop on "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," was to examine some of the new types of genes being used for plant genetic engineering with regard to implications they may have for field trial practices and confinement procedures. Because the mode of action of many of the new genes differs from those in current commercial production, this workshop asked whether such genes pose new questions or require new considerations.

As of October 2001, the genes that have been incorporated into transgenic crops and approved for deregulation by USDA (the last step required by USDA prior to commercialization) fall into a narrow range of categories (Table 1). Of the first genetically engineered crops to be commercialized, 75% were engineered for herbicide resistance or Bt-mediated insect resistance; greater than 99% of the global acreage planted to transgenic crops in 2001 had herbicide resistance, insect resistance, or a combination of the two. Importantly, with regard to the questions to be addressed at this workshop, for this first wave of genes, it is the immediate protein product that confers the desired phenotype. For example, herbicide resistance genes either produce a protein that degrades the herbicide or provide an alternate, non-herbicide sensitive target molecule; Bt genes produce a protein that is toxic to certain classes of insects.

In contrast to the first wave of genes, many new genes are being utilized for which the connection between the gene, the protein it produces, and the desired phenotype is less direct. Table 2 shows a sampling of some of the new genes being tested in field trials; examples include genes involved in transcription, signal transduction, or metabolic engineering.

Table 1. Traits incorporated into genetically engineered crops deregulated by USDA (as of 10/01)

Trait	Number	%	Global Ha 2001 (x 10^6)[1]
Herbicide Resistance	24	49%	32.7 (74%)
Insect resistance	16	26%	8.4 (19%)
Herbicide + insect resistance			3.1 (7%)
Male sterility	7	12%	
Altered ripening	6	10%	
Virus resistance	5	8%	
Altered oil	2	3%	

From: James, C. 2000. ISAAA Briefs No. 21. Global status of commercialized transgenic crops.

For these categories of genes, multiple steps can occur between the protein product and the ultimate desired phenotype. For example, transcription factor genes encode a protein whose function is to activate transcription (expression) of numerous other genes and so can be used to induce expression of an entire pathway or gene cascade, as was described by Mike Thomashow (Michigan State Univ.) and Xinnian Dong (Duke Univ.) with regard to cold tolerance and disease resistance. The key advantage to the use of transcription factors for genetic engineering purposes is that a single gene can induce a range of responses without requiring the introduction of each participating gene one at a time. This advantage, however, also raises the question of whether secondary changes would be more likely.

Similar types of questions could be raised for signal transduction factors. For a plant to respond to changes in its environment, whether they be external (e.g., temperature, pathogens) or internal (stage of development, carbohydrate status), the

Table 2. Examples of genes tested in recent field trials (USDA-APHIS, 2001-2002).

Regulatory, Transcription
Agamous-like, B1 regulatory gene, C transcriptional activator, C1 regulatory gene, CRT/DRE binding factor, Knotted-1, LEAFY, microtubule associated protein, negative R transcription activator, negative C transcription activator, Pti4 transcription factor, nucleosome assembly factor, histone deacetylase, DNA methyltransferase
Signal transduction
Cyclin dependent kinase, ethylene receptor protein, protein kinase, receptor kinase, rol hormone gene, SAM carboxylase
Metabolic engineering
Isopentenyl transferase, kaurene synthase, starch synthase, palmotyle thioesterase, ADP glucose pyrophosphorylase, glucosyl transferase

pertinent signal must be perceived and transmitted to facilitate the appropriate response. Signal transduction refers to the process leading from perception to response and can involve a number of steps. Specific molecules are needed to perceive the signal, others transmit the signal (e.g., hormones, peptides, small carbohydrates) either by movement within in the cell or systemically through the plant, and others cause molecular modifications of key proteins (e.g., the kinase cascades involving series of phosphorylations and dephosphorylations) that in turn modify cellular activities. Ultimately, a signal transduction pathway can lead to changes in gene expression. Thus signal transduction pathways cannot be completely separated from transcriptional modification. In many cases, there also appears to be cross-talk among different signal pathways, resulting in different plant responses. Harry Klee (Univ. Florida) and Peter McCourt (Univ. Toronto) examined aspects of signaling involving hormone perception and action and interplay between different hormone signals.

The third category examined contained genes intended to alter plant metabolism, whether by increasing production of a specific compound of interest or by introducing the ability to produce novel compounds such as vitamins, biodegradable plastics, or specialty oils. Because cellular metabolism is comprised of interconnected biochemical pathways with regulatory and feedback mechanisms and key intermediates that can influence relative flux through different pathways, modifications in one pathway have the potential to influence activity of other pathways. Metabolic changes also can affect signaling or gene expression. John Ohlrogge (Michigan State Univ.) described experiences in metabolic engineering and interplay among interconnected pathways.

The complexities of the interactions associated with transcription, signal transduction, and metabolic engineering genes raise questions that were addressed at the workshop, such as, does introduction or modification of these genes have implications for field trials, especially confinement considerations? The workshop included two phases. The first day was a series of plenary talks. The morning talks gave an overview of general procedures and goals of field testing. David Heron from USDA discussed regulatory aspects and considerations and Chuck Mihaliak from Dow AgriScience described an industry perspective on performing field trials with transgenic crops. Two speakers from academia, Steve Strauss (Oregon State Univ.) and Allison Snow (Ohio State Univ.) discussed biological and ecological aspects and considerations for field testing. In the afternoon, talks described specific types of metabolic, signal transduction, and regulatory genes, as mentioned above. Each of the speakers was asked to examine what is known with respect to cross-talk or interaction, or lack of interaction, for the different examples.

On the second day, participants worked in breakout groups to examine specific examples of genetic modifications using regulatory, signaling, or metabolic engineering genes. Case studies were chosen on the basis of two criteria: type of gene and type of trait. Because these two aspects (types of genes and phenotypes) are highly interwoven, they may be hard to separate. Thus an important question is, is the type of gene or the phenotype (or both) the critical issue? There is clearly more than one way to reach a given phenotype. Questions to examine included: Is modifying expression of multiple genes different than modifying a single gene? Is the range of primary and secondary phenotypic effects likely to be different with different types of genes? For example, is there a difference if ripening is altered by reduced polygalacturonase, as has already been done for the previously commercialized FlavrSavr tomato, or by modified ethylene pathways? Do the differ-

ences in mode of action of the introduced gene have new/different implications for containment when we refer to issues such as pollen movement, plant seed movement, or reproductive capacity? An intuitive answer may be that modifying expression of multiple genes would be different than modifying a single gene; such response is what prompts the questions being posed here. Alternatively, the key issue may be the final phenotype, regardless of how it is achieved. The intention of this workshop was to bring together molecular biologists, physiologists, agriculturalists, and ecologists from academia, industry, and government, to share their expertise to critically address these questions.

REPORT OF THE ALTERED FLOWERING GROUP[1]

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PHASE I. GENERAL DATA NEEDED

"Given the regulatory criteria of field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?"

The regulatory criterion of field testing most relevant to this discussion is that plants are not allowed to persist in the environment following the field test, including through volunteers or escapes, or through progeny resulting from pollen flow to nearby plants of the same or related species. Also relevant is that the criteria for field testing under notification require that the function of the introduced genetic material is known and does not result in plant disease, cause the production of an infectious entity, or encode substances likely to be toxic to nontarget organisms.

With this in mind, the "altered flowering breakout group" identified a number of changes that could impact confinement of transgenic plants during field testing. Although many of the changes discussed were related to potential effects of altering the flowering time or flower morphology, which was the topic of our case study, a few were different. Biochemical or physiological changes that might lead to these phenotypic changes were difficult to discern.

Changes related to altered flowering included:

- Altered flowering time, which could affect temporal isolation from other crop varieties or wild relatives during field testing.
- Changes in flower morphology (e.g., flower color and patterns, anther or stamen shape or size, pollen availability) that might alter pollinator behavior or increase the number of pollinators.
- Changes in the number of nectaries or the nutrient or sugar content of nectar that might affect pollinator behavior.
- Changes in pollen morphology, amount, shed duration, and viability.

Some of the other kinds of changes discussed included:

- Changes in seed composition or seed morphology that could potentially affect seed dormancy and seed dispersal (attractiveness, palatability, or digestibility by predators, i.e., birds or small mammals).
- Changes in seed size or shape that might alter aerodynamics.
- Changes in shattering mechanisms.
- Hormonal changes that could affect seed dormancy. Altering levels of ABA (abscisic acid) and GA (gibberellic acid) might increase or decrease dormancy.
- Altered stress (cold, heat, salt, drought) tolerance that would allow the plants to be grown where they were not grown before, in approximation to wild relatives.
- Increased toxins that could affect non-target organisms. Altering metabolic pathways with

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

the intention of, for example, increasing vitamin content might unintentionally increase a toxic substance at the same time.

Some of these changes might be detected during gene characterization prior to field testing. As a result of the group's discussion, it became apparent that "gene characterization" occurs at different stages, including characterization based on gene sequence information, characterization in the greenhouse of plants transformed with the gene, and characterization of the plants during field testing. It should be possible to make some predictions about secondary effects of genes based upon the known activity of the gene, or the known function of a gene with a similar sequence in another organism, although this would depend a great deal on how well the interaction among the genes or gene pathways are understood, and in many cases this understanding is just emerging. Obvious morphological changes (e.g., significant changes in seed morphology) should be identifiable in the greenhouse, if plants are observed in the greenhouse before field testing. However, many of the changes discussed would not be noticed until plants are grown in the field, and even then might not be noticed unless someone is looking for them (e.g., changes in pollen viability or pollinator behavior).

Many of the changes would not affect confinement because current protocols for confinement are already adequate, e.g., when plants are not allowed to flower or produce pollen, fields are screened for volunteers for multiple seasons following a field test, or field tests are not conducted anywhere in the vicinity of wild relatives. Some of these changes might, however, require confinement protocols to be changed, and this would particularly be true for crops that have wild relatives. This group noted that, given the regulatory criteria for field testing, many of the changes discussed might have little impact at the small scale of a field test, but could have ecological impacts once plants are released without confinement. There may also be secondary changes that are not listed here because they would have no effect on confinement, but could have quite serious ecological effects outside of confinement, e.g., altered flowering could secondarily change the female flower receptive period.

"Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring these changes?"

The current standards and methods for "gene characterization and identification" of secondary effects may not encompass the kinds of phenotypic changes discussed in Question 1, unless careful consideration is given to the potential for these effects based on the known activity of the gene and the pathway it affects, and/or unless careful attention is given to the morphology and performance of the transgenic plant relative to the nontransgenic plant in the greenhouse and the field. However, as discussed in Question 1, the current regulation and management of field trials should be adequate to address many of these phenotypic changes even when they are unanticipated. Any unintended or unanticipated effects should be included in field data reports and considered during scale-up and commercialization.

In order to make gene characterization more effective with regards to secondary effects, this group, particularly the individuals in the group from industry, suggested that the regulatory agencies could put forth some very general guidance to the industry and scientific community: "Before field testing an engineered plant, if the engineered gene will affect regulatory, metabolic, or signaling pathways, consider whether the primary (intended) changes due to that gene might affect confinement of the plant during field testing, or whether there might be any secondary (unintended) changes due to altering a pathway that might affect confinement during field tests." In addition to a general guidance statement such as this, the regulatory agencies could provide examples of genes that raise a concern for secondary effects. The case studies selected for breakout group discussions at this workshop are good examples, and the proceedings of this workshop might be cited as useful additional guidance.

The regulatory agencies could provide this guidance to prompt the industry to remind its researchers to think about possible impacts of altered pathways. This guidance would be equally as important, perhaps even more so, to academic scientists who do not benefit from the already conscientious internal regulatory oversight of the industry.

"What are the strengths of the industry approach (described in morning plenary session) to characterize genes from plant genomics projects? Are there areas where the approach should be improved?"

A number of strengths of the industry approach to characterizing genes from plant genomics projects were identified, although these strengths were considered primarily in comparison to genomic projects by the academic sector, and not so much with regards to identifying secondary effects of regulatory or metabolic genes that alter pathways. Some of the strengths of industry compared to academic ventures included the industries strong bioinformatics capabilities to determine the potential functions of gene sequences, as well as extensive access to databases to screen for potential toxin and allergen properties. A primary safety feature for gene isolation from genomics projects is the limited pool of organisms from which genes are sourced, which is restricted to avoid the selection of genes that might directly cause human or plant diseases, for example. From a general safety perspective, the industry representatives in the breakout group suggested that strengths of the industry include a very conservative and conscientious approach during product development, perhaps more so than academic researchers whose approach is more basic than applied. Industry has a prominent concern with cost and liability issues, as well as a general responsibility for stewardship.

In terms of identifying secondary effects of genes from genomic projects, one member of industry in the group noted in particular that industry has a significant advantage when it comes to characterizing gene effects at the field evaluation stage because the products being developed will be evaluated in the field by a team of interdisciplinary scientists, including breeders, pathologists, entomologists, and others. A potential improvement for identifying secondary effects from genes in the field might be to include ecologists (some companies already do) among this cadre of specialists.

It seemed, however, that little thought has been given to potential secondary changes due to genes affecting regulatory or metabolic pathways, and a simple but effective improvement would be to encourage the scientists in industry who are working with these genes to be thinking about potential gene effects, in addition to the desired or intended effects. As the information on pathway interactions progresses, industry and academic scientists should continue to rethink the potential for secondary effects. Industry should, therefore, ensure that scientists have continuous updates as information on interactions among pathways becomes available. One area of improvement for academic scientists would be guidance on how to predict which proteins may have toxic effects, e.g., what databases to search.

"Do any new environmental issues relevant to field testing releases and management arise when considering emerging genes and the phenotypes they affect?"

The concerns associated with emerging genes that alter regulatory or metabolic pathways and the phenotypes identified by this group that the genes might affect do not raise any new environmental issues. They pose the same risks to the environment that are generally associated with transgenic plants. These genes may, however, increase the potential for risk because the changes that might affect the environment may not be intended and so may not be considered in a risk assessment. Another exception might be when new metabolic pathways unique to plants or to a particular plant family are introduced from a foreign source.

The general consensus among the members of this breakout group was that regulation and management of field trials is currently adequate to safeguard the environment from risk, and the field testing phase should be used to gather additional data on secondary effects. If a potential secondary effect is identified, current field protocols for confinement should be reviewed and modified if necessary. Secondary effects may not have an impact during small, controlled field tests, but when secondary effects such as those discussed above are identified, these should be given more serious consideration before plants are released into the environment on a large scale.

PHASE II: THE CASE STUDY — ALTERED FLOWERING

Altered flowering background information

Although a great deal of progress has been made in recent years toward understanding the genetic control and regulation of flower development, it is expectedly a complex system and our understanding is only beginning to emerge. Much about the complexity of flowering has been learned from studying the transition from the vegetative to the reproductive phase in plants. This transition is regulated by a complex genetic network, and genetic analysis has identified some 80 genes placed in multiple pathways that control floral transition (Araki, 2001).

Studies in the model annual plant *Arabidopsis* have defined at least three post-embryonic phases: a juvenile vegetative phase; an adult vegetative phase; and a reproductive phase (reviewed in Simpson *et al.,* 1999). *Arabidopsis* and other plants progress in a coordinated manner through vegetative maturation to the reproductive phase, resulting in a clear separation of vegetative and reproductive phases. However, the relationships between vegetative phase change and reproduction are variable among species and are particularly complex in trees (reviewed in Jones 1999). Studies in a variety of plants indicate that the transition to flowering is under multifactorial control (Bernier *et al.,* 1993). Different factors of this regulatory network are predicted to become limiting in different species or genotypes, or in a given genotype grown under different environmental conditions. The floral transition is regulated both by transmissible signals originating outside the shoot apical meristem (SAM) and by competence of the SAM to respond to these factors (reviewed in Levy and Dean, 1998).

Most of the recent advances in unraveling the genetic networks that interact to control flowering have come from studies of the facultative long-day plant, *Arabidopsis* (reviewed in Simpson *et al.,* 1999; Araki, 2001). At least four pathways regulate flowering time in *Arabidopsis* (Simpson *et al.,* 1999). Plants measure day length by integrating signals from photoreceptors and an endogenous circadian clock, and long days promote flowering via this photoperiodic pathway. Extended periods of cold temperatures promote flowering in many ecotypes via the vernalization pathway. Genes in the autonomous pathway probably respond to an internal 'developmental clock'. Under short-day photoperiods, flowering depends on a gibberellic acid (GA) signal transduction pathway. Ultimately, the interplay among flowering pathways activates floral meristem identity genes and the competency of the SAM to respond to floral induction signals (Blazquez and Weigel, 2000; Colasanti and Sundarasen, 2000).

Quantitative regulation of gene expression and redundancy are important features of the flowering regulatory system. Additional characteristics of this regulatory network are that it includes both suppressors and promoters of the floral transition, that related genes might have opposite effects, and that regulation involves transcriptional, post-transcriptional, and epigenetic mechanisms. These pathways include genes, such as photoreceptors, that regulate a wide variety of plant responses as well as genes that appear specific to the floral transition. In addition, downstream genes that integrate multiple flowering pathways have been identified. Still unknown is the extent to which genes that regulate the floral transition in *Arabidopsis* also regulate this transition in other plants. Only a few flowering-time genes have been cloned from other species, but it appears that the function of at least some genes is generally conserved among divergent species (Yano *et al.,* 2000).

Much of what is known about floral organ development is based on the 'ABC' model of flower development, which describes classes of floral homeotic genes: A genes (e.g., *APETALA1*) specifying sepals; A and B genes (e.g., *APETELA3*; *PISTILLATA*) together specifying petals; B and C genes (e.g., *AGAMOUS*) together specifying stamens; and the C genes specifying carpels (Ng and Yanofsky, 2000). These genes, members of the MADS-box family, are expressed only in regions of the developing flowers that require their activity. Many of the genes involved in regulating the ABC genes have been identified, with names such as *UFO, LEAFY, CAULIFLOWER, CURLY LEAF, SUPERMAN, and LEUNIG.* Floral specification and inflorescence architecture are regulated mainly by interactions between *TERMINAL FLOWER1 (TFL1)*,

LFY, *APETALA1 (AP1)*, *CAULIFLOWER (CAL),* and *FRUITFULL (FUL*) (Liljegren *et al.,* 1999; Ratcliffe *et al.,* 1999; Ferrandiz *et al.,* 2000).

Many of these genes may be candidates for engineering, but the mechanistic roles of these regulatory genes are not all clearly defined, and our understanding of the transcriptional regulation of the flowering genes is far from complete. There have been very few requests for field testing of plants that have been engineered for "altered flowering" or "altered flowering time." Field test applications are on record for poplar, apple, and walnut, as well as an herb plant called Clary, engineered with the *LFY* gene (ISB database: www.isb.vt.edu). *LEAFY* (*LFY)*, the floral meristem identity gene that is a direct target of both the long-day and GA promotion pathways and whose overexpression accelerates flowering in *Arabidopsis* (Weigel and Nilsson, 1995; Blazquez and Weigel, 2000), is perhaps the most studied flowering gene and the primary candidate for engineering. The goal of this type of engineering is most likely to be an attempt to reduce juvenility, manipulate maturity, or alter fertility, particularly in trees. In the future, requests for field tests of trees genetically engineered with these genes may be necessary in order to simply evaluate the phenotypic effects of these genes. Genes conferring altered flowering time and altered flower morphology are also being tested in corn functional genomic field trials.

"Does this gene/trait differ from currently commercialized genes/traits (e.g., Bt, herbicide tolerance, virus resistance, delayed fruit ripening) in ways that are relevant to regulatory criteria for field testing?"

This question can be answered in two parts. First, engineering plants with genes that alter flowering is different than currently commercialized genes/trait such as Bt, herbicide resistance, or virus resistance because changes that affect flowering have the potential to impact pollen movement. Therefore, inserting regulatory genes that control flowering could potentially result in unintended changes that might affect confinement. (See examples of evidence below.) As noted in the summary of the group's discussion in Phase I, a number of changes potentially related to altered flowering were identified that could impact confinement of plants during field tests, but they would only have an impact if they were unnoticed and if the protocols for confinement were not already adequate.

However, the second notable part of the answer to this question discussed by the group is that, since intended or unintended changes resulting from altered flowering do not raise new environmental issues compared to currently commercialized genes, the regulatory *criteria* (i.e., plants are not allowed to persist in the environment, the function of the gene is known, etc.) for "altered flowering" is not different from currently commercialized traits. The criteria are the same, yet the protocols to meet these criteria may need to change. As a fairly obvious example, if the protocol for confinement of a transgenic plant relied solely on the use of temporal isolation from other compatible plants (*although the group acknowledged in its discussion that temporal isolation, when used in field tests of transgenics, is almost always used in combination with some other measure for containment*), but the insertion of the altered flowering gene resulted in a change in flowering time so that flowering of compatible plants overlapped with the transgenic, the protocol for confinement during the field test should be modified to mediate the movement of pollen (i.e., use of isolation distance, bagging or removing male flowers, etc.).

The potential secondary effects of altered flowering identified by this group are changes that would impact movement of pollen at the field test stage. This would primarily be a concern in plants that have wild relatives in the vicinity of the field test. Most of these plants are already grown under very stringent confinement measures. For example, Bt- and Roundup-resistant poplar field tests have typically been terminated before the trees reach flowering age. Poplars with sterility transgenes have been allowed to extend to flowering age, but even these field tests have only been conducted using transgenic poplar species that are not sexually compatible with the native poplar species in the region of the field tests. These confinement protocols have also typically been the same for female and male poplars, because not only pollen but also the wind-dispersed seed can travel considerable distances.

"Is there evidence to indicate engineering the pathway under consideration may produce effects (either directly or secondarily) that impact confinement of field trials?"

Most of the work that has been done to date with genes that affect flowering in plants has been done in *Arabidopsis*, and most of the observations have been made in the laboratory or the greenhouse. There has been almost no experience growing altered flowering transgenics in the field, thus no opportunity to find "evidence" that an observed change has indeed impacted the confinement. There have certainly not been any studies designed specifically to test for changes in, for example, pollinator behavior or patterns of pollen movement related to altered flowering in transgenic plants. However, there is evidence that engineering plants with genes that control flowering time or flower morphology do have unintended, sometimes unpredictable, secondary effects. Some examples are discussed below.

Some of the best examples of attempts to alter flowering in plants other than *Arabidopsis* have been in poplar and citrus trees, with the goal of shortening juvenility or inducing early maturity via the *LFY* gene. It is worth noting that the *Arabidopsis* floral meristem identity *LFY* gene, inserted into poplar and citrus, had very different effects in the two species, even among genotypes within the species. Overexpression of *LFY* induced the formation of flowers in transgenic poplar shortly after transformation indicating that flowering in trees might be usefully manipulated (Weigel and Nilsson, 1995). However, these flowers were not entirely normal—trees were dwarfed and highly branched, and additional studies showed that *LFY*'s ability to induce early flowering in poplar was highly dependent on genotype (Rottmann *et al.,* 2000). In contrast to poplar, overexpression of either *LFY* or *AP1* accelerated normal flowering and fruit production in a citrus cultivar (citrange—a hybrid: *Citrus sinenis* L. Osbeck x *Poncirus trifoliata* L. raf; Pena *et al.,* 2001). Developmental differences between subtropical evergreen citrus and temperate deciduous poplar were suggested as possible reasons for the different responses. Another possibility is that unlike forest trees, fruit trees are likely to have undergone some selection for early and intense flowering, and thus may be more competent to respond to these genes. Transgenic *35::LFY* citrus trees also displayed altered growth (*e.g.,* SAM abortion). 35S*::AP1* plants generally exhibited normal growth, but rapidly showed vegetative characteristics typical of adult trees, such as reduction in the size and number of thorns. The early-flowering trait was stably transmitted to offspring, and transgenic trees appeared to remain responsive to internal and environmental signals regulating flowering, such as the response to photoperiod.

There was one unintended effect reported in the study by Rottman *et al.* (2000) that has relevance for the issue of confinement. Overexpression of the homolog of *LEAFY/ FLORICAULA* in poplar did not lead to a predictable shortening of the juvenile phase. Flowering was induced earlier in some, but not all, transformants. In addition, some female transformants (poplar trees are dioecious) were morphologically male. The gender-changed flowers had extremely limited pollen viability, which, when combined with already strict confinement protocols for field tests of poplar, means that confinement for this specific example would most likely not be a problem. This example does illustrate, however, how an unintended effect may affect the pollination biology of a plant and so have implications for confinement. The change in gender was an obvious morphological switch, but many traits that affect pollination (discussed in Phase I) could be less apparent to researchers or not be traits that are actively being scored, and so be of more concern during field tests.

Genes that affect nectary formation (reviewed in Baum *et al.,* 2001) could also produce secondary effects that could impact confinement during field trials. The formation and location of functional nectaries could influence attractiveness to pollinators and whether the pollinator would pick up pollen while in the act of collecting nectar. These would in turn affect pollinator efficiency and specificity. Unlike floral organs, whose relative positions are conserved across angiosperms, nectaries are not located in the same floral position in all plants. In *Arabidopsis*, nectaries are normally present on stamens that occupy the third whorl. Studies with *Arabidopsis* mutants have shown that nectary formation is independent of the ABC floral homeotic genes that specify the four groups of

floral organs (sepals, petals, stamens, and carpels); but they are always restricted to the third whorl domain, which is in part established by the action of the *UNUSUAL FLORAL ORGAN (UFO)* and *LEAFY (LFY)* genes.

While nectary gland formation does not depend on stamen development, aberrant nectary morphology or nectary secretion was associated with nectary formation in floral mutants lacking stamens. The *CRABS CLAW (CRC)* gene has been shown to be necessary, though not sufficient, for nectary formation. *CRC* mutants lack nectaries, but no ectopic nectaries are formed in plants expressing *CRC* from a *35S* promoter. Nectary formation in *35S::CRC* plants was normal. This suggests that other factors are necessary for their formation. It is not known whether *CRC* is universally used to promote nectary differentiation in other species, but transgenic expression of *CRC* in other species should be examined for effects on nectary formation. In *Arabidopsis SUPERMAN-1 (SUP-1)* mutants, the third floral whorl is reiterated several times, resulting in multiple whorls of stamens with associated nectary glands, which are greatly enlarged in the outermost stamens. A similar pattern was seen in plants expressing *UFO* from a *35S* promoter. These latter two types of genetic transformations could possibly lead to increased attractiveness to pollinators and more pollen/flower, which could be available for outcrossing.

"Are there areas that would benefit from additional research? What data or experiments would address these areas?"

Very generally, since our understanding of the complex genetic control of flowering is still emerging, the most useful contributions from research will come from continuing to study the genes involved, how they are controlled, and how they interact with each other to affect the various pathways.

Identification and testing of potential secondary effects of transgenes related to flowering is needed in order to gauge the likelihood of such effects. For example, as corn lines transformed with *LFY* are developed, data on the amount of viable pollen in different transformed lines compared to nontransformed lines would provide useful baseline information to indicate the potential of a secondary effect of *LFY* on pollen production in that crop.

Since different factors of the regulatory network controlling flowering are predicted to become limiting in different species or genotypes, or in a given genotype grown under different environmental conditions, and because related genes may have opposite effects, key research will involve the observation of differences in effect of a single transgene in different species and in different genotypes within a species. This will be especially useful information when transgenes are inserted into crops that have wild relatives and in which exists the potential for the transgene to be transferred via pollen flow to a similar but different genetic background.

PHASE III. RETURN TO THE BIG PICTURE.

"Has discussion of the case study altered any of the answers to the general questions posed in Phase I?"

Because many of the potential changes in phenotype identified in Phase I of this discussion were relevant to altered flowering, the answers to the general questions in Phase I did not change. The risk related to secondary effects of genetic engineering with genes intended to alter flowering is not likely to have a significant effect when plants are grown at the small scale of a field test, given the regulatory criteria for confinement and the current standards and methods for regulation of these plants.

However, the group agreed that researchers working with genes known to specify flowering time or floral organ and nectary development should be mindful of potential secondary effects that could impact confinement and should be monitoring the effects of any unintended alterations. Potential unintended effects related to altered flowering should be given careful consideration while developing protocols for reproductive confinement in field tests, particularly for plants with compatible wild relatives. The field testing phase should be used to gather information on the occurrence of secondary changes and their effects, and the potential effects of any

identified changes should be examined before the plants are considered for scale-up or unconfined release into the environment.

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REPORT OF THE ALTERED RIPENING WORKING GROUP[1]

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PHASE I: GENERAL DATA NEEDED

"Given the regulatory criteria of field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?"

The focus of our group was on altered fruit ripening. Fruit ripening facilitates the dispersal of seed through attractiveness to herbivores and/or shedding of seed via abscission and senescence. Biochemical changes in transgenic plants that alter patterns of fruit ripening or senescence could affect seed dispersal through changes in attractiveness (scent, color, or flavor) to herbivores, thus affecting seed dispersal. Similarly, alterations to the development, maturation, and dormancy of seeds within the fruit could affect confinement of transgenic plants through persistence of seeds in the environment. Modifications to signaling or metabolic pathways that alter these processes could be associated with altered fruit ripening due to cross-talk among hormonal response pathways. This is particularly true for ethylene and abscisic acid (ABA), which are known to play roles in fruit ripening and seed development, respectively. Finally, alterations in transgenic plants that affect pollen shed, morphology, and/or viability could impact confinement decisions. It is not clear that alterations to fruit ripening would translate to changes in pollen biology.

Detection of reproductive changes that potentially affect confinement of transgenic plants should be possible through careful observation of early generation plants in controlled environment growth conditions. Fruit development, fruit ripening, pollen shed, and seed viability are all traits that are readily apparent in early investigations of confined plants prior to field testing.

"Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring these changes?"

Yes. Current standards call for knowing the sequence of the inserted gene(s), with clear evidence for stable incorporation into the genome of the recipient plant. Careful observation of transgenic plants for intended consequences should include monitoring secondary effects on reproductive development.

"What are the strengths of the industry approach to characterizing genes from genomics projects?" Are there areas where the approach should be improved?"

Industry is moving forward rapidly to identify genes critical to crop improvement. The strength of the industry model is high throughput analysis leading to gene discovery. However, industrial science quickly focuses on genes and traits of commercial interest, with little opportunity for the detailed study of secondary effects. In this regard, cooperation with scientists within academia and from other research centers would increase the

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

value of early discoveries from genome projects. Concern over protection of intellectual property could limit this approach, however.

"Do any new environmental issues relevant to field testing releases and management arise when considering emerging genes and the phenotypes they affect?"

The complexity of signaling pathways in higher plants is in the very early stages of description. The cross-talk among these pathways is clear and could lead to secondary effects in transgenic plants altered in signaling components. In many cases, secondary consequences might include reduced fitness, given the critical role of signaling in all aspects of plant development, disease resistance, and resistance to environmental stresses. In such cases, new environmental issues associated with these transgenic plants would not be needed.

PHASE II: ALTERED FRUIT RIPENING

"Does altered fruit ripening differ from other commercialized traits in ways that are relevant to regulatory criteria for field testing?"

No. Indeed, altered fruit ripening has been commercialized as a transgenic product. In addition, natural genetic variants that are known to affect signaling pathways leading to alterations in fruit ripening have been commercialized for many years. The development of new transgenic plants altered in fruit ripening would not likely differ substantially from those already commercialized, at least in species where ethylene is the hormonal signal regulating the ripening pathway. The question remains open for those fruit that ripen in an ethylene-independent manner.

"Is there evidence to indicate engineering altered fruit ripening may impact confinement of field trials"

Ethylene is a plant hormone that plays a critical role in a number of processes including fruit ripening, seed germination, abscission, senescence, root formation, and disease resistance. Given this, secondary effects as a consequence of modifications to ethylene synthesis or response would be expected. Indeed, transgenic petunias expressing a mutated version of the ethylene receptor gene *Etr*1 clearly exhibited altered patterns of ripening as expected. In addition, these plants revealed a number of secondary effects that reduced plant fitness. These included, reduced rooting of cuttings, increased incidence of disease, brittle stems, and prostrate growth habit. In these cases, tissue specific promoters could limit the secondary effects by confining the expression of the transgene to the tissue of interest.

"Are there areas that would benefit from additional research? What data or experiments would address these?"

Yes. Perhaps the most critical area of research is in control of gene expression by promoters. The use of tissue-specific and/or developmental stage-specific promoters could limit secondary effects resulting from the introduction of signaling or metabolic pathway transgenes. The discovery and characterization of these promoters is a critical research need. In the case of fruit ripening, the understanding of ripening in fruit that do not respond to ethylene (non-climacteric) is very limited. There is some evidence for involvement of other hormones (auxin, cytokinin, methyl jasmonate, gibberellic acid and ABA), but much work is needed to clarify their roles. Given the complexity of signaling pathways in higher eukaryotes, continued research to identify key regulatory molecules and their role in plant growth and development is essential. The National Science Foundation has embarked on a project to identify all of the proteins in *Arabidopsis* by the year 2010. This project promises to shed considerable light on the signaling and metabolic pathways in higher plants, opening up many opportunities for the use of transgenic technology to improve plant performance and add value to crops.

In light of the potential for improving crops through genetic engineering, it is essential that USDA and other federal agencies continue to invest in research that assesses the risks associated with release of transgenic plants into the environment and into our food chain.

Finally, application of transgenic technology to specialty crops (non-program crops) is very lim-

ited. Current investments in genomics and agricultural biotechnology are largely limited to those species that occupy significant acreage in the United States. Industry is not likely to invest significantly in specialty crop biotechnology, and thus public sector research will be critical to advances in this area.

PHASE III: RETURN TO THE BIG PICTURE

"Has discussion of the case study altered any of the answers to general questions posed in Phase I?"

Consideration of the altered fruit ripening case study has not altered our response to questions in Phase I. While modifications that affect fruit ripening have been shown to lead to secondary effects, it is not clear that these changes would warrant increased confinement of the transgenic plants.

REPORT OF THE COLD TOLERANCE GROUP[1]

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REVIEW OF REGULATORY CRITERIA

To clarify our understanding of the regulatory criteria for genetically modified (GM) crops, we discussed the regulation of current GM cold tolerant plants. Since notification became an option in April 1993, all genetically modified (GM) cold tolerant plants have qualified for release under the notification procedure. The crops concerned are cotton, oilseed rape, persimmon, potato, and tomato. Some cold tolerant phenotypes were obtained by overexpression of enzymes, such as sorbitol synthase, which increase the accumulation of osmotically active compounds in the cell and hence reduce damage from the desiccating effect of ice formation in the intercellular spaces of the plant. Another mechanism is the overexpression of superoxide dismutase, an enzyme involved in the removal of free radicals generated following the exposure of a plant to biotic or abiotic stress factors. Increased cold tolerance has also been achieved by the over expression of genes that regulate multiple stress responses and pathways; the transcription factor CBF1 (C-repeat binding factor) named for the DNA element it binds, the C-repeat, from *Arabidopsis* expressed in oilseed rape is an example of this method.

All cold tolerant phenotypes have qualified for notification because the species were not weeds in the release area and the modifications were of genes of known function. It was made clear that lack of knowledge about which genes, if any, might be regulated by the transgene was not necessarily a barrier to notification. However, in the case of the CBF and other regulatory genes, an important proviso is that the gene was isolated from *Arabidopsis* and used to transform another crucifer species. If the gene had been isolated from, for example, an exotic bacterium, a permit may have been required.

"What changes might have impacts on confinement?"

We decided unanimously that the gross phenotype had to be the level at which changes were considered. Changes in gene expression, protein synthesis, or production of metabolites are, of themselves, not something that should trigger a reassessment of confinement. However, biochemical changes might be useful if they are correlated with alterations of plant phenotype that are of concern and could be used to predict the behavior of plants in field trials (see below).

It was suggested that if a genetic modification was associated with changes to any life history characters that have the potential to alter transgene confinement (e.g., OSTP, 2002) then confinement measures might need to be revised. We then considered whether it would be possible to predict these changes before the plants were grown in field trials.

We thought that prediction of the field performance of transgenic plants from laboratory data would be very difficult. One could determine if the DNA sequence of the transgene has homology to genes known to control life history characters. An obvious problem with this strategy is that most

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

genes will be poorly characterized. Transgenic plants could be screened using a variety of methods to characterize the phenotype in terms of mRNA, proteins, or metabolite profiles. Because of the huge numbers of molecules tested, it is likely that transgenic plants will differ from controls in some way. However, at the present time we do not know the biological significance of those differences and do not know whether the differences are predictive of changes in life history characters in the field.

There are a huge number of problems to be overcome if an RNA or metabolite-profiling approach is to ever work:

- What is the biological relevance of experimentally determined differences in terms of pest potential and meaningful ecological impact?
- What is an appropriate experimental approach —should comparisons be made to isogenic or near-isogenic controls such as the immediate nontransgenic progenitor or some broader representation of the range of variation present in the crop?
- A prohibitive number of replications may be necessary to deal with the likely variation from experimental error and biological variation depending on the experimental approach and relevant endpoints chosen.
- A related problem is to determine the correct level of statistical significance for differences in expression of individual genes given the enormous number of simultaneous tests.

Interpretation will be very difficult. Life history characters often show quantitative variation and in the field will likely be determined by a complex interaction between alleles at many loci and a fluctuating environment. Therefore, models of the molecular mechanisms underlying plant phenotypes (produced in the laboratory or glasshouse) are unlikely to be much help in identifying molecular markers that will be associated with changes that significantly affect confinement in the field.

Logistics An empirical approach to the problem of interpretation would involve correlating molecular data collected early in the characterization of the transgenic plants with variation in life history characters in the field. However, this would involve redesigning current field testing strategies. If it were required, extensive characterization information would necessitate the collection of sufficient information on nontransgenic plants prior to field release. Thus, researchers would have to spend much more time and money to establish a baseline for comparison from traditional crops. The alternative might be a change in USDA requirements from confined trials to contained trials. This too is not practical.

Cost If detailed genomics data are required before field trials can begin, universities, other public sector institutions, and smaller biotech companies could find regulatory and compliance costs prohibitively high.

An important point is that with current biotech products almost all ‘unusual’ transformation events are eliminated before any field trials take place (this may not be the case with novel traits). Although this does not eliminate the possibility that biologically significant changes in life history characters might be expressed in the field, it does reduce the variety of material in test programs. Also, initial field trials tend to be on a small scale and so confinement can be very efficient. Data from these trials might be used to try to find molecular markers that predict phenotypic change, but this should be on a ‘nice to know’ rather than a ‘need to know’ basis. A final point is that pre-field testing data requirements should not be so strict that they can never be met.

Identification and reporting of secondary effects

There is a legal obligation to notify APHIS of ‘any unusual occurrence’ in field trials (defined as "excessive mortality or morbidity, or unanticipated effect on non-target organisms"). The key question is how to implement this requirement because the regulatory process will be overwhelmed if all variation is reported. The first filter on the amount of information reported is the distinction between unintended and unanticipated effects. Much variation will be unintended, but not unanticipated. For example, changes in phenotype that follow tissue culture can be regarded as ‘normal’ even though they may be undesirable and unpredictable. Such unanticipated effects need not be reported.

A second limitation is created by the pragmatic decisions of individuals who report, or require reports, on only very unusual events. Research scientists may be concerned about particular traits and not others, depending on the purpose of the experiment, while for regulators it might be interesting but not essential to know about minor unexpected variations in phenotype. The scale of the trial may also play a part; large unexpected variation in a $1m^2$ plot of *Arabidopsis* could be regarded as less worthy of note than small, unforeseen changes to 1000s of hectares of maize. Finally, the experimental conditions may limit variation. For instance, if plants are treated with insecticide, unexpected effects on non-target insects are unlikely to be detected (or measured).

The attitude of researchers to unexpected events can vary. To industry scientists, unexpected variation can be a problem. For example, an unexpected loss of confinement in a trial could adversely affect factors such as the power of the experimental design or the purity of harvested seed. This should lead to an element of industry self-regulation over unexpected effects. To academic researchers, unexpected effects may provide a fascinating new direction of inquiry, and so self-regulation might be less significant. Nevertheless, the size of experiment is likely to be smaller than a field trial prior to commercialization.

It might be useful for APHIS to give guidance on the kind of change that should be reported during, or on completion of, a field trial. This could be in the form of a checklist of life history characters that potentially affect confinement (see above). Applicants could be asked to complete a simple questionnaire to confirm that no unexpected variation was observed in these characters.

Environmental issues, new genes and phenotypes

The production of stress tolerance traits by modifying metabolic and signaling pathways raises no new issues for field testing. However, it is possible that unpredictability (range of possible phenotypes) might be increased and therefore procedures may need a change of emphasis. We may need to focus equally on both the intended trait along with likely pleiotropic effects. Traditional breeding for stress tolerance and information about common molecular processes underlying different tolerances can give clues to the correlated changes in phenotype that we should monitor.

One example is the correlation between increased cold tolerance and enhanced tolerance of salt. If cold tolerant rice were to be field tested next to a salt marsh, this prior knowledge might lead us to monitor the marsh for feral populations of rice, or for USDA-APHIS to restrict these locations from field trials. We would probably not monitor if the rice were modified for herbicide tolerance.

It is possible that genetic modification of metabolic pathways might create different correlated changes from those observed in traditional breeding. However, the consensus was that such changes would probably have a negative effect on plant performance.

Cold tolerance, regulatory criteria and confinement

Inference from molecular biology and traditional breeding suggests that modification of cold tolerance is more likely to affect life history traits than are modifications such as insect resistance via expression of Bt endotoxins or current methods of herbicide tolerance. However, this does not alter the regulatory criteria. Nevertheless, additional relevant data may be desirable based on familiarity with the phenotype. In the case of cold tolerance, we may need more studies on cold tolerant turf grass than on oilseed rape because this crop has already been extensively studied.

Phenotypes that are most likely to result from modification of cold tolerance and affect confinement are increased seed dormancy, increased vegetative persistence (of tubers, rhizomes, corms, bulbs, etc.), changed timing of seedling emergence, and changed flowering time brought about by altered vernalization requirements. Pollen viability might also increase through improved desiccation tolerance, though it is doubtful whether this would have much effect on outcrossing rates.

Research requirements

For improved risk assessment and regulation of cold tolerant phenotypes the group recommended the following research requirements:

- Measure seed dormancy and seedling emergence in the laboratory (studies could be done in parallel with small-scale field trials)

- If significant changes are found in dormancy or emergence, carry out a study of seed bank dynamics
- Measure pollen longevity

  But how will we use such information? What kind of tier II study would be triggered by observing increased longevity?
- Investigate whether it is possible to predict changes in phenotype that affect confinement from molecular data
- Determine if a pre-field test screen for altered life history characters possible and practicable
- Examine the consequences of cold tolerance for weed population dynamics
- Ask whether cold limits the abundance or distribution of volunteers, feral populations, or sexually compatible wild relatives of the cold tolerant crop
- Determine if gene excision technology, which removes the transgene(s) from pollen, would improve the predictability of containment/confinement. However, the general ecological impact of this technology would also have to determined

To assist the commercial introduction of cold tolerant phenotypes, consider the following:

- Will cold tolerance improve agricultural sustainability?
- What are the wider ecological and social implications of introducing crops with enhanced cold tolerance?

  For example, the change from spring to winter-sown cereals had a profound effect on farmland ecology in Europe. What can we learn from changes in patterns of conventional agriculture to help us predict the long-term impacts of GM plants?
- Studies of the molecular mechanisms of cold tolerance may reassure the public that we can predict the ecological impacts of cold tolerant crops and allow us to alter the smallest number of genes in order to have the desired effect.

Reference

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REPORT OF THE DISEASE RESISTANCE GROUP[1]

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PHASE I: GENERAL DATA NEEDED

"Given the regulatory criteria of field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?"

First we consider a situation where sexually compatible wild relatives are growing sympatrically with a genetically modified crop and the genetic enhancement is not a pharmaceutical product. The most critical issues relative to gene flow are alterations in pollination-related traits. This includes alterations to the anther, tapetum, or locule, which results in production of larger quantities of pollen, alters the longevity (viability) of the pollen, or enhances the distance that pollen can disperse. Alterations in floral morphology would similarly be a potential issue of concern in crops that are pollinated by insect vectors. Morphological changes that could alter gene flow include, but are not limited to, increasing the number of stamens or petals, modifying the height or position of stamens, changing the number or attractiveness of floral nectaries, modifying the coloration or corolla to enhance attractiveness to pollinators, and altering the phenology of anthesis (e.g., length of pollen shed, synchrony with style receptivity).

In species that rely on vegetative propagation, any traits that alter tillering, rhizome or stolon formation, and rooting capacity of any vegetative tissues could potentially enhance the survival of hybrid progeny resulting from gene flow. Such changes could also affect the persistence of plants in the field plot, which may increase the likelihood of cross pollination with compatible plants in close proximity.

It is plausible that the transgene of interest may be unrelated or unlinked to the morphological alterations discussed above (i.e., they may be the result of pleiotropic, epistatic, or position effects). Predictability of these types of changes is difficult at best. Hence, it is necessary to closely monitor for floral changes in the greenhouse or other containment facility prior to field testing. For more subtle effects, such as the viability of pollen over time, direct experimentation is required to detect such changes. In general, it was felt that alterations in floral morphology of major potential concern would be those that exceed the range of natural variation across the germplasm base; subtle morphological changes do not necessarily need to be monitored closely. Critical to all of these types of assessments is the creation of a database of information on characteristics of interest (e.g., floral morphology, pollen production, etc.) in non-genetically engineered varieties of the crop. A complete assessment of all possible changes within the modified plant would be prohibitively complex. Minor alterations in morphology would fall within the natural variation of the crop and thus would not significantly alter gene flow characteristics. Moreover, highly detailed greenhouse studies of minor changes perhaps negate the intended purpose of a field trial—to experiment and observe.

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

When changes in factors that might affect gene flow or gene persistence are observed in greenhouse trials, the adequacy of confinement protocols should be considered. These are based on the crop biology and may include increasing isolation distances, temporal isolation, and length of time for monitoring volunteers. Other strategies that alter pollen flow may be used in instances where seed increase is not required, such as detasselling, bagging of pistils, or emasculation.

"Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring these changes?"

Current methods and standards for gene characterization are considered adequate. Some of the considerations listed in the response to the first question (above) need to be taken into account when characterizing and identifying potential secondary effects of new transgenic crops. Monitoring or observation of unexpected or unintended changes in the transformant is required to adequately detect changes that may affect gene flow. In instances where the toxicity of the gene product is of concern, the assessment of toxicity may be obvious from literature or may need to be performed directly through testing.

"What are the strengths of the industry approach to characterize genes from plant genomics projects? Are there areas where the approach should be improved?"

In general, it is thought that industry based efforts will be better funded and rely on a more substantial database relative to genomics information. Sequences selected for field testing that are based upon comparison to known gene homologs with described functions are likely to be well characterized before industry expends effort on field testing. On the other hand, annotated sequences or expressed sequence tags may or may not represent genetic elements of potential concern (i.e., toxicity) and are not necessarily well characterized. The testing of essentially unknown open reading frame sequences, transcription factors, or other elements affecting signaling pathways does raise the level of concern relative to containment of field plots. The potential for uncharacterized sequences to outcross with crop or seed production fields should be treated differently from the case involving characterized genes of known function. It is anticipated, however, that field testing of uncharacterized sequences will be on a very small scale compared to those of known function (i.e., dozens of plants per test vs. thousands of plants per test).

The sharing of information by some in the biotechnology industry is laudable and will likely impact the rate of advancement of genomics as a whole.

"Do any new environmental issues relevant to field testing releases and management arise when considering emerging genes and phenotypes they affect?"

This was largely addressed in the answer to the second and third questions. With some of the proposed novel gene combinations to be field tested in the near future, it is plausible that the phenotypic effects may be less readily predicted or at least less obvious. While the effects may be more complex to decipher at the biochemical level, the environmental issues of most concern remain the same (e.g., afford a selective advantage to a recipient species or adversely impact the biodiversity of smaller populations).

Transgenic crops with new genes of pesticidal intent (e.g., insect or disease resistance) require special consideration. Outcrossing of these new varieties to food crops, or to wild relatives that may subsequently backcross with crop plants, may result in that commodity and any of the food products derived from such plants being considered as adulterated under the Federal Food, Drug and Cosmetic Act. Such foods or feed would then be subjected to seizure and removal from the food supply by the FDA unless a previous tolerance action was already in place for that specific gene product. This is particularly problematic when breeding or seed production nurseries are in close proximity to experimental research plots.

PHASE II: DISEASE RESISTANCE

"Does this gene/trait differ from currently commercialized genes/traits in ways that are relevant to regulatory criteria for field testing?"

When considering the phenotype (e.g., disease resistance) and the genes of interest (e.g., signal transduction modifiers), persistence in the environment needs to be considered since the disease resistance phenotype could afford an enhanced persistence in the receiving gene pool should gene flow occur. This is true for both currently deployed disease resistance genes and those considered herein. However, the complexity and breadth of the disease resistance provided by enhanced systemic acquired resistance (SAR) expression could conceivably alter the reaction of the host plant to a variety of seemingly unrelated disease organisms as well as result in some pleiotropic or epistatic effects. If a disease was a limiting factor for proliferation of a recipient species, and the newly acquired SAR modifications (e.g., *NPR1* / *NIM1*, *DTH9*, jasmonic / ethylene mutants) provided for tolerance or resistance to this disease, there would be a clear selective advantage for the members of the population carrying this gene(s). In instances in which a wild relative is known to have no viral disease of a limiting nature, the introgression of a viral coat protein gene into the wild population would likely have little effect on the receiving species in terms of selective advantage. If the introgressed resistance mechanism is of a broader nature, as may be the case with a SAR inducer, resistance may be provided to other pathogens (e.g., bacteria, fungi, nematodes, etc.). To adequately assess the risk potential in this situation, a greater knowledge of the host:parasite biology of the wild relative is required. This is, unfortunately, often lacking. In the event of broad resistance gene transfer, such as SAR, the normal evolution of virulence (*avr*) and resistance (*R*) genes in the pathogen and host genomes, respectively, may be altered if the resultant phenotype is beyond the bounds expected from transgressive segregation of the native population's genome.

With respect to criteria for field testing, it is conceivable for the cases involving these broad alterations to pathways that there may be pleiotropic effects that would influence confinement-related characteristics as outlined in the first question. These will require evaluation similar to that discussed above with some of the less well characterized traits.

"Is there evidence to indicate engineering the pathway under consideration may produce effects (directly or secondarily) that impact confinement of field trials?"

It is most probable that any secondary effects resulting from alteration of the SAR or other disease resistance pathways will be detrimental to the plant and not result in a selective advantage *per se*. This does not mean that it is out of the realm of biological plausibility that such an occurrence (i.e., a secondary effect resulting in a selective advantage) could take place, it is just that the likelihood is exceedingly low. The probability that pleiotropy results in increased pollen production, altered nectary production, or other characteristics that might enhance gene flow is considered low, though not without possibility. These types of alterations would be addressed in the greenhouse (pre-field) phase of evaluation and observed further in the field test plots.

In cases where the novel phenotype is constitutively expressed, as may be the case with modified signal transduction pathways for SAR, the metabolic cost to the recipient may be greater than any selective advantage provided by the novel trait, especially under low disease pressure. The effects on fitness and persistence of the gene(s) in the affected population are difficult to predict.

"Are these areas that would benefit from additional research? What data or experiments would address these areas?"

Building a database of information detailing the natural variation seen in crop plant characteristics that may be related to gene flow would be a good starting point. Such studies would include measurements for each crop of interest for characters like stamen length, pollen production, viability of pollen under the range of 'typical' field conditions, phenology of anthesis, attractiveness to pollinators, and any other trait of potential importance to gene transfer.

Determining the status of disease resistance in the wild populations potentially affected by gene flow from a modified crop would also aid in risk assessment. For example, if the basic phenotype of disease resistance already exists in the population, there is a reduced probability of selective advantage being afforded to recipients of gene flow. Such studies may be a complex undertak-

ing, however, since the apparent phenotype as measured by reaction to a pathogen may not tell the whole story of net impact on a species (e.g., other effects unrelated to the disease reaction may occur).

Further characterization of isolation distances needed for specific crops in various geographic areas is also needed. Basic information on pollen biology, pollinators, sexual compatibility with related species, and phenology would be helpful in creating a thorough risk assessment for both the crops species and wild or feral species of relevance.

PHASE III: RETURN TO THE BIG PICTURE

"Has discussion of the case study altered any of the answers to the general questions posed in Phase I?"

No. Given the complexity of interactions possible with the alteration of pathways such as SAR (e.g., salicylic acid production, oxidative burst, apoptosis, hypersensitive response, shikimic, terpenoid or phenolic pathways, kinase cascades) and other disease mechanisms involving signaling pathways, the ability to presciently determine the plausible secondary effects on a recipient plant is severely limited. Hence, the basic criteria needed to assess containment or confinement conditions of a research plot still need to be based upon the primary concerns outlined in the answer to the fifth question. A trait transferred via gene flow to a compatible recipient, introgressed, and properly expressed in the progeny, which provides a selective advantage to the progeny of such an event, is still subject to the basic evolutionary pressures of the natural environment, regardless of gene type. As mentioned in the answer to the fourth question, the potential for unintended secondary effects that impact gene flow rates or other more subtle physiological changes will need to be described through pre-screening in the greenhouse and careful field observation.

REPORT OF THE LIGNIN MODIFICATION WORKING GROUP[1]

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BACKGROUND TO CASE STUDY

The lignin biosynthesis pathway is of great interest given the importance of lignin for digestibility of forage crops, conversion of lignocellulose for bioenergy products, and for wood quality and paper-making. At the June 3–4, 2002, ISB workshop, seven scientists with diverse affiliations and backgrounds were asked to examine a case study of lignin modification in which genes are introduced via transgenic methods to decrease lignin content in trees or crops. Background papers, which summarize the use of several transgenes for lignin modification, were provided. For example, partial sense or antisense constructs have been used to inhibit the biosynthetic enzymes involved in the phenylpropanoid or monolignol-specific pathway (Boudet, 2000).

The working group was asked first to examine the general issues associated with field testing metabolically engineered plants (Phase I), and second, to highlight field testing issues specific to modifications in lignin content (Phase II). Then, the group was asked to compare and contrast its thoughts about the general issues and the case study (Phase III). Key questions posed to the working group and its ideas are summarized below.

PHASE I: GENERAL DATA NEEDED

"Given the regulatory criteria of field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?"

The working group reviewed USDA's six criteria for field testing and discussed potential changes due to metabolic engineering that might impact confinement. There was consensus in the group that because lignin is a fundamental biochemical present in virtually all plant cell walls, its modification—whether via conventional breeding or genetic engineering—could have diverse effects on plant development, including on traits related to dispersal such as seed dormancy, fertility, and vegetative persistence. These characteristics could be changed by modifications of the lignin biosynthetic pathways. However, the members agreed that significant natural variation in these traits exists within natural populations and that breeders have substantially changed lignin content and dispersal traits via conventional breeding. This record and the existing range of natural variation should provide an important context for evaluating potential confinement issues associated with metabolically-engineered transgenic plants. All agreed that more information on natural variation of seed dormancy, fertility, and vegetative persistence, as well as the effects of this variation on confinement, would be useful. Some information is already available in diverse and often regional plant breeding and ecology literature (a.k.a. "gray literature"). Many group members thought that more information, or a cataloging, is needed to make regulatory decisions about field trials, but others did not think that this information was needed for regulatory decision-making.

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

"Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring these changes (that impact confinement)?"

Prior to field testing, phenotypic changes related to confinement could be monitored in greenhouse settings but some conditions may not be appropriate for some species (e.g., those affected by limited light, root development, etc.). Greenhouse studies might be appropriate for evaluating flower structure and pollen production in transgenic lines and comparing them to control plants. Some members of the group were unsure whether studies such as these are systematically or routinely done, but felt that they could be incorporated in some circumstances. Other members thought that for some crops—such as trees—a regulatory requirement for such studies would be onerous. For such crops, which show high gene dispersal in nature without transgenic modification, some members thought that greenhouse characterization would be of little value. Instead, breeders would prefer to assume an absence of containment and instead assess the consequences of release.

The group discussed the potential powers of DNA, RNA, protein, and metabolic profiling (e.g., via microarrays and chip technology), and noted that, in the future, such analyses might be helpful for predicting changes that impact confinement. In order to make such predictions, extensive field experiments that measure confinement parameters would be needed to correlate them with biochemical or physiological characteristics. Currently, the basic physiological and biochemical factors affecting confinement are not well understood. Some members felt that adding more information on the expression of thousands of genes at various stages of development—and in diverse environments—would pose significant experimental and statistical challenges and would be extremely costly. These members felt strongly that molecular approaches would always have significant limitations in predicting confinement changes, in comparison to direct studies of confinement traits and consideration of the invasion potential of the transgenic modifications. Other members were hopeful that with rapid advances in the field of molecular biology, these approaches could become routine and relatively inexpensive.

"What are the strengths of the industry approach? Are there areas where the approach should be improved?"

The group spent considerable time discussing how industry evaluates transgenic plant varieties at early stages of product development (i.e. before field testing). The methods used for gene discovery, such as microarray analysis, analysis of different metabolic pathways, and mutant analysis, are similar in industrial and academic laboratories. Research capabilities and interest begin to diverge as gene validation (connecting function with sequence) becomes the focal point of research. Generally, industrial laboratories are better equipped and more inclined to carry out larger-scale experiments using transgenic and other genetic evaluations than most academic laboratories. Past the discovery point, industry also tends to have a greater focus on the eventual product development, the marketability and value, and the product's safety for consumers. Industry is more concerned about biosafety and regulatory issues than the academic community, given its focus on product development. Therefore, most members felt that industry will tend to investigate unintended metabolic changes at earlier stages.[1]

Other potential industry strengths that were identified include 1) better access to genomic information and databases (at least for large corporations with active genomics programs), 2) better access to transformation and analytical technology, 3) better documentation, and 4) a high level of interaction between molecular biologists and conventional breeders. For example, with access to better analytical methods such as microarrays, industry has an increased ability to detect unintended biochemical effects in metabolically engineered plants.

Some members of the group noted that industry could improve 1) the transparency of its process (e.g., by making the R&D process and safety testing protocols understandable to the interested public and by making more data/information available) and 2) its communication with the public at early stages of product development. How-

[1] The group based its concept of an industry approach on views from a representative from one company. In fact, industry approaches vary and there is no single "industry approach."

ever, such communication might not be appropriate in light of confidential business information and the fact that few products make it to commercialization. This communication could also potentially provide terrorists with information to target research facilities.

"Do any new environmental issues relevant to field testing releases and management arise when considering emerging genes and the phenotypes they affect?"

In order to think more deeply about this question, the members identified three categories for metabolically engineered plants: those in which 1) an endogenous gene is up or down regulated (e.g., new promoter, knockout, or antisense regulation); 2) a gene from another species is added, but this gene shares a high degree of functional and sequence similarity with one in the host plant (e.g., bacterial version of a plant gene); and 3) a novel gene is added (e.g., a gene for a biosynthetic enzyme not found in the host plant species).

In the limited discussion time, members of the group examined whether generalizations about the type and magnitude of environmental risk could be made for each category. Some group members felt that for the first two categories, metabolic changes would likely fall within the range of natural variability, given the biochemical checks and balances in the host and the abundance of regulatory and loss-of-function polymorphisms found in large population surveys. Therefore, the third category should receive the greatest scrutiny as it could give rise to ecologically novel phenotypes with respect to invasion of wild population (e.g., novel pest defensive chemistries). Other members disagreed, given the greater number of pleiotropic effects that could arise from the first two categories (i.e., endogenous or homologous genes could interact with a greater number of other genes/enzymes in the host). Regardless, the group reached consensus on the point that existing variation in metabolic profiles for various host taxon is an important context by which to measure whether an engineered plant is of concern.

For transgenic plants with novel genes or with metabolic changes that fall outside the range of natural variability, many of the group members agreed upon two main issues on which to focus environmental assessments—the fitness of crop/wild hybrids and potential non-target effects (if the gene is introgressed into wild populations or if the field trial(s) encompass a large area). Overall, many in the group agreed that no new categories of environmental issues would arise from metabolic engineering of plants. The potential environmental risks would still depend on the biochemical or physiological change in a particular product and on the environment into which it is introduced.

PHASE II: LIGNIN MODIFICATION

"Does this gene/trait differ from currently commercialized genes/traits (e.g. Bt, herbicide tolerance, virus resistance, delayed fruit-ripening) in ways that are relevant to regulatory criteria for field testing?"

Some in the group noted that, so far, transgenes used to modify lignin content in crops or trees fall into categories 1 and 2 (i.e., endogenous or homologous genes added). In light of this, some group members felt that metabolic or phenotypic changes in low-lignin transgenic plants would fall within the range of natural variability for that species, and therefore at the field testing stage, would not be of any greater concern than changes resulting from conventionally bred low-lignin plants. However, some members noted that lignin is ubiquitous in the plant body, and therefore by modifying its content, unexpected metabolic or structural effects which impact confinement or non-target species could occur.

Overall, the group agreed that field testing criteria for low-lignin transgenic plants would be similar to criteria for currently commercialized transgenic plants. However, one member felt strongly that transgenic modifications in expression of native lignification genes could be considered at a lower level of scrutiny at the field testing stage, and even exempted from the need for confinement and regulatory oversight.

"Is there evidence to indicate engineering the pathway under consideration may produce effects (either directly or secondarily) that impact confinement of field trials?"

The group identified several potential ways in which lignin modification could impact confinement, including possible changes in seed dormancy and flower morphology (although group members could not cite direct evidence for such effects). It was stated that, in most cases, decreased lignin should lead to decreased fitness, given the structural and pest-resistance roles that lignin plays. Members wanted to emphasize that lignin modification is of greatest interest in tree species, which are highly outcrossed. One member of the group stated that tree species being used for such modification (e.g., Poplar) have wild relatives in the US, and, therefore, some level of gene flow is a certainty, as is a high degree of dilution of transgenic propagules by wild sources when trees are allowed to flower. However, transgenic trees are often harvested prior to flowering in field trials, and trees that have strong engineered or natural fertility mechanisms are under development. Nonetheless, some in the group felt that the primary focus of biosafety assessments for field trials should be shifted from changes in confinement traits to the potential impacts of gene escape and introgression, should they occur.

"Are there areas that would benefit from additional research? What data or experiments would address these areas?"

The group identified the following potential research needs:

- Database or compilation of the range of natural variability in phenotypes and metabolic profiles of engineered plant species, and for metabolic profiles of concern
- Correlation of phenotypes and metabolic profiles to performance in field trial settings
- Basic research to understand biochemistry of lignin production so that there is a larger suite of options for transgenic plants with altered lignin (i.e., safer options)
- Microchip technology to profile biochemical changes that affect plant health, confinement factors and non-target risks
- Evaluation of the effects of gene flow from crop to wild relatives—determine how particular traits affect fitness and investigate the effects of introgression on non-target species, in both conventionally bred and transgenic varieties
- Field monitoring for gene escape and its consequences (e.g., in low risk situations where flowering is permitted)
- Examinations of whether seed dormancy or flower morphology are altered by lignin engineering in such a way as to increase, rather than decrease, persistence, outcrossing, and plant competitiveness
- Cost and risk/benefit analyses on transgenic plants with decreased lignin
- Long term, public research trials on lignin modified plants to assess their production value compared to possible liabilities due to increased biotic or abiotic stress susceptibilities
- Limitation of expression to particular tissues (e.g., expression in wood or secondary xylem), but not reproductive structures (e.g., primary xylem, seed coats, etc.)

Members of the group also highlighted the need for improved interactions between plant breeders and biotechnologists (especially in academe), more public support for traditional agricultural research, and large field trials in the public sector so that important academic questions can be addressed.

PHASE III: RETURN TO THE BIG PICTURE

"Has the discussion of the case study altered any of the answers to the general questions posed in Phase I?"

The group quickly reexamined its responses to the questions in Phase I and determined that they were still accurate. Overall, some members in the group voiced the opinion that only modest changes to confinement for metabolically engineered plants (including transgenic lignin altered plants) would be needed, and that phenotypic changes would likely fall within the range of natural variability. Some in the group felt that 1) for most crop species, but particularly for trees, some level of gene flow is a certainty (i.e., 100% containment would never be achieved if flowering is permitted); 2) biosafety assessments should focus on whether the "escapes" from field trials are likely to give rise to invasive propagules; and 3) if so, the fitness of the crop/wild hybrids and potential effects on non-target species if introgression occurs should be focal points of assessment. Some group members

felt that environmental consequences will rarely, if ever, be of concern for lignin modified transgenic plants at the field trial stage, as they are highly unlikely to confer increases of invasive capability. Other members disagreed and emphasized the need for further studies of phenotypic and fitness consequences of lignin modification.

REPORT OF THE OIL MODIFICATION WORKING GROUP[1]

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INTRODUCTION

Oilseed production represents a major sector of U.S. and world agricultural output. The most important oilseed crops are soybean, oil palm, rapeseed/canola, and sunflower. Fatty acids from such plants are a vital component of the human diet and can provide up to 25% of the caloric intake in developed countries. In addition, plant fatty acids have many industrial applications such as soaps, detergents, lubricants, biofuels, cosmetics, and paints. There are at least 200 different types of fatty acids that have been identified in plants, but the most abundant are linoleate, palmitate, laurate and oleate. The end use of the plant oil dictates which fatty acids are most desirable. Genetic engineering presents opportunities to modify oil content. This may be done to increase the proportion of "healthy" fatty acids in an oil, improve oil stability, expand the range of fatty acids that can be produced at low cost, and increase oil content to reduce cost. We considered two case studies in which oilseed crops were genetically engineered. In both cases, the inserted genes were under the control of seed-specific regulators.

The first case study was soybeans, genetically engineered for high oleic acid content. Commodity soybean varieties have over 50% linolenic which is an omega-6 fatty acid. Oleic acid is a monounsaturated fatty acid. Evidence is accumulating that incidence of coronary heart disease might be reduced by consuming a lower amount of omega-6 fatty acids than is typical in many western diets. The omega-6 content was reduced and oleic acid was increased by transformation with a delta-12 desaturase enzyme from soybean. The promoter was a sequence from beta conglycinin, a seed storage protein also from soybean. The inserted gene then inhibited synthesis of the target desaturase through a process known as sense suppression. As a result oleic acid content was greatly increased and omega-6 fatty acids were greatly decreased in the seeds. Dupont submitted a petition for determination of non-regulated status to APHIS, which was granted in 1997.

The second case study was canola, genetically engineered to accumulate laurate in seeds. Laurate is an important fatty acid for production of soaps, shampoos, and detergents. A thioesterase gene from California bay (*Umbellularia californica*) was inserted under the control of the napin storage protein promoter from rapeseed. This enzyme cleaves lauroyl-ACP to yield free laurate. The bay thioesterase is related to the native canola enzyme but has different specificity for fatty acid chain length, and, under the napin promoter, laurate accumulates in the seed. The accumulation of laurate induced several biochemical pathways to become active. It was shown that the ß-oxidation pathway for breakdown of lipids, the glyoxylate pathway for fatty acid carbon re-utilization, and the fatty acid synthesis pathways all showed increased activity, giving rise to a futile cycle in seeds during the time period when the promoter was active. Interestingly, total oil yield was not reduced, indicating that increased fatty acid synthesis was able to compensate for any breakdown. Calgene submitted a petition for determination of non-regulated status to APHIS, which was granted in 1994.

In addition to these two case studies, the groups also considered possible future engineered oil modifications to plants for producing better food or animal feed or for industrial uses. In such discussions, we generally assumed that future modifica-

[1] Group Report from "Criteria for Field Testing of Plants with Engineered Regulatory, Metabolic, and Signaling Pathways," held in Washington, DC, June 3 – 4, 2002. Sponsored by Information Systems for Biotechnology.

tions would likely still involve seed-specific promoters, which may narrow the focus of concern to mostly seed factors.

PHASE I: GENERAL DATA NEEDED

"Given the regulatory criteria for field testing, what biochemical, physiological, or phenotypic changes may impact confinement of transgenic plants? How might these changes be detected prior to field testing?"

There was general agreement that certain phenotypic changes could have an impact on confinement of transgenic plants. However, such changes would need to be large in magnitude to really have the potential to affect confinement measures. Phenotypic changes that might impact confinement are typically factors that influence dispersal of plants such as:

- Pollen: amount, longevity, dispersal distance, factors influencing self-incompatibility
- Floral traits: morphology (e.g., anther extrusion), development, number of flowers, spatial separation of floral organs, attractiveness to pollinators, flowering time
- Seeds: number, longevity, dispersal distance, persistence
    - Vegetative propagation: extent, dispersal

If the transgene is intended to induce such changes, current procedures would deal with the increased risk in the permit process. Small, unintended changes may be difficult to detect, no matter whether they are induced by a 'first generation' gene (such as Bt or RR) or from one of the 'second generation' genes (such as transcription factors, genes with a regulatory function), or by traditional breeding. On the other hand, large phenotypic changes would be likely identified in greenhouse or growth chamber trials already and would not progress to field testing given the typically applied screening procedure.

Because some changes will not be detected during growth chamber and greenhouse trials, field trials are an essential tool for detection of such important changes. Because initial field trials are quite limited in scale, the risk is considered to be minimal. Furthermore, most unintended effects are likely to disadvantage the plant with regard to fitness.

It was also agreed within the group that there is no such thing as absolute containment during a field trial. However, current procedures are adequately addressing this point. Regulation of field tests involves a risk-based approach for categorizing transgenic plants, such that suitable confinement conditions can be imposed.

Under the current procedure, the size of the field trials is not limited. It was noted that it is hypothetically possible that transgenic plants could be tested under large scale field trials that have not been previously tested under small scale trials. However, as a matter of practice, this is highly unlikely to happen, as there is no incentive to commit the necessary financial resources to perform such large scale field trials without prior screening in smaller trials. Once an event is put on a commercial track, large scale field trials usually become necessary. However, during these trials, a whole battery of regulatory questions has to be addressed, assuring that all the above-mentioned changes in phenotype will be captured and addressed.

"Do existing standards and methods for gene characterization and identification of secondary effects encompass monitoring for these changes?"

In general, yes. Small-scale trials are unlikely to negatively impact confinement issues. Larger-scale trials (e.g., 100 acres—the group knew of no basis defining a limit) would likely be established for commercial-track events. Evaluation of such events would adhere to current industrial guidelines consistent with evaluation for potential unintended effects (description of traits/phenotypes analyzed has been presented elsewhere). Industry and non-industry groups share processes/approaches toward identifying unintended effects. Different species may require different acreages, although this should be considered more the exception than the rule. Characterization of transgenic plants is typically initiated in the first generation, and intensified in subsequent generations. Events requiring planting on large acreages would already be substantially characterized upon such planting.

"What are the strengths of the industry approach (described in the morning plenary session) to characterize genes from genomic projects?"

The team decided to redefine the scope of this question to consider genes and phenotypes relating to regulatory, metabolic, and signaling pathways, rather than those from genomics projects. We agreed that there are many new types of genes that may be incorporated into field released plants over the next few years. Some of these plants may contain genes for which the function is not as well characterized as those in the first generation of de-regulated plants such as Bt or EPSPS genes.

Several strengths of the existing industry approach were noted. Early safety evaluations through reviews by an internal biosafety committee would potentially identify any genes or phenotypes that would require more stringent field testing requirements. Early consultation with USDA-APHIS also allows the researcher to gain valuable insight of potential risks or hazards that should be considered.

Extensive gene characterization prior to field testing is not always feasible (since some of the characterization requires growth under field conditions). However, some key information is usually available prior to a release. APHIS requires that gene function be known for genes tested under notification and has provided guidance on determining gene function. An example of information that should be considered prior to a release is the comparison of the protein to databases of known allergens and toxins. Information collected prior to field testing should be sufficient to conclude that the protein is not likely to be toxic to non-target organisms if the field test is to be authorized under the notification procedure.

Another important aspect of the industry approach is a commitment to stewardship. A stewardship program often includes committing dedicated resources to ensure that the proper procedures are in place and that protocols are strictly followed and documented. Utilization of the established and well-tested protocols will greatly minimize the potential for gene flow out of the experimental trial.

"Are there areas where the approach should be improved?"

Those in industry, who have gained considerable experience in producing and developing transgenic plants for commercial use, could offer to train others in the approach that has been successfully deployed in the past. Trainees might include those in academia or even emerging small companies. The training could encompass identification of the type of data that is typically needed at the various stages of development and the methodologies that have been used for gathering such data.

"Do any new environmental issues relevant to field releases and management arise when considering emerging genes and the phenotypes they affect?"

No, the phenotypes of these plants are not expected to be different enough from other GM plants to result in new types of environmental risks. We felt that existing regulations cover questions about gene escape and non-target effects that could occur during field testing. Existing procedures should be sufficient for field tests of plants with GM pathways. Due to the limited scope of this workshop, we did not consider environmental issues that should be evaluated prior to deregulation.

PHASE II: OIL MODIFICATION

"Does this gene/trait differ from currently commercialized genes/traits (e.g., Bt, herbicide tolerance, virus resistance, delayed fruit ripening) in ways that are relevant to regulatory criteria for field testing?"

No, as with previously commercialized genetically engineered plants, plants engineered for oil modification are not expected to be drastically altered relative to the fitness characteristics listed in our response to the first question. Therefore, we do not see a need to change the regulatory criteria for field testing. Most examples of plants genetically engineered for oil modification, including our two case studies, involve modifications to existing pathways to increase the amount of a certain fatty acid or to effect accumulation in certain tissues. Current and near future technol-

ogy will not likely increase total oil levels dramatically. In addition, we have no reason to believe that modified oils will be inherently more toxic to non-target organisms.

In anticipating new risks, it is important to consider promoters. For oil modification, we anticipate that new genes will always be under seed specific promoters, as in the case studies. This helps to focus the risk evaluation to seed factors. As new plants engineered for modified oil content progress into large scale field testing toward commercial development, changes in seed parameters that may have smaller effects on fitness should be examined. These factors could include: duration of seed production; seed dormancy; and germination and emergence under various conditions.

"Is there evidence to indicate engineering the pathway under consideration may produce effects (either directly or secondarily) that impact confinement of field trials?"

Unlike the other case studies presented at the workshop, the engineered plants considered in the modified oil case studies have been deregulated by APHIS. They have already been field tested extensively and have been found to be no different than their nontransgenic counterpart with respect to characteristics that may impact confinement. In addition, we have a long history of traditional breeding and release of oilseed crops with modified oil quality or quantity. These practices have not led to the identification of any phenotypic changes of the type and magnitude that would affect confinement as in the case of field testing of regulated articles.

"Are there areas that would benefit from additional research? What data or experiments would address these areas?"

Several areas of research were identified that might produce information useful in helping to assess the risks of plants intended for commercialization. We noted that much of this information was not critical to field testing. Areas discussed were that following:

- Effects of distance and other parameters on pollination frequencies for various crops—such data would be helpful in validating current isolation distances required for isolation distances required for various crops engineered with various categories of transgenes.

- Baseline data for ecological studies—this type of data is necessary to interpret ecological changes that may be detected during field testing. Such data would help to define normal ranges in agricultural settings and would be useful in determining whether such changes are beneficial, neutral, or deleterious.

- Transcript profiling—appears to be very useful as a tool for academic research, but we do not see an immediate application as a screen for safety.

CONCLUSION

As a general conclusion, we do not see a need for changing the regulatory criteria for field testing based on our case studies of crops genetically engineered for altered oil content. This does not mean that modification of lipid pathways or other metabolic pathways cannot have effects that can alter plants phenotypes in ways that may affect their ecology, but rather that we see no indication that such changes would be of the magnitude that they would significantly affect containment in field trials. As field testing is scaled up, as a result of favorable results and predictable behavior in initial trials, more extensive data should collected to detect smaller changes prior to commercialization.

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PLENARY PAPERS

USDA REGULATION OF AGRICULTURAL BIOTECHNOLOGY

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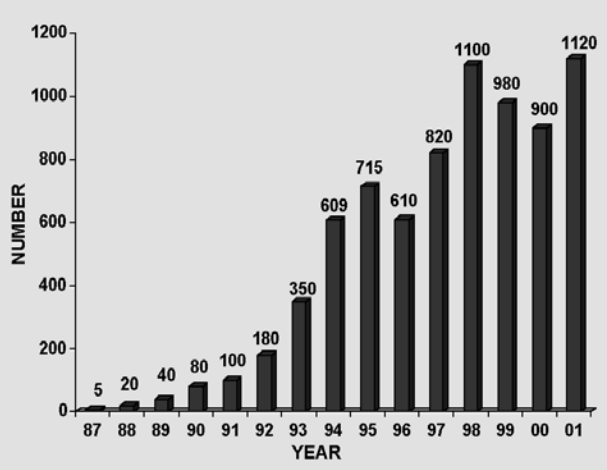
In the United States, three agencies have regulatory oversight at the federal level over genetically engineered plants—USDA-APHIS is one of those three. The EPA becomes involved in the field testing process for pesticidal plants, or, more correctly, plant-incorporated protectants, once field tests reach the 10-acre limit. From a regulatory standpoint, agencies have very specific questions to consider as they make their decisions. What follows is an overview of APHIS regulatory authority and procedures and their application to laboratory work in industry and academia. Also, how the new genetic constructs and approaches to modifying plants fit into that overall regulatory scheme will be discussed.

Much of this workshop will focus on some of the earliest stages of field testing. Figure 1 represents the field tests that have been performed since 1987, and a clear, increasing trend is evident. Plant biotechnology is a very active area of research. In recent years, APHIS typically processed around 1200 – 1400 authorizations for field tests; this past year the number was again about 1400. Since 1987, over 8,700 field tests have been authorized at over 30,000 sites. Additionally, a single authorization from APHIS might cover several field sites. A wide diversity of crop plants (36 species), grasses (10 species), trees (13 species), and ornamentals (9 species) has been field tested since 1987.

A comparison of the types of genes used in 1988 with the types currently used suggests that in 1988 the state of the art of plant bioengineering was fairly straightforward. Currently in 2002, a lot of the plant engineering work is generated by the plant genome projects. In addition, a number of different systems are emerging in which researchers are using animal models and developing plant analogs to answer some very interesting questions about plant biology. These new and diverse systems prompt APHIS to question the assumptions we have previously made about regulating field tests. Are those assumptions still valid when we examine these "newer" types of gene constructs?

Figure 1. Number of field trials from 1987 to 2001.



From Information Systems for Biotechnology field testing database accessible at http://www.isb.vt.edu

APHIS uses two separate mechanisms for authorizing field tests: permits and notifications. They have slightly different requirements, which may present important considerations for this workshop.

APHIS regulations are administered under the Plant Protection Act of 2000, which is a consolidation of several acts including the Plant Pest Act, the Federal Plant Quarantine Act, and the Noxious Weed Act. The regulations have not been changed since the consolidated act was instituted. APHIS regulations were first promulgated in 1987, and then amended in 1993 to outline the system for notification procedure and also to describe the process for granting nonregulated status. In 1997, we amended the regulations again to broaden the eligibility for notifications to virtually all plants, as long as the plants are not noxious weeds in the release area.

The permit is the original mechanism APHIS used to regulate field tests. It is a fairly straightforward and paper-intensive procedure. The notification procedure is for plants only; whereas permit authorizations can be used for all regulated articles: plants, microbes, and arthropods.

The time required for review is longer for permits than notification. For field testing, review is a 120-day process under permits and a 30-day process under notification. Importation approvals require 60 days under permits, versus 30 days for notification. Interstate movement approvals take 30 days under permits versus 10 days under notifications. Importation and interstate movements are defined as transport from one contained facility to another. The regulated article must remain in containment during the entire trip so that there is no chance of environmental release. Under both systems, permits and notifications, APHIS interacts with the States so that they have the opportunity to concur with the proposed APHIS authorization.

Under a permit application, there are no restrictions imposed on the types of traits that can be approved for testing, but under the notification procedure certain genes or traits are not eligible. APHIS has had less experience over the years evaluating those traits that are considered ineligible for notification are or those that might pose a higher risk or whose risk is less well characterized.

A notification contains basically two main categories of information: eligibility criteria and performance standards. The eligibility criteria are shown in Table 1. To be eligible, the recipient plant cannot be a noxious weed or weed in the release area. Also, the inserted genetic material has to be stably integrated and its function known.

The phrase "function is known" as it relates to the eligibility criteria for the notification procedure was applied when APHIS was first developing the regulations in 1992. Researchers who were working with disease resistant response genes and who were evaluating these genes in field tests authorized under APHIS permits did not know precisely what the genes did (i.e., what the gene products were). However, they knew that expression of these genes was increased when the plants were inoculated with pathogens. Therefore, as APHIS was writing the eligibility criteria for the new notification procedure, they decided that the level of characterization of gene function for the disease resistant response genes would not meet the criterion for "gene function is known." These plants can still be field tested, but an applicant must apply for a permit rather than utilize the notification procedure. Conversely, the Bt genes are examples of genes whose function is known and therefore qualify for the notification procedure.

Table 1. Brief summary of the six eligibility criteria for notification.

1. Recipient is not a noxious weed, or a weed in the release area
2. Stable chromosomal integration of the genetic material
3. Function is known; does not result in plant disease
4. Genetic material does NOT:
   - cause the production of infectious entities,
   - result in toxic effects on associated nontarget organisms, or
   - encode substances intended for pharmaceutical use
5. Plant-derived virus sequences must be:
   - regulatory sequences of known function,
   - sense or antisense genes from prevalent & endemic virus that infects the recipient plant and
   - not functional noncapsid cell-to-cell movement genes
6. No animal or human pathogen sequences that are: Viral coding sequences of a likely causal agent of disease

Although the same level of biological confinement occurs under both systems, the mechanisms differ in the types of information required to be submitted to the Agency and States. When applying under a permit, the applicant must provide a detailed list of the protocols used to achieve biological containment. Under notification, built in to the regulations is the stipulation that the applicant must meet performance standards. The type of design protocols used may vary from experiment to experiment, and the applicant is not required to submit a written protocol at the time the request is made for a notification. Rather, the applicants periodically must submit a set of de-

sign protocols that they may use at one of their test sites. APHIS reviews the design protocols for adequacy to ensure that the applicant has a high likelihood of meeting the performance standards if the protocols are followed. Under both systems, field sites are periodically inspected by APHIS personnel and relevant State Department of Agriculture officials.

An additional eligibility criterion stipulates that the genetic material cannot cause the production of infectious entities that could produce toxic effects on associated non-target organisms. Under this criterion, the Bt genes would qualify for the notification procedure. Other excluded classes include those substances intended for pharmaceutical use. Another eligibility criterion pertains to plant virus sequences and is intended to reduce the chance of generating new viral components during field testing. The final criterion addresses animal and human pathogen sequences that are likely to cause disease.

Performance standards comprise the second part of the notification procedure. Basically, performance standards require that plants in a field test are grown so that nothing is left behind in the environment when the test is completed. This stipulation has been used in field testing in the U.S. and elsewhere, whether the test is conducted under a notification procedure or under permits or some other procedure.

To achieve confinement of transgenic plants, scientists must consider the potential for outcrossing and pollen and seed dispersal by either biological or physical mechanisms. Some methods for achieving confinement at the test site include: termination of the test prior to flowering; male sterility; inhibiting or removing flowers; bagging; spatial separation (using isolation distances such as those proposed by the Association of Official Seed Certifying Agencies (AOSCA)) from sexually compatible species; and temporal separation of flowering cycles. Additional mitigation measures may be taken such as planting windbreaks or border rows. A key to establishing confinement measures in the United States and elsewhere is to take the knowledge gained from traditional plant breeding for minimizing the persistence of material in the environment and apply it to genetically engineered plants. Beginning in 1987, in the course of preparing an environmental assessment for each field release permit, APHIS discussed how to apply the knowledge underlying the AOSCA seed purity standards used to maintain seed stock purity to design appropriate confinement measures for field testing. This approach has been recognized within the scientific community as a good place to start and is used around the world in designing confinement measures for field testing plants.

Regulatory compliance mechanisms include inspections and reporting requirements. Records, facilities, and sites are inspected during planting and harvesting of the plant material and following the field test. In addition, a field test report has to be submitted to the agency following the test. If anything unplanned or unusual occurs during the field test, the responsible party must notify APHIS. APHIS has the authority, under the Plant Protection Act, to levy substantial fines for non-compliance, if necessary. Fortunately, compliance is very high, so the agency rarely has to impose fines.

The following is a summary of the context in which regulatory decisions are made by APHIS when considering the field testing of plants engineered with these newer, "complex" genes (an imprecise term chosen to simplify the discussion).

First, do these genes meet the eligibility criteria? That question has two components: (1) is the function known and (2) is it unlikely to affect non-target organisms? These two key questions determine whether the field test will be handled under the notification or permitting system.

Second, what are the confinement protocols? Performance standards stipulate that the genetically modified material has to be confined during the test and that essentially nothing is left behind when the test is over. Confinement is the issue, whether the field test is handled under a notification or a permit. Likewise, the likelihood for the impact on non-target organisms is an issue for both mechanisms. To restate this second point about the confinement, the determination depends on the assumptions we have made. When making a relatively small genetic change, the assumption has been that, for the most part, the plant is going to behave in the environment like

the unmodified parent plant. It will likely flower about the same time and have the same interaction with other organisms. Issues affecting confinement such as reproductive biology and changes in the vegetative biology are important considerations. Another consideration is the likelihood that the modified plant will impact non-target organisms.

One of the questions APHIS needs to answer when considering some of these unusual constructs, or "complex genes" as they are referred to, is "Should our assumptions be the same or different from the assumptions that have been widely used for the less complex genes?" We want to consider how we reach our conclusion, i.e., what scientific information is available to inform our assumptions.

PREPARING AND CONDUCTING FIELD TESTING: AN INDUSTRY PERSPECTIVE

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INTRODUCTION

The variety and diversity of potential products described during this workshop illustrate the rapidly expanding breadth of emerging applications of plant biotechnology. The success of both academic and industry scientists clearly demonstrates that the next generation of biotechnology-derived plant products will likely include plants with modified regulatory, metabolic, and signaling pathways. Field testing is a necessary step to further develop these products and to increase our understanding of the potential benefits and environmental risks.

Current commercial products, which have primarily incorporated traits such as insect resistance and herbicide tolerance, have formed much of the basis for evaluation of the potential environmental risks associated with conducting field tests of transgenic plants. The regulatory system for evaluating the environmental safety of plant products developed using recombinant DNA technologies has been extensively evaluated over the past ten years. Several reviews have been published that describe the current status of this regulation (NAS, 2000, FAS/WHO 1996, OECD 1997, OECD 1993, NAS, 1987). These studies have all drawn a general conclusion: environmental risks posed by transgenic plants are similar to unmodified organisms or those modified by non-recombinant techniques. Familiarity of any new plant product is also an integral part of the basis by which USDA-APHIS assesses the risk posed by any new transgenic plant (Hokanson *et al.,* 1999).

The regulatory approach for transgenic crop products in the United States is managed under a Coordinated Framework of three agencies: Environmental Protection Agency (EPA), USDA Animal Plant Health Inspection Service (USDA-APHIS) and the Food and Drug Administration (FDA). Under the coordinated framework, many statutes and their implementing regulations and guidelines are invoked (Table 1). There are specific governmental regulations at every stage in the development of transgenic crop product. Regarding environmental release and field testing, some of these statutes apply only to specific types of products or activities and are administered by only one agency, while others apply across-the-board and thus pertain to all or virtually all agencies.

Table 1. Regulatory Statues Governing Plant Biotechnology Products in the United States

United States Department of Agriculture:

Plant Protection Act (PPA), 7 U.S.C. 7701-7772, which consolidated several previous statutes that APHIS used to regulate genetically engineered organisms, including the Federal Plant Pest Act (FPPA), 7U.S.C. 150aa-150jj, the Plant Quarantine Act (PQA), 7 U.S.C. 151-164a, 166-167, and others.

Environmental Protection Agency:

Federal Food, Drug, and Cosmetic Act (FFDCA), 21 U.S.C. 321, 346a et seq., as amended by the Food Quality Protection Act (FQPA), Pub. Law 104-170 (1996).

Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 7 U.S.C.136-136y, as amended by the Food Quality Protection Act (FQPA), *supra,.*

Food and Drug Administration:

Federal Food, Drug and Cosmetic Act (FFDCA) 21 U.S.C. 321-397

The focus of this paper is to provide a perspective on the industry approach to preparing and conducting field tests with transgenic products. Testing a new transgenic plant product under field conditions has been, and will continue to be, an integral part of the development process.

DEVELOPMENT OF A TRANSGENIC PLANT PRODUCT

The commercial development of a new transgenic plant product is a multi-year, multi-generation process. The process can be divided into several steps, beginning with discovery of a gene that affords the desired trait, and culminating with the commercial sale of the new product. (Fig. 1) Each step may take one to several years, depending on the complexity of the product and the technical success in completing each phase. Regulatory agencies are engaged in the development process beginning at a very early stage.

Commercial development starts by identifying a target product concept and finding a gene (or genes) that results in expression of the desired trait or phenotype. Plant transformation is performed to create tens to hundreds of unique transgenic events containing the gene(s) of interest. Once the transformants (transgenic events) have been generated, a selection process is initiated to identify the most promising events for the trait or phenotype. The ultimate goal of the event selection process is to identify a single event that will become the commercial product. During early generations of the event selection process, plants from each event are individually evaluated under greenhouse conditions to assess expression of the desired trait(s) as well as certain agronomic qualities. Seed is collected from these plants for future testing. Subsequent generations are evaluated for a variety of performance and agronomic characteristics while selection for the commercial event continues. After one to a few generations are grown under greenhouse observation, the events are evaluated under field conditions. Performance of the trait and evaluation of the agronomic properties under field conditions are critical elements in making the final event selection decision.

During the event selection process, it is also necessary to initiate breeding and variety development programs to incorporate the gene of interest into commercial seed lines. Variety development is an integral part of testing the event since it is important to ensure that the desired trait delivers the expected phenotype across a wide range of varieties of the crop of interest. Larger scale testing in multiple geographic locations is conducted to ensure that the gene is performing in the commercial varieties in multiple locations and under a variety of environmental conditions. Once the event selection and variety development are complete, production of commercial seed will begin in anticipation of marketing the final product.

SAFETY ASSESSMENTS OF BIOTECHNOLOGY DERIVED PRODUCTS

Full characterization of a specific transgenic event includes conducting a variety of studies that are used to evaluate the food, feed and environmental safety of the product. The safety evaluation of a new transgenic product begins at an early stage in the product development process. Internal corporate Biosafety Committees are involved during the discovery and development stages to ensure the safety of scientists working with early phase materials and to ensure that containment procedures are properly developed and followed (Traynor *et al.*, 2001). As the product advances through the development process, additional safety and characterization studies are performed, including those required to fulfill regulatory requirements in the USA and other countries. The safety assessment includes a thorough assessment of the potential risks to humans, animals, and the environment.

Data collected for evaluation of plant biotechnology product safety can be generally categorized as: 1) data and information that are either specific to the protein(s) or to the gene; and 2) data and information that are specific to a transgenic event. Safety information about the gene and protein (which are not event-specific) includes gene source(s) and sequence(s), protein sequence(s), in-vitro digestibility, and allergenicity assessments. Non-event specific data collection also includes protein toxicity testing on mammalian and non-target organisms. Most of the protein safety data are generated using a microbially-derived source of protein that has been demonstrated to be equivalent to the transgenic plant-expressed protein. Use of a microbial protein source is necessary for these studies since expression levels in the plant are usually too low to allow for purification of adequate amounts of the proteins for the dose levels to be attained in acute toxicity tests.

An extensive molecular characterization is performed to determine the number of copies of the gene(s) and associated regulatory elements as well as the stability of the insertion both within and across generations. Additional analyses are performed to search for and characterize any open reading frames that may have been unintentionally incorporated during transformation. Protein expression is measured in all tissues, at various developmental stages, in plants grown in multiple geographic locations. Agronomic parameters are compared between the event and relevant nontransgenic control(s) in multiple geographic locations (Table 2). The nutritional composition of the grain is similarly evaluated relative to conventional controls grown under common conditions in several locations. Depending on the nature of the new trait, additional environmental assessments are conducted (i.e., non-target organism field surveys and insect resistance management studies are conducted on new insect resistant products).

Table 2. Examples of agronomic parameters that are evaluated during field testing of a transgenic corn product.

Stand establishment	Leaf color
Early plant vigor	Silk color
Leaf orientation	Tassel color
Silk date	Late season stay-green/appearance
Ear height	Root strength
Ear tipfill	Reaction to fungicides
Ear shape	Reaction to herbicides
Tassel size	Susceptibility to pathogens/pests
Dropped ears	Plant height
Stalk rating	Yield
Above ear intactness	Weediness Potential

THE ROLE OF FIELD TESTING IN TRANSGENIC PLANT DEVELOPMENT

There are three major components to field testing during the development phase of a commercial transgenic crop prior to final regulatory approval. Event selection and biological characterization of the crop are conducted to ensure that commercial levels of performance (i.e., efficacy) are attained and that agronomic performance of the varieties is maintained. Some field tests may be carried out with only a few plants in a single location, while others require much larger areas and multiple locations. Field testing of a new transgenic product usually begins within the first few generations after transformation. Limitations on the availability of seed usually restrict testing of any given event to a few rows and one or a few geographic locations. The trials are all conducted under USDA permits or notifications that place restrictions on planting locations and establish other performance standards designed to eliminate unintentional release and persistence in the environment. These performance standards also address areas such as shipping and storage of transgenic crop seed, and means to maximize confinement and minimize the possibility of pollen dispersion and gene flow. As the event selection process continues and additional seed is available, trials are expanded to slightly larger plots (e.g., four row strip plots) and more locations. The testing of an individual event is typically restricted to a relatively small area (cumulatively less than a few acres) for several generations. During this time, the sorting and selection of events is still occurring. As the event selection process narrows the number of choices for commercialization, the size of the field trials with the remaining events increases.

The safety of the event to humans, animals, and the environment is also evaluated concurrent with product development. The field portion of safety testing includes field studies to measure possible effects on non-target species as well as production fields to prepare seed and grain for various safety and characterization studies (e.g., animal feeding, compositional analysis, and processing studies) to fulfill regulatory and product stewardship needs. Most of the events that are not candidates for commercialization have been eliminated by this point in the development process. The regulatory and development costs associated with launching a new product onto the market (NAS 2000) require diligent selection of the events prior to completing these trials. Additionally, many of the studies require significant quantities of seed of the appropriate quality and zygosity. Other studies require seeds from multiple generations to measure genetic stability. Comparisons are usually made to isoline or germplasm sources that are considered equivalent to the transgenic material. Since the tissues utilized in transformation are not identical to elite

germplasm, several generations of back-crossing may be necessary before it is possible to perform direct comparisons between the transgenic and control plants. Thus, events must be well advanced into variety development in order to obtain meaningful results from field trials intended to characterize their safety or to fulfill regulatory requirements.

The third component of field testing includes variety development and the scale-up of seed production in anticipation of obtaining final regulatory approval. Eventually, the scale of evaluation and seed production may reach hundreds or thousands of acres. These trials are conducted under USDA permits as well as Experimental Use Permits (if the trait is pesticidal) from EPA until final de-regulation and registrations are granted by USDA and EPA, respectively.

FIELD TRIAL STEWARDSHIP

Proper field trial stewardship is an integral part of any field testing program. Proper stewardship is critical to ensuring that all field research with transgenic plants is conducted in a manner that meets the obligations of the Plant Protection Act (Table 1). A full-time, dedicated staff is responsible for managing the field trial process. Additionally, all researchers involved in conducting field trials are provided with training and documentation to ensure a high level of compliance with the rules and regulations governing the field trials. Compliance validation through audits conducted by dedicated biosafety personnel ensure strict adherence to release (planting) conditions.

Detailed compliance manuals, which describe how the trials are to be conducted, are provided to each field trial manager and cooperator. Information in the manual includes instructions on proper shipping and storage of transgenic seed and plant material. It also includes a description of all planting restrictions necessary to properly establish isolation (distance and/or time isolation) zones and buffer rows, bagging and detasseling instructions (when appropriate), procedures for destruction and disposal of viable tissues upon completion of a trial and monitoring the field for volunteer plants during subsequent field seasons. In addition, planting and monitoring reports are included to allow for documentation of all phases of the trial from receipt of the seed through post-trial monitoring and reporting of volunteers.

CONCLUSIONS

Developments in plant biotechnology over the next several years are likely to generate new commercial crop varieties with modified regulatory, metabolic, and signaling pathways. The path to commercialization is likely to closely follow the approach taken to develop today's products such as insect resistant and herbicide tolerant corn, cotton, and soybean.

Field testing of plants engineered with modified pathways will be necessary to evaluate the efficacy and safety of these new products. Typically, the field trials will begin with a few plants from several events planted in one or a few locations. As product development progresses, event selection will reduce the number of events while the size and number of tests of each remaining event increases. Safety evaluations will begin soon after the first field testing and will continue until human, animal, and environmental safety is thoroughly characterized and regulatory trials are completed. Proper stewardship of the field trials will need to follow the standards and practices that have been established during development of existing transgenic products.

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POSSIBLE PHENOTYPIC EFFECTS OF GENETICALLY MODIFIED PATHWAYS ON GENE FLOW FROM FIELD TESTS

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INTRODUCTION

A major goal of this workshop was to determine how new types of genetically modified (GM) pathways might affect the extent of gene flow that occurs during field trials. This requires an examination of current methods used to minimize gene flow from field-test plants, as well as speculation about the phenotypic effects of GM regulatory, metabolic, and signaling pathways that might affect the extent of gene flow. Few plants with these types of GM pathways have been developed to date, so it is difficult to generalize about their phenotypic characteristics. Some general questions about plants with GM pathways include:

- Will hormonal and developmental effects of GM pathways be more complex than those of plants that are nontransgenic or have other GM traits?
- Will some phenotypic effects of GM pathways change during the plant's development?
- Will these phenotypic effects vary a great deal due to variable environmental conditions in the field?

Characterizing the phenotypes of plants with GM pathways should involve consideration of these types of questions, especially if any of these phenotypic changes might affect gene flow.

Gene flow from GM plants can take place via the dispersal of pollen, seeds, and vegetative propagules, so it is appropriate to focus on phenotypic traits that could affect these features of a plant. For example, a GM plant that produces greater quantities of pollen or much longer-lived pollen than other types of GM plants may require greater precautions to minimize gene flow during field tests. Another hypothetical example would be a GM trait that results in longer seed dormancy, which would require monitoring field sites for volunteer progeny over longer periods of time than is necessary for other types of GM plants. If such differences are possible, we should also ask whether they can be predicted on the basis of preliminary studies of greenhouse-grown plants, prior to the field testing stage. These are some of the topics we discussed at the workshop. As background information for this discussion, I reviewed various plant characteristics that affect the extent of gene flow from field plots of GM crop plants.

BACKGROUND ON CONFINEMENT AND FIELD TESTS

Field tests of GM plants require prior approval from USDA-APHIS and stipulations about what measures will be taken to prevent gene flow to other plantings of the crop, feral crop plants, or sexually-compatible wild relatives. It is generally recognized that strict containment of all pollen, seeds, and vegetative propagules within the field test area is not always possible (e.g., OSTP 2002), so the term "confinement" is used instead of "containment." The goal of confinement is to minimize gene flow out of the field test plot to the greatest extent that is feasible for a given crop. To achieve this goal, one of the major considerations for each crop is the isolation distance that has been established for producing certified seed for seed markets (USDA 1994). With conventional plant breeding, seed companies and others use standard isolation distances to prevent pollen contamination, i.e., pollen from undesirable sources. Pollen from wild relatives or pollen from other varieties of the crop could compromise accepted standards for seed purity. Isolation distances vary a great deal among crops and are greatest for outcrossing species such as sun-

flower, squash, melon, corn, carrot, and many wind-pollinated grasses and trees. Crops such as soybean, rice, and wheat are primarily self-pollinated, so less isolation is needed to produce certified seeds.

Isolation distances such as those published by USDA provide useful guidelines for the relative extent of pollen flow between fields, but they are not intended to provide rigorous information about variable, long-distance pollen dispersal from a given crop. Although most pollen is deposited within a limited, predictable distance of the crop, a small fraction can often travel much farther. For example, a recent study of canola, which has high rates of self-pollination, reported very rare instances of pollen dispersal that were 2 – 3 km from the source (Rieger *et al.,* 2002). The occurrence of cross-pollination between different nontransgenic crop varieties has not been a concern in the past. However, in the context of field tests involving GM plants, extra care is taken to ensure that novel, unapproved transgenes do not enter the food supply or persist in the environment. It is likely that low levels of gene flow from GM field tests have already occurred because of rare, long-distance pollen dispersal (OSTP 2002). For the purpose of this discussion, however, I will not address the question of whether existing confinement practices are adequate. Instead, I will focus on how general characteristics of plants with GM pathways might enhance their ability to disperse pollen, seeds, and vegetative propagules.

VARIATION IN OUTCROSSING RATES

In botanical terminology, "outcrossing" has two definitions. Strictly speaking, outcrossing refers to the proportion of a plant's seeds that result from cross-pollination with other plants, as opposed to self-pollination. Thus, outcrossing rates are inversely proportional to selfing rates, so an outcrossing rate of 1.0 means that all seeds on the plant were sired by non-self pollen, and a selfing rate of 1.0 means that all seeds result from self-pollination. Species with separate male and female individuals, such as asparagus and holly, always outcross because they are incapable of self-pollination. Outcrossing rates are also high in species that have separate male and female flowers on the same plant (e.g. corn, squash). At the other extreme are the flowers of species such as soybean and rice, in which the male and female parts of the flower are so close together that self-pollination predominates. A second definition of "outcrossing" refers to the plant's ability to disperse pollen and sire seeds on other plants in the population. This is also known as the plant's male fitness or male reproductive success. These two definitions are related because plants that outcross a lot tend to produce much more pollen and sire more seeds on other plants than those that are primarily self-pollinated.

Before considering this second definition of outcrossing, we can ask whether plants with GM pathways could have lower selfing rates, and, if so, whether this would affect the extent of pollen-mediated gene flow from field-test plots. In my view, changes in selfing rate are largely irrelevant to gene flow from field tests to surrounding areas. Whether a GM pathway causes a plant to self-pollinate less than its non-GM counterpart is not important because the main question is how such changes would affect pollen *leaving* the plot. It is possible that receiving more incoming *wild* pollen would make crop seeds more likely to persist as feral plants, but this can be considered on a case-by-case basis. Therefore, the rest of my discussion about pollen-mediated outcrossing will focus on pollen dispersal away from the plot (i.e., variation in male reproductive success).

AMOUNT, DISPERSAL, AND LONGEVITY OF POLLEN

A GM pathway that causes the plant to disperse greater amounts of pollen than non-GM plants could lead to greater levels of gene flow from field trials. This could occur due to several mechanisms, including GM pathways that result in:

- More flowers per plant
- More pollen per flower
- More pollen per anther
- More anthers per flower
- Floral changes that increase the release and dispersal of pollen
- Greater exertion of anthers to expose them to air currents and pollinators
- Greater attractiveness of flowers to insects and other pollinators

- Greater attractiveness of flowers to long-distance pollinators such as honeybees

In addition, certain characteristics of pollen grains could affect their ability to disperse, such as their size and shape. For example, corn pollen has unusually heavy pollen grains compared to other monocots. Smaller, lighter pollen grains might disperse farther from the plant. In many species, such as corn and rice, pollen grains generally do not remain viable for more than thirty minutes or even shorter periods of time. If a GM pathway causes pollen grains to have greater longevity, it is possible that this also could result in longer distances of gene flow to plants outside the field test plot.

Some of these phenotypic effects would be easy to observe in greenhouse-grown plants prior to field testing. However, others may involve subtle changes that are very difficult to detect, even under field conditions. Plants with GM pathways could have multiple phenotypic changes during the plant's development, and these might have inconspicuous but important effects on gene flow.

An example of unexpected effects of transgenesis on gene flow is found in a study of male reproductive success in *Arabidopsis thaliana*, which is a diminutive weed and a model species for molecular biologists. Bergelson and Purrington (1999, 2000) compared different *Arabidopsis* lines with the same transgenic construct for herbicide resistance to see if these lines differed in the numbers of seeds they sired on other plants (Table 1). This research was carried out in small, outdoor experiments where syrphid flies were observed visiting the flowers. Nontransgenic *Arabidopsis* are highly selfing, but a small amount of outcrossing can occur when the flowers are visited by insects. The four transgenic lines in this study exhibited a great deal of variation in the number of seeds sired on other plants (Table 1). This variation among these lines might occur due to position effects of the insertion site or somaclonal mutations during the transformation process. Although the genetic and phenotypic mechanisms for these differences are not known, it is clear that the amount of pollen dispersed to other plants varied markedly among the transgenic lines. This illustrates how small, unidentified differences among transgenic lines potentially can have substantial effects on gene flow.

Table 1. Variation in male outcrossing in artificial populations of *Arabidopsis thaliana* from four transgenic events involving the same transgenic construct for herbicide resistance (pGH8). From Bergelson and Purrington (1999, 2000).

Type of Plant	Percent of seeds sired on other plants
"Normal", nontransgenic	< 1.0 %
Event 1, transgenic	12.4 %
Event 2, transgenic	1.3 %
Event 3, transgenic	8.6 %
Event 4, transgenic	1.9 %

While it may be useful to check for these types of phenotypic changes in plants with GM pathways, it is also important to note that other types of GM and nontransgenic plants could also have these features. For example, transgenic plants with higher yields might also have more flowers per plant, regardless of whether a GM pathway is involved. In summary, any transgenic plant may have greater potential for gene flow than its nontransgenic counterpart, although this is not usually expected. In the case of plants with GM pathways, the potential for enhanced gene flow during field tests should be considered if the GM pathway might affect the amount, dispersal, and longevity of pollen.

AMOUNT, DISPERSAL, AND PERSISTENCE OF SEEDS AND VEGETATIVE PROPAGULES

Gene flow also occurs when seeds or vegetative propagules (e.g., grass tillers, banana clones, strawberry clones) are dispersed out of the field test area and persist or reproduce. These types of feral and volunteer plants could then flower and cause a greater spread of the transgene via both pollen and seeds. For most row crops, vegetative propagation is unlikely to be important, although tubers from potatoes often persist as volunteer weeds (e.g., Boydston 2001). Seed dispersal and persistence is a much more common route of gene flow in row crops, especially since many species have long-lived seeds that can be dis-

persed over very long distances by people, wind, water, and animals. The seeds of most crop plants lack innate dormancy, which is more common in wild species, but dry or buried seeds can sometimes remain viable for several years.

In the context of field testing, it is usually recommended that fields where transgenic crops have been grown be monitored for volunteers for one or two growing seasons so transgenic volunteers can be killed. However, this precaution does not address the possibility that seeds from GM crops could disperse away from the plot that is being monitored and therefore escape detection. This may be especially difficult in large-scale field tests of more than ~5-10 acres in size. Even when efforts are made to prevent seed dispersal, strong winds and storm events potentially could carry seeds outside the plot.

As with pollen-mediated gene flow, seed-mediated gene flow could be enhanced by GM pathways if this leads to greater numbers of seeds per plant, greater potential for dispersal, or longer viability of seeds under field conditions. Thus, the extent of seed dispersal out of confined field tests could become greater due to:

- More seeds per fruit
- More fruits per plant
- Changes in seed shattering
- More seed shed prior to harvest
- Seed ripening more staggered, so harvesting is less efficient
- Greater dispersal by animals, wind, water, people

As with pollen-related traits, these types of changes are also possible with other types of transgenic and nontransgenic breeding.

CONCLUSIONS

Plants with GM pathways could exhibit many phenotypic changes that result in greater levels of gene flow from field testing plots, as discussed above. Therefore, it is appropriate to ask how much of an increase would be cause for concern? Also, could the potential for unacceptably large increases in gene flow be predicted or noticed prior to the start of the first field trial? These are difficult questions, especially given that the isolation distances and other confinement strategies that are currently used to minimize gene flow from field trials are unlikely to achieve complete containment of transgenes. This raises another challenging question, which is how much gene flow from regulated GM plants is acceptable? Without more precise knowledge of the amount of ongoing gene flow from field-testing plots of various sizes and in different geographic areas, it is hard to identify new concerns that are unique to plants with GM pathways. All of these questions deserve further consideration as we become more familiar with the phenotypic characteristics of plants with GM pathways.

In the meantime, on a very practical level, it seems likely that most plants with GM pathways will not exhibit dramatically increased levels of gene flow. Many of these plants will probably be similar to other types of transgenic and conventionally bred cultivars that exhibit small phenotypic changes in agronomically important traits. Although the phenotypes of these plants may be more complex than those of other types of GM plants, there are several opportunities to examine these changes. First, many GM plants are carefully observed in greenhouse trials that precede plans for field testing. Also, selected GM plants can be studied when they are grown on successively larger plots each season, while seeds are bulked for commercialization. Concerns about gene flow will be magnified when larger and more numerous field plots are proposed. GM plants that obviously produce much greater amounts of pollen and seeds or show greater likelihood of long-distance gene dispersal or persistence may be candidates for stricter confinement methods.

Some types of phenotypic alterations that affect gene flow could escape the notice of plant breeders, e.g., changes in pollen longevity or seed dispersal properties. Therefore, it is prudent to remain open-minded about unanticipated effects of GM pathways, and to encourage innovative research on these questions by researchers who are able to “think outside the box.” Previous experience and a fundamental understanding of plant biology, crop breeding, and ecology will undoubtedly be useful in this regard.

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A BIOLOGICAL VIEW OF FIELD TESTING DOMESTICATION TRANSGENES: FAMILIARITY AND SCALE PROVIDE HIGH LEVELS OF ENVIRONMENTAL SAFETY DURING FIELD TRIALS OF RMS[1] TRANSGENIC PLANTS

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SUMMARY

Transformation is the most powerful and precise tool of functional genomics because it allows the effects of specific genes on organismal phenotypes to be unambiguously determined. It also permits the vast databanks of gene sequences to be rapidly translated into new kinds of transgenic plants for research and commercial application. If innovation is not to be stifled, the diversity of transgenes, plant species, and traits in the genomics pipeline requires new classifications of risk that are based on biological principles. Most importantly is whether the transgene is likely to result in its own amplification via increased invasiveness in wild or feral populations after small-scale releases from confined trials. I propose several guiding principles, a classification scheme, and a risk-assignment flow chart to aid regulatory decision making.

INTRODUCTION

Compared to the first wave of transgenic traits—predominantly the pest management traits herbicide and pest resistance—RMS alterations differ in several ways that require a new, more discriminating approach to field trial regulation. First, many of the traits have more subtle and physiologically complex effects. The first wave of traits was largely gain-of-function genes whose action was virtually independent of plant metabolism. In contrast, RMS traits such as altered wood chemistry are likely to have subtle effects, whose expression interacts with genetic background and environment and has significant impacts on many facets of plant metabolism. Second, the phenotypic effects of RMS alterations will often require long-term growth under relevant (farm-like) environments for characterization. This is most clear in perennial crops such as trees where economic tissues (e.g., wood) or developmental behavior (e.g., flowering) take many years to express themselves, and do so very differently depending on growth environment. Thus, observing even the basic intended phenotypes is likely to require field trials, whereas laboratory or greenhouse studies often sufficed for initial characterization of the first wave of transgenic crops. Third, large genomics databases provide many avenues (i.e., many gene targets and methods of their modification) for influencing similar kinds of traits, and new transgenic methods of gene tagging provide many routes for random transgenic mutagenesis on a large scale to discover novel gene-trait associations. It is therefore desirable to analyze large numbers, and diverse kinds, of transgenic plants—which preclude contained studies in most species due to expense.

The need for field trials of diverse materials for RMS transgenes makes it important that regulatory requirements do not impose costly hurdles if not essential for environmental safety. Thus, intensive pre-release testing and metabolic characterization of each new transgene, as was common with the first wave of transgenic crops, would be likely to preclude a significant portion of functional genomic research and products, particularly from public sector researchers and small companies. Several biological considerations suggest that most of the RMS transgenes under development can be safely tested without intensive characterization or containment measures.

[1] RMS refers to plants with engineered regulatory, metabolic, and signaling pathways—the subject of the workshop for which this paper was prepared.

GUIDING PRINCIPLES FOR RISK CLASSIFICATION OF FIELD TRIALS

I propose several principles based on population genetic theory, breeding experience, and molecular biology that can serve as guides to help categorize risks of field trials.

1. Invasive Probability is the Key Determinant of Environmental Risk.
The main environmental concern at the field trial stage is from plants whose new traits are likely to cause a significant increase in fitness in wild or feral populations. It is biological amplification that enables the development of a substantial environmental impact from small field trial releases. As discussed below, most RMS transgenes are likely to have domesticating rather than fitness-enhancing effects.

2. Domestication Traits Greatly Reduce Risk.
Field tests of plants with engineered RMS pathways are largely based on modifications of native gene expression. These traits will therefore tend to be inherently domesticating because they move plants outside, or to the extremes, of the wild phenotypic distribution produced by balancing selection (Bradshaw and Strauss, 2001). Clear examples include plants with reduced stature or dwarfism that cannot compete for light in the wild; plants with complete or partial sterility that reduces dispersal potential; plants with highly modified tissue chemistry or structure that reduces pest resistance or tolerance of environmental stress; and plants with altered ripening whose hormonal signaling pathways are impaired in the ability to sense and respond to environmental and developmental cues.

3. Trait Familiarity Provides Safety Despite the Diversity of RMS Transgenes.
Because most wild and bred species show very large variance among varieties, the new traits that result from engineered RMS pathways will often be familiar in kind if not in precise phenotype or genotype. Thus, despite their diversity and less intensive characterization than the first wave of commercial transgenes, this class of traits is likely to be considerably safer at the field trial stage compared to novel, ecologically significant transgenes resulting from long-distance phylogenetic transfer (e.g., novel anti-pest toxins). For example, varieties and wild populations of plants vary widely in lignin content (Boudet, 2000) and are the subject of conventional selection in many species. In some cases the same genes that were modified via transgenesis were found to also be the cause of commercially significant variation in conventional breeding programs (Sederoff *et al.,* 1999).

4. Developmental and Ecological Complexity Defies Metabolic Predictions of Environmental Risk.
Methods such as metabolic profiling and microarray analysis are useful for monitoring changes in physiological health of transgenic organisms. However, because of the complexity of fitness and invasion potential at organismal, population, and ecological levels, there are unlikely to be any useful predictive methods provided by metabolic profiling in the foreseeable future. Even traits directly related to invasive potential themselves usually provide only modest predictors of invasion of exotic species (e.g., Reichard and Hamilton, 1997)—and the challenges to biological interpretation and prediction from even highly focused microarray experiments are great (Lockhart and Winzeler, 2000; Gifford, 2001). The cost of the extensive studies required given the diversity of environments, varieties, and developmental stages that would need study is also daunting—likely requiring several million dollars per transgenic product. Environmental risk and thus regulatory decisions would be most accurate and cost-effective based on population genetic principles and trait assessments, with required monitoring in uncertain cases.

5. Pleiotropy is Abundant both in Conventional Breeding and Genetic Engineering of RMS Traits, but Does Not Constitute an Environmental Risk at the Field Trial Stage.
In this context pleiotropy refers to a genetic alteration that is intended to affect one trait having unintended effects on other traits or metabolic processes. Pleiotropy must be carefully analyzed to avoid yield drag and increased pest susceptibility as fundamental physiological processes such as lignin deposition and hormone reception are engineered toward domestication goals. It can be viewed as a physiological disturbance, whose effects are far more likely to be enfeebling than to promote fitness and thus invasive potential

(see principle 1). Pleiotropy therefore does not represent a significant environmental risk at the field trial stage.

6. The Limited Scale of Release from Field Trials is a Major Safety Buffer for RMS Transgenes. For a transgene to invade and thus have a significant environmental consequence it must overcome the huge numerical obstacle provided by extant wild and domesticated gene pools (Strauss *et al.,* 1999). Because most RMS transgenes are expected to be deleterious, neutral, or only mildly beneficial (see above), their spread will be mostly determined by genetic drift (Li and Graur, 1991). The probability of fixation is thus a function of its initial frequency (approximately the inverse of twice the effective population size of the wild/feral species), which should be extremely low ($<< 10^{-6}$) from confined field trials.

A PROPOSED BIOLOGICALLY BASED CLASSIFICATION SYSTEM FOR REGULATORY DECISIONS ON FIELD TRIALS OF TRANSGENIC PLANTS

As discussed above (see principle 6), the scale of release is important to environmental risk (Table 1). Most early stage field trials of RMS genes are very small ("type 1") and exploratory, and thus unintended releases will also be very small.[1] The large majority of these trials will not go beyond this stage toward commercial development. On the other hand, large scale trials (type 2) are generally carried out by industry and generally signal the intention to eventually commercialize a new trait. Their scale and intent signal more attention to environmental risks, including the possibility that strongly domesticating genes could negatively impact nearby small-scale populations of interfertile species or wildlife. These issues are generally required for consideration by regulatory agencies before permits for large trials are issued.

Table 1 also categorizes transgenes by their biological and ecological novelty. They propose that RMS transgenes, the subject of this symposium, are inherently low risk compared to two other

Table 1. Proposed categories of risk for large- and small-scale transgenic field trials.

Containment Level	Type 1 Field Trials (Exploratory) <10 acres	Type 2 Field Trials (Pre-Commercial) >10 acres	Examples
High	A / B: Biologically and physically contain - detailed monitoring		Pharma
Medium	C: Confine, basic data	D: Confine, detailed data	Novel anti-herbivore cmpds. & herbicide resistance genes
Low — Domestic.	E: Exempt	F: Confine, basic data	RMS genes
Low — Fitness?	Confine, basic data	Confine, detailed data	

Basic data = Degree of confinement via general observation;
Detailed data = Vigor, fitness, spread, non-target effects

classes of genes for which major commercial efforts (or uses) are underway (discussed under principles 2 and 3). For RMS genes that have a clear domesticating phenotype (see principle 2) and are in a small-scale trial, the degree of environmental safety seems sufficiently high that such trials could be *exempt* from regulatory oversight to reduce costs and terrorism risks. A similar principle would apply where a large-scale random mutagenesis experiment is carried out (many mutants, each on a small scale), as new mutants virtually always have neutral or reduced fitness compared to wild type.

The flow diagram in Figure 1 shows how an experiment might be categorized with respect to the risk levels shown in Table 1, and thus containment or confinement goals. RMS transgenes are shown under item IV and below on the chart. For RMS projects that attempt to improve traits like abiotic stress tolerance, more scrutiny may be required to determine if the traits provide a substantial improvement in the wild, or if they only apply to cultivated fields or are inherently domesticating due to unfavorable pleiotropic effects on traits important to wild fitness (e.g., rate of growth). Adaptation to stress in wild plants often involves highly complex, multifactor responses to several stresses, in contrast to the simple transgenic modifications being sought (Iba, 2002), suggesting that substantial fitness benefits for wild plants will be rare. The tables also suggest where obtaining data on confinement/fitness might be warranted, versus simple observations

[1] The definition of "small" and "large" scale field trials (Table 1) as below and above 10 acres is arbitrary; other cutoffs could be appropriate depending on the gene and environment.

to make sure confinement factors have operated as expected.

CONCLUDING REMARKS

The United States National Research Council has twice issued major reports that identified the new traits, rather than the method of production, as the key factor for consideration of risks of transgenic plants (NAS 1987, NRC 2000). Until recently this distinction was mostly academic as there were very few transgenes and most conferred novel, or at least uncommon, phenotypes and physiological mechanisms. Genomics is changing this scene significantly. It is allowing breeders to generate similar traits to those sought conventionally, but often to do it more precisely or efficiently by targeting the underlying genes. These kinds of RMS traits—particularly those which impart domestication phenotypes—would seem to require far less oversight by government regulators, *if any*. On the other hand, transgenes that effectively introduce a novel physiological mechanism such as a new secondary compound pathway (Tattersall *et al.,* 2001), might be of more concern than the simple, single gene pest management traits that have been the mainstay of the first wave of commercialized transgenic crops.

If field testing regulations are to protect the environment while allowing the innovative use of genomic information via transformation, they will need to explicitly address the very different levels of environmental risk expected from the cornucopia of transgenes coming through the genomics pipeline. The failure to do so may frustrate attempts to leverage the major public investments in plant genomics for public good via crop improvement.

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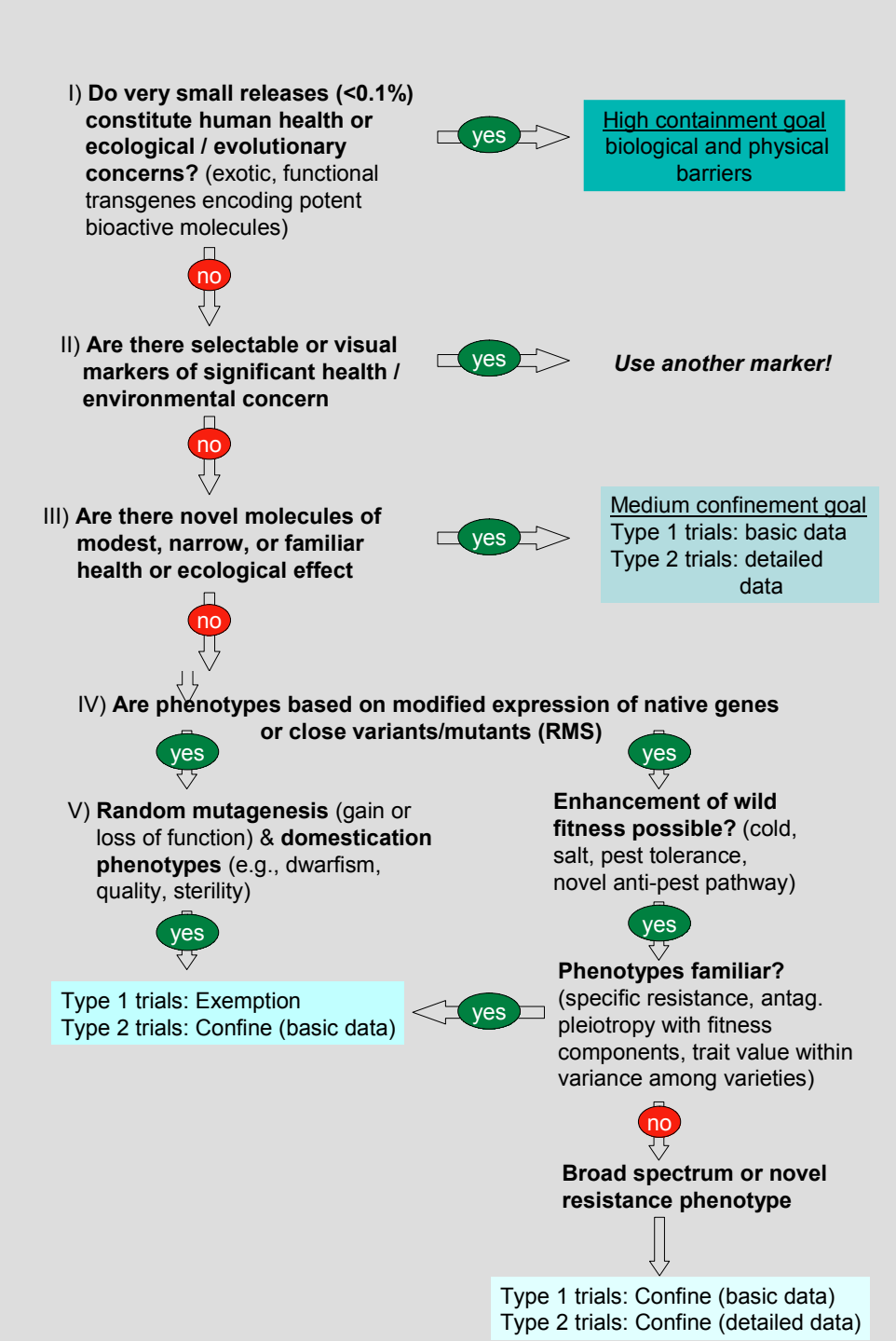
Figure 1. Decision tree to guide risk level assignments (Table 1) for transgenic field trials.



METABOLIC ENGINEERING OF FATTY ACIDS AND SECONDARY EFFECTS

John Ohlrogge
Michigan State University

Vegetable oils are a major commodity with a well-developed commercial infrastructure for production and use, supplying both food and industrial needs. World vegetable oil production from soybean, palm, rapeseed (canola), and other crops is over 100 million metric tons per year and is valued at approximately US$50 billion in annual oil sales. The primary use for vegetable oils today is in the food industry and includes salad oils, margarine, and oils used in frying and baking. In most plants, the same five or six fatty acid structures that are also found in the phospholipids in the cell membranes occur in the triacylglycerols found in seeds. These 16- and 18-carbon fatty acids (primarily palmitic, oleic, and linoleic acids) are the major constituents of the vegetable oils that are consumed as foods.

In addition to food uses, about 30% of vegetable oils produced today are used by the oleochemical industry for hundreds of products such as soaps, detergents, paints, lubricants, and polymers. In many cases, the fatty acid composition of the oils used for these applications differs from that found in edible oils, and these different structures lead to special applications. For example, the tropical oils from coconut and palm kernel are rich in lauric acid, which is a 12-carbon saturated fatty acid. The properties of lauric acid lead to a balanced solubility in water and oil, which makes it ideal for production of soaps and detergents. As a result, the United States imports up to US$400 million of these tropical oils for use largely in soap and detergents. Thus, although these oils are edible, their major use is not for food.

In this presentation, I will review two examples of genetic engineering of plant oils that have led to commercial products. The first involves down-regulation of an endogenous fatty acid desaturase gene from soybean. The second case involves introducing a thioesterase gene from a wild plant species (California Bay tree) into canola to produce an oil with very different composition than available in canola.

HIGH-OLEIC SOYBEAN OIL: A GENETICALLY ENGINEERED PRODUCT WITH CONSUMER BENEFITS FOR BOTH FOOD AND NONFOOD USES

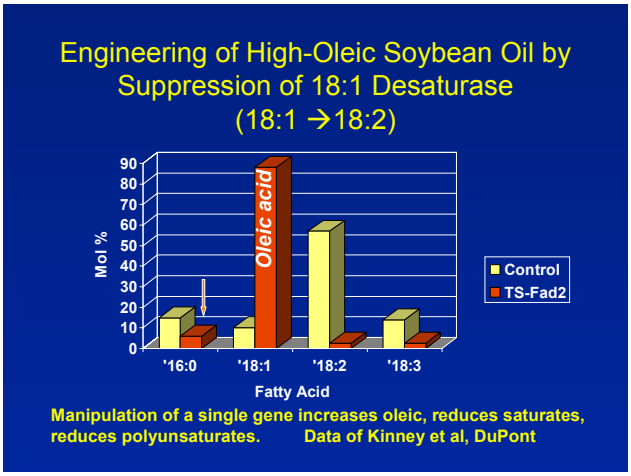
Soybeans are the largest source of vegetable oils in the world, and in the United States soybean oil accounts for about 70% of vegetable oil consumed. Most soybean varieties produce an oil rich in polyunsaturated fatty acids (about 50% linoleic acid or 18:2 and 10% linolenic acid or 18:3), and these fatty acids make the oil unstable and easily oxidized. When heated, the oil develops objectionable flavors and odors. Thus, unprocessed soybean oil is unsuitable for many applications, and for most edible uses it is chemically hydrogenated. This process adds to the cost of the oil and also introduces side reactions such as conversion of double bonds from the *cis* to *trans* configuration creating *trans*-fatty acids.

The biosynthesis of polyunsaturated fatty acids in plants is catalyzed by a series of enzymes with the first step carried out by an enzyme that converts oleic acid (18:1) to linoleic acid (18:2). In 1994, the gene (*FAD2*) for this enzyme was isolated in *Arabidopsis* by screening mutants generated by T-DNA insertions. Shortly afterward, molecular biologists at DuPont succeeded in isolating and suppressing the expression of the gene in soybean. This strategy led to a major decrease of the 18:1 fatty acid to 18:2 conversion step and almost completely eliminated polyunsaturated fatty acids in the soybean oil (see Figure 1).

The new transgenic soybean oil has 85% oleic acid, one of the highest oleic acid contents found in nature. The absence of polyunsaturated fatty acids eliminates the need for hydrogenation to stabilize the oil. Furthermore, an unanticipated

benefit of the oleic increase was that the saturated fatty acid content of the oil fell from approximately 15% to less than 8%. The new soybean oil has a composition similar to olive and other high-oleic oils, which are considered to provide health benefits, compared to other plant and animal oils. The fatty acid trait was stable in field trials, and the oil yield of the crop was identical to the control lines. Thus neither the transformation process nor the major change in fatty acid composition was detrimental to the high yield of the soybean line. This example is also instructive because it demonstrates how quickly some discoveries can be translated into new crops. With the resources of a major corporation, genetic engineers only needed five years from gene isolation to a field-tested transgenic soybean crop ready for commercialization as an industrial product.

Figure 1. Genetic engineering of fatty acid composition in soybean by suppression of the oleate desaturase.

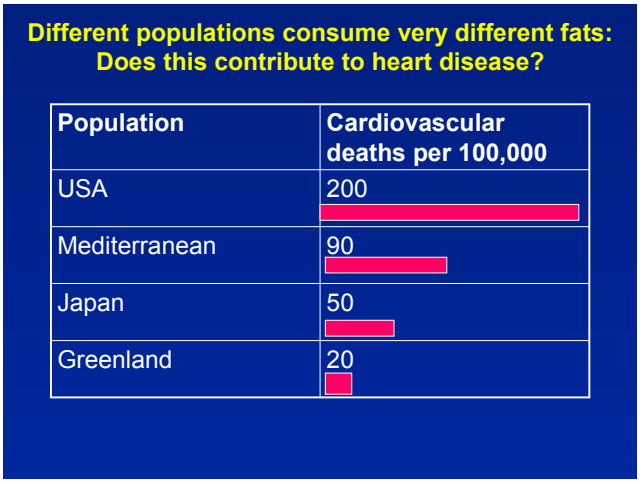


POSSIBLE HEALTH BENEFITS OF HIGH-OLEIC SOYBEAN OIL

Current medical understanding indicates a strong impact of dietary fatty acids on cardiovascular disease and human health. Consequently, there is much interest in tailor-producing healthier vegetable oils, and such products may help to balance consumer opposition to "GMO" foods. As mentioned above, health concerns regarding vegetable oil-derived foods include the presence of saturated (particularly palmitic) and *trans*-unsaturated fatty acids. Industrial hydrogenation increases saturated fatty acid content and also results in production of *trans*-isomers of unsaturated fatty acids that are normally not found in vegetable oils and have been associated with coronary heart disease. For many food applications, vegetable oils with a reduced amount of *trans*-unsaturated and saturated fatty acids are desirable to improve human health. The transgenic soybean oil composition shown in Figure 1 provides these benefits in a crop that provides the major source of fatty acids in American diets.

One added consumer benefit to wide future use of the engineered high-oleic oils may be reduction in the pathologies associated with high omega-6 fatty acid consumption. In recent years, evidence has accumulated that the balance of omega-3 and omega-6 unsaturated fatty acids in diets influences a wide range of human physiological responses including coronary heart disease (CHD). The dominance of plant oils with high omega-6 18:2 in many diets has led to omega-6/omega-3 consumption ratios near 10:1 whereas populations that consume ratios near 1:1 (e.g., Greenland, Japan) have strikingly lower incidence of CHD. These different diets may be associated with the very different CHD levels shown in Figure 2.

Figure 2. Deaths from Cardiovascular Disease in various regions.



Non Food Benefits: Vegetable oils have long been known to have useful properties as lubricants, and, because they are biodegradable, are ideal for applications where harm to the envi-

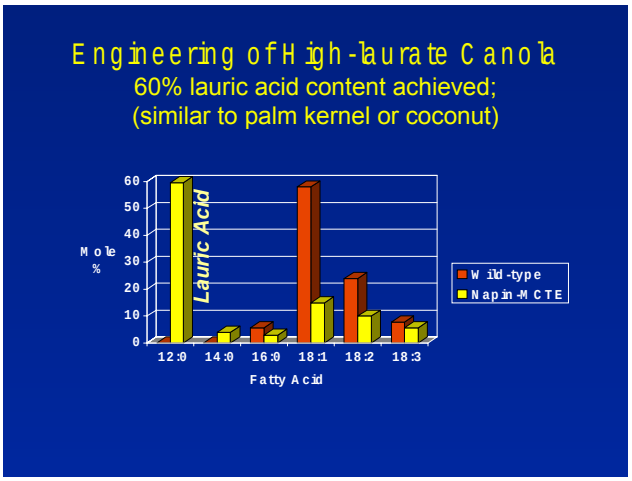
ronment must be avoided. However, the tendency of the oils to break down or polymerize as a result of oxidation limits their use. With its very low polyunsaturated fatty acids, high-oleic soybean oil has an oxidative stability more than 10 times greater than most vegetable oils. As a result, it can substitute for mineral oil in many applications such as marine engines, chain saw lubricants, and other applications where oil spills are particularly damaging. A number of other industrial applications may also become possible, because chemical additions to the double bond can lead to polymers and other products that have desirable properties for certain plastics.

High-Lauric Canola Oil: A Success Story in Genetic Engineering of Oils and a Test Case for Secondary Effects

The first commercial product to result from changing the composition of a plant seed via genetic engineering is high-lauric-acid canola oil. Lauric acid is a 12-carbon saturated fatty acid found at a high level in tropical oils, such as coconut and palm kernel. However, until recently much skepticism existed about whether temperate crops could be genetically engineered to produce the same high level of this 12-carbon fatty acid instead of the 18-carbon fatty acids normally found in temperate oilseed crops. Scientists at Calgene, a California biotechnology company, discovered the biochemical pathway responsible for lauric acid synthesis. They used extracts of seeds from the California Bay tree; like the tropical trees, these seeds also accumulate high levels of lauric acid-containing oils. Calgene researchers cloned the gene for the critical acyl-ACP thioesterase enzyme in the pathway, introduced this gene into canola, and dramatically changed the spectrum of fatty acids produced in the canola seeds (Fig. 3). In 1995, industry achieved the first commercial production of a genetically engineered oil by extracting 500 tons of oil from canola seeds engineered to produce an oil with 40%–50% lauric acid.

Secondary Effects: An unexpected lesson learned from study of the laurate producing transgenic plants described above was that high level production of novel fatty acids can induce a futile cycle of fatty acid synthesis and degradation (Fig. 2). By analyzing hundreds of independent transgenic lines, workers at Calgene observed that laurate production in canola seeds

Figure 3. Genetic engineering of canola oil that is high in lauric acid, a fatty acid with 12 carbon atoms. By introducing a single gene from the California bay tree, the canola oil was changed from containing 60% oleic acid to 60% lauric acid. This new canola oil resembles the oil found in coconut and oil palm.

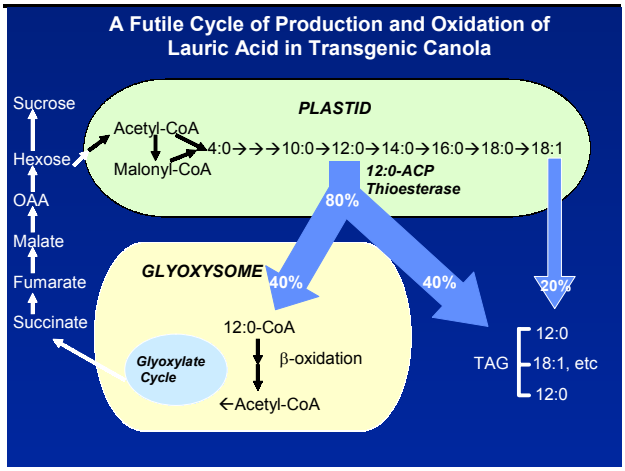


increased linearly up to about 35 mol% with increased lauroyl-ACP thioesterase expression. However, to achieve 58 mol% laurate required 10 fold higher levels of the introduced enzyme raising the question about what limits higher laurate accumulation. Michigan State researchers examined these high laurate canola seeds and found that enzymes for medium chain fatty acid beta-oxidation were increased several fold, as were malate dehydrogenase and isocitrate lyase, which participate in the glyoxylate cycle for fatty acid carbon re-utilization. These and other results led to the conclusion that high production of unusual fatty acids in transgenic hosts can induce pathways for their breakdown. Surprisingly, seed oil yield was not reduced which led to the additional discovery that the FAS pathway was also induced, presumably to compensate for the loss by oxidation of medium chain fatty acids.

Microarray Analysis: Microarray technology can be a particularly useful diagnostic tool to analyze transgenic plants during the development of commercially valuable products such as high-laurate canola. The introduction of a single transgene may have far reaching consequences at the level of metabolism and ultimately at the

level of transcription that need to be understood before commercial production of many products

Figure 4. Proposed futile cycle which occurs when transgenic canola produce high levels of lauric acid. Up to 50% of lauric acid produced in plastid may be subject to beta-oxidation because the capacity of acyltransferases or other enzymes involved in oil synthesis cannot accommodate very high lauric acid production.



in a transgenic plant is possible. Microarrays may provide the diagnostic means to uncover futile cycles and other possible secondary effects in transgenic plants that will have to be addressed to realize the potential of crop metabolic engineering. As shown in the table below, our microarray analysis of high-laurate developing canola seeds demonstrated that isocitrate lyase and malate synthase were both induced at the mRNA level, in agreement with the earlier biochemical studies. It is important to note, however, that 98% of the genes measured showed no changes in expression of greater than 2.5 fold. Thus, despite the very major changes in metabolism in these seeds, only a very minor fraction of genes changed expression.

Microarray analysis of high-laurate canola.

Protein	Expression Ratio (high laurate/control)
Glyoxylate cycle:	
Isocitrate lyase	4.5
Malate synthase	3.1

Conclusions

Over 95% of the transgenic crops that are planted for commercial production have been genetically engineered to provide either herbicide or pest tolerance. These traits represent the first phase of crop metabolic engineering. The traits now available in most transgenic crops provide farmers with either lower costs of production or higher yields or both. For example, glyphosate or other herbicide tolerant crops require less overall herbicide use, and often have higher yields due to less competition from weeds.

Phase two of plant metabolic engineering is just beginning but can be expected to have an even larger eventual impact on agriculture than phase one. Phase two can be considered the engineering of plants, not for higher yields, but to provide new or improved products or more complex traits. This second phase promises to have a larger economic impact on agriculture because it will provide farmers the opportunity to produce higher-value products for new markets. The genetic engineering of fatty acid composition of oilseeds has provided the first example of successful modification of the major components in plant seeds. These new engineered plants, such as high-oleic soybean oil described above, may provide new health benefits to consumers from GMO food. Oil modifications efforts such as the development of high-laurate canola have also shown that major changes in seed fatty acid composition and introduction of novel traits can at least sometimes be obtained without loss of yield or crop performance. These metabolic engineering strategies sometimes may lead to secondary effects on metabolism and gene expression. As briefly described above, *Arabidopsis* cDNA microarrays can be used to study gene expression in transgenic rapeseed and these studies show that the great majority of genes change very little despite major changes in metabolism. Thus, microarrays are useful to diagnose results of metabolic engineering experiments and can provide reassuring information regarding the overall stability of seed metabolism.

ENGINEERED CHANGES IN ETHYLENE SIGNAL TRANSDUCTION PATHWAYS

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INTRODUCTION

The purpose of this presentation is to illustrate that manipulation of complex signal transduction pathways in plants can indeed lead to significant and unpredicted alterations in plant growth. However, we should not consider these potential alterations as problems since in virtually every case the plant is severely compromised in its ability to survive. While it is relatively easy to engineer profound alterations in growth of a plant, it is exceedingly complex to do so in a way that improves the vigor of that plant.

THE EXPERIMENTAL SYSTEM

The principal pathway on which we focus is that of ethylene signal transduction. Ethylene is an extremely important phytohormone that plays critical roles in many aspects of development (Abeles *et al.,* 1992). It also is a key mediator of both biotic and abiotic stresses. When a plant is stressed in any number of ways, it produces ethylene. This ethylene is a signal to the plant to mount defenses appropriate to the signal. For example, ethylene is critical in pathogen defense where it induces many defense genes. In terms of development, most people are familiar with its effects on ripening of climacteric fruits and in fact a number of products involving tomatoes, some of which I have been involved with myself, have been deregulated. Although it was technically successful in tomato, economically it was not. This technical success likely indicates that ethylene inhibition will be commercialized in the future in other species, such as tropical fruits with short shelf lives, where it makes much more economic sense. In addition to effects on ripening tomato, ethylene is very important in many aspects of flower development, and, there again, we are likely to see commercialization of ornamentals in the fairly near future. Ethylene controls floral abscission in many different plant species such as petunia, geranium, impatiens, and carnation. It also influences the ability of the plant to senesce.

We have engineered multiple plant species to either produce less ethylene or not respond to it. The plants we have produced are altered in the desired traits. We have produced plants with fruits that have greatly extended shelf lives. We have produced ornamentals with flowers that last much longer than their nontransgenic controls. But we have discovered along the way that the plants that we have engineered are altered in their abilities to respond to a number of environmental cues. Some of those alterations were predictable and some of them were not.

When we think about biotechnology and ethylene, two complementary technologies come to mind; one technology revolves around blocking the ability of a plant to produce ethylene and the other involves preventing the plant from responding to it. Most of the deregulated products address ethylene synthesis. The two technologies are somewhat complementary and which one makes more sense is determined by the commercial target. For example, one does not want to make tomato fruits that never respond to ethylene because they will never ripen. Rather, the target that makes sense is a fruit that makes very little or no ethylene but is capable of responding to it when a ripe product is desired. You want it to ripen at the appropriate time. Thus, the emphasis has been to target the synthesis pathway. If we block synthesis, the fruit doesn't ripen. When we add ethylene back, we get a fruit that ripens. On the other hand, a product such as a flower never needs to see ethylene, and it makes more sense to knock out the ability to ever see ethylene; thus, a non-senescing flower.

In terms of controlling ethylene synthesis, there are multiple approaches and all of them work (Oeller *et al.,* 1991; Hamilton *et al.,*1990). We have evaluated most of them and made tomatoes

that do not ripen or overripen. We have used antisense technology to shut down the two enzymes involved in ethylene synthesis, ACC synthase and ACC oxidase. We have also over-expressed a bacterial enzyme that we isolated from a soil *Pseudomonas*, ACC deaminase, which degrades ACC, shunting it away from ethylene synthesis (Klee *et al.,* 1991).

The ability of the plant to respond to ethylene can be manipulated in a number of different ways. We have focused principally on the *Arabidopsis thaliana* ethylene receptor, *ETR1*, where we have mutations that change the form of the receptor so that it cannot see the ethylene (Wilkinson *et al.,* 1997). These mutations are dominant, meaning that we can take the mutant gene from *Arabidopsis* and put it into a variety of different species to make plants that no longer see ethylene. We have also evaluated genes that are downstream of the ethylene receptor in the signal transduction pathway, including *EIN2* and *EIN3* (Tieman *et al.,* 2001). With these two genes, their products must be removed via either antisense or co-suppression technologies to achieve ethylene insensitivity. Removal of either of these steps in signaling effectively controls the ethylene response. So the bottom line here is that there are multiple approaches to control ethylene synthesis and perception, and to some degree, they all work…sort of.

Although we readily accomplished our stated goals—delayed ripening of fruits and long lived flowers—there were unintended and unpredicted consequences. The first indication surfaced during field studies of ACC deaminase-expressing tomato lines in Florida. At a fairly mature age, the transgenic plants succumbed to *Fusarium* crown rot. This was not a major problem since there are effective resistance genes that can be bred in to eliminate this problem, but the result was completely unpredicted. We saw enhanced disease susceptibility in the transgenic line, and we still do not fully understand the molecular basis for this enhanced susceptibility.

We have subsequently spent much effort characterizing ethylene insensitive tomato and petunia lines. Here, we have used both transgenic and nontransgenic plants to evaluate the consequences of ethylene insensitivity. An important point to make is that the transgenic lines behave exactly like the nontransgenic ethylene insensitive line of tomato, *Never ripe* (Lanahan *et al.,* 1994). *Never ripe* is a point mutation, a single nucleotide change in the tomato genome. It was identified in a commercial field in 1950 (Rick and Butler, 1956) as a mutation in a single nonripening fruit in a field of ripe fruits. We identified a single nucleotide change that confers ethylene insensitivity to the tomatoes throughout the entire plant. The transgenic plants were made essentially doing the exact same thing—taking a receptor, changing a single nucleotide that makes that an ethylene insensitive receptor, and putting that into the plant.

When we evaluated the ethylene insensitive lines of both tomato and petunia, we again saw unexpected consequences. An excellent example of unintended consequences was the observation that the insensitive lines do not make adventitious roots (Clark *et al.,* 1999). We discovered this while attempting to propagate the lines through cuttings. We discovered that the ethylene response is absolutely needed for making adventitious roots in both species. While adventitious rooting is not important for tomato, it is extremely important for many crop species that are vegetatively propagated. The dogma for years was that auxin is the "rooting hormone." Horticulturists have for years applied auxin powders to stem cuttings to induce rooting responses. A role for ethylene in this phenomenon was not predicted. We also observed that roots of ethylene insensitive plants have difficulty penetrating heavy soils. Ethylene must be important for facilitating root penetration. In soft soils this is not an issue. But in a more challenging real-world environment, the ethylene insensitive plants would be at a severe handicap in terms of competition.

In parallel with tomato, we have been working with the model ornamental species, petunia (Wilkinson *et al.,* 1997). Here, constitutive expression of the mutant *Arabidopsis* ethylene receptor (i.e., everywhere in the plant) results in flowers that last substantially longer; the average is about 9 to 10 days after pollination versus two days in controls. This is a remarkable increase in flower longevity that would be highly desirable to home gardeners and landscapers. They look wonderful. We have a product everyone will want in their yards. Then we asked a simple question: How do these plants perform horticulturally? Will the plants actually thrive in the real world? Unfortunately, the answer is, probably not. In petunias, we saw disease

problems. Our transgenic plants in the greenhouse succumbed to *Botrytis*. This organism is not normally a significant pathogen in petunia but it is to the transgenic plants. *Botrytis* is an opportunistic pathogen. Transgenic plants also exhibit the rooting problems observed in tomato. This is a significant problem in petunia because many of the commercial varieties are vegetatively propagated. These problems clearly rule out this iteration of ethylene-insensitive plants as products.

As part of the overall evaluation of transgenic plants, we examined petunia seed vigor. While it is not a fleshy fruit, petunia, like tomato, is a solanaceous plant. The seed capsule is the equivalent of a tomato ovary. As in the ethylene-insensitive tomato, the ovary of the transgenic petunia is much slower to develop, which had a very significant consequence on seed maturation and subsequent germination. Seeds from the ethylene insensitive lines germinated very poorly. This could be overcome by applying gibberellic acid, but the transgenic lines would not germinate very well by themselves. Again, this is a significant unintended consequence of engineering mutations in the ethylene signaling pathway, but one that clearly would impair the ability of the transgenic plants to survive in nature.

Clearly, if we consider unintended consequences of transgene manipulation in the context of escape, the issue becomes the ability of the engineered plant to compete in the wild. In our specific case study, problems with germination, rooting, and disease susceptibility would strongly suggest that those plant are not going to do very well. In fact, ecophysiologists have examined the ability of transgenic ethylene-insensitive tobacco plants to compete and found that they indeed do extremely poorly (R. Voeseneck, personal communication). If you germinate them separately and just measure growth rates, you see that they grow quite well in monoculture. In the mixed culture, they just do not compete.

CONCLUSIONS

How do we get beyond this stage of development? We believe that the answer is targeted expression. Our current efforts are aimed at identifying transcriptional promoters that give us the tissue and temporal specificity for engineering ethylene responses. We needed to do the field studies to evaluate the current generation of plants. We then return to the lab and fine-tuned the product to perform better. Some of the unintended consequences were obvious with extensive greenhouse evaluations. But the hampered fitness of the transgenic lines was obvious in the real world. This is and must remain an integral part of the evaluation process. We have not produced super-weeds. We have produced the opposite—plants that are at a severe handicap in the real world. The bottom line to me is that we have engineered a complex pathway. It is naïve of anyone to think he is going to engineer a signal transduction pathway that has evolved over the past 50 million years and expect to produce a super plant. Our research is a good example of this. We can manipulate ethylene pathways but the plants are worse off. The plants are clearly unable to compete in a wild environment. I believe that this decrease in viability is going to be the norm as we move beyond simple single gene traits such as insect and herbicide resistance. To engineer a plant that is altered in yield or even in its response to environmental stresses will be a real challenge that will keep us quite busy for the foreseeable future.

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PLANT HORMONES, COORDINATION OF DEVELOPMENT, AND INTERACTIONS AMONG SIGNALING PATHWAYS

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INTRODUCTION

Most plant hormones are small organically-based compounds that work at low doses to elicit a variety of responses. Because they can have profound effects on growth and development, it is essential that we understand the molecular basis of how a hormone is converted into a cellular response, as these compounds are excellent targets for biotechnological manipulation. With the advent of genomics and, in particular, complete sets of mutant knockouts lines and global transcript profiling, we are entering a stage in which all the genes involved in these pathways will be known. Although this information has now been applied to genes that modulate hormonal responses, the information indicates that the mechanisms by which these compounds work involve complicated networks of cross interaction, which will need to be dissected if we are to have any success.

"THE GREEN REVOLUTION" YESTERDAY

Although many groups are now trying to manipulate genes that regulate hormonal responses in plants, historically the biological properties of these compounds have been selected for in traditional breeding programs. One of the best known examples of a biotechnological application of a plant hormone response was Dr. Norman Borlaug's "Green Revolution" breeding program of the 1950's (Peng *et al*., 1999). During this era, it was calculated that if food production did not increase, a large portion of the developing world would starve within 20 years due to exponential population growth. Borlaug and a team of breeders were given the task of improving crop yields in the developing world, and, with selective breeding programs in wheat and rice, this group doubled production over a 20-year period. For this achievement, Norman Borlaug was awarded the Nobel Peace prize in 1970. To put this accomplishment in perspective, presently 600 million hectares of wheat are grown worldwide. If we were to limit ourselves to the same wheat strains that were available in 1965, we would need an additional 850 million hectares of land to grow the same amount of wheat (Borlaug, 2000). The scientific breakthrough of "the Green Revolution" was the selection for semi dwarfed varieties with strengthened plant stems, which in turn made them more resistant to adverse weather conditions. However, although the developmental changes were obvious, the mechanistic basis of Borlaug's breeding program was unclear. Borlaug had succeeded, but little was known about what gene combinations created these semi-dwarfed varieties. Thus, there was no foundation to reproduce these effects in other plant species.

This changed in the 1990's when Nick Harberd's group at John Innis Center at Norwich, England, using the model genetic plant *Arabidopsis thaliana*, cloned the genes that Borlaug's team had been selecting for during the Green Revolution (Peng *et al.,* 1997, Peng *et al.,* 1999). Subsequent work demonstrated that these genes were involved in gibberellic acid (GA) signaling. GA is a small steroid-like plant hormone, which is required for cell elongation in plants. When GA action is inhibited, plants grow as semi dwarfs. At a mechanistic level, the Borlaug genes were shown to be encoded transcription factors that repressed GA action.

Interestingly, one rice variety that was resistant to Borlaug's breeding program was basmati rice. However, with the availability of a dwarfing gene, it was now possible, in principle, to inhibit the same gene in basmati rice. When done, this led to the production of new semi-dwarf varieties of basmati rice (Peng *et al.*, 1999). Although this example shows the power of molecular genetics to speed up traditional plant breeding, it also brings up another issue. In principle, aside from the

method of production, GMO-generated basmati rice is genetically identical to Borlaug's wheat and rice varieties. However, if the same level of regulation on GMOs was applied to Borlaug's semi-dwarfed varieties, we would have never had the Green Revolution. Many more people in the developing world would have starved, while we in the developed world were regulating.

HORMONE SIGNALING, TODAY

As can be seen from the above example, molecular genetics can circumvent many of the resource problems inherent in traditional breeding programs in a more laser-like, efficient way. However, it requires that we fully understand the molecular basis of the genes we want to manipulate. This has now been applied with great effort to another hormone with biotechnological potential, abscisic acid (ABA). ABA plays a major role in protecting plants against drought and other environmental stresses such as cold and salt stress. Thus, manipulation of ABA signaling may allow the expansion of where and when we can grow crops. The approach used to identify genes involved in regulating ABA responses, again, has used the model plant *Arabidopsis* (McCourt, 1999). One gene, identified through genetic screens and designated *ERA1*, is required to attenuate the ABA response of the plant (Cutler *et al.,* 1996). By inhibiting the *ERA1* gene, it is possible to increase the ABA response of the plant, which in turn increases the plant's drought avoidance (Pei *et al.,* 1998).

The success of the *ERA1* gene in improving drought avoidance in higher plants encouraged the screening for more mutants involved in this pathway. This was done by identifying second site mutations that either suppress or enhance the *era1* mutant phenotype (McCourt, 1999). For example, *era1* mutants are hypersensitive to exogenously applied ABA, so new mutations that make the plant more or less sensitive to the hormone are relatively easy to find. The unexpected consequence of these genetic screens was that many of the genes identified were involved in other hormone response pathways such as ethylene and GA. The situation is even complex when a single gene is studied in more detail. For example, allelic mutations in *ABI3*, a gene involved in ABA signaling, results in a variety of phenotypes. This is because proteins like ABI3 are modular in design and hence have multiple functions encoded on different parts of the protein. Single mutations that change a function can often have specific phenotypes unique to that allele. In summary, it appears that single modular proteins in a hormone-signaling pathway may have more than one function and, secondly, extensive cross-talking is occurring between various hormone response pathways. Although this information reflects what plant physiologists have observed for years that single hormones can do many things and different hormones can carry out similar functions, the difference is, now that the genes are in hand, we can clearly determine how these interactions occur.

GENOMICS, TOMORROW

As can be seen from the previous section, hormones are not transduced into a cellular response through a simple linear pathway but require complex networks of interaction. With the advent of genome projects, it may soon be possible to apply some of this knowledge to unravel how genes work in networks. The goal of plant genomics is to understand the function of all genes, and, again, *Arabidopsis* has led the way in being the first plant genome to be fully sequenced. *Arabidopsis* has a relatively small genome of about 25,000 genes compared to other plants (*Arabidopsis* Genome Initiative, 2000). The genome can be subdivided into various categories such as genes involved in transcription or metabolism, for example. Interestingly, 33% of the genome encodes genes of unknown function. Even with this large number of unknown genes, the *Arabidopsis* genome project was successful because it is one-dimensional. That is, the information gleaned from sequence analysis could be directly translated into protein function. However, as can be seen from above, proteins and the pathways they regulate are not one-dimensional but function as networks. Furthermore, an understanding of this protein network or proteome is an order of magnitude more difficult because it has four-dimensional structure, and this structure will change depending on the development of the plant and the environment in which it is growing. An understanding of the proteome network is in its infancy, but model systems, such as the yeast *Saccharomyces cerevisiae*, are allowing us to construct proteome interaction maps. In one study, a

map was built by taking a single mutant in yeast and crossing to all 6000 knockout mutants that basically encompass all the genes of yeast (Tong *et al.,* 2001). This type of map gives us some hints at to how dynamic the genetic network will be in higher plants. Early results from these and other projects suggest the proteome is very flexible and will require new methods of data representation and thinking before the massive amounts of data will be useful.

These types of studies, whether biochemical or genetic, give us some idea of the nodes and interactions that can occur in a cell. However, when thinking about a network, the topology is as important as the individual nodes and the distances between nodes. For example, if a spider's web is to function well, the spider must be able to tell the difference between wind blowing through the web and an insect being caught in the middle. If the web moves due to the wind, the spider does not want to waste time coming down to check things out. But if an insect is caught, the spider wants to know. An insect caught in a web oscillates the web at a different frequency than wind blowing through the web, thus it is actually the oscillations of the web that contain the information and not the individual nodes. The oscillations are not due to any one particular node but to the interactions between them. In signaling, it is possible we will have to understand how a group of signaling pathways causes an oscillation in order to understand how the signal is transduced. The genetic or proteome interaction alluded to above represents the identification of nodes but says little about the oscillations of the network. It will be an understanding of how these genes contribute to the oscillations that will give us a sense of how a plant reads an environment and makes the appropriate decisions.

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ENGINEERING DISEASE RESISTANCE AND CROSS-TALK

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All living organisms have to live in association with other organisms including microbes. Many microbial organisms are pathogenic, so the host-pathogen interaction is a constant struggle. This struggle is a dynamic process; it can change during evolution over many generations or change within a generation if inducible responses occur in either or both partners. Both the host and the pathogen are under selective pressure. It seems that it would be to the benefit of the host to maximize resistance and to the benefit of the pathogen to overcome resistance. However, a host with the highest level of resistance is often not the fittest to survive selection nor is the pathogen with the highest level of virulence. Because a resistance response often involves activation of many genes, it is energy consuming. In order for the host to be fit, it not only has to maximize resistance but also minimize the cost of resistance. For scientists who are attempting to engineer disease resistant crops, it is easy to focus on the first part and ignore the second part of the balance.

Several explanations for the fitness costs of resistance have been proposed. First, resistance responses often involve activation of many genes. Turning on resistance will therefore result in diversion of resources. Second, turning on one disease resistance mechanism often leads to suppression of a different defense pathway through cross-talk. Third, activation of resistance pathways often results in changes in developmental processes. Fourth, activation of resistance responses usually has ecological consequences. Because resistance responses can be costly, scientists argue, many resistance responses are inducible.

Fitness cost caused by resistance is often assumed but rarely quantified. The susceptible and resistant lines used in experiments are usually not isogenic, and the genetic difference between the two lines is often unknown. Under these circumstances, it is difficult to distinguish fitness costs caused by a resistance response from those caused by other genetic traits. Furthermore, to measure the costs of an inducible resistance, pathogen infection or chemical treatment is often used for induction. Treating plants with a pathogen or a chemical may induce not only resistance responses but also other physiological reactions, making it difficult to really assign fitness costs to the resistance response.

To overcome the problems mentioned above, we utilized a genetic approach. Using *Arabidopsis thaliana* as a model system, we identified resistance-compromised mutants that only differ from wild type in a single gene. We also obtained mutants that cause constitutive induction of a resistance response to eliminate the need for pathogen infection or chemical treatment.

Even though *Arabidopsis* is a weed with no agronomical importance, it is an ideal system for genetic studies of plant biological processes. *Arabidopsis* can be grown not only in the laboratory but also in the wild. In fact, wild *Arabidopsis* habitats have been found in the coastal plain of North Carolina.

In the past thirteen years, we have identified pathogens in all major categories that infect *Arabidopsis* and studied all major pathogen defense responses in *Arabidopsis*. Among the various plant defenses, the *R* gene-mediated hypersensitive response (HR) is triggered by an interaction between a signal produced by the pathogen and its corresponding receptor in the host. HR often involves rapid cell death at the infection site and other biochemical responses that restrict the growth of the pathogen. *R* gene-mediated resistance is heritable and has been used in plant breeding programs to introduce disease resistance from resistant cultivars into agronomically important cultivars. There are also inducible defense responses studied in *Arabidopsis*; these include salicylic acid-(SA)-mediated systemic acquired resistance (SAR) and ethylene/jasmonic acid (JA)-mediated responses.

The focus of our research is on the mechanism of SAR. SAR is normally induced after a local infection. At the site of infection, a systemic signal is produced, leading to induction of a battery of genes known as pathogenesis-related (*PR*) genes. As a consequence of this coordinated gene expression, resistance to secondary infection is elevated. SAR is broad-spectrum and long lasting.

Salicylic acid, which is the active ingredient of aspirin, is an endogenous signal of SAR. Inhibition of SA accumulation blocks the onset of SAR (Gaffney *et al.,* 1993). SA is a sufficient signal for SAR; spraying plants with SA or an SA-analog such as INA or BTH induces SAR without any pathogen infection (White, 1979; Métraux *et al.,* 1991; Görlach *et al.*, 1996; Lawton *et al.,* 1996).

SAR induction involves up regulation of many downstream genes. Using microarray technology Maleck *et al.,* (2000) found that among the 30% of *Arabidopsis* genes surveyed under 14 different SAR inducing conditions, 413 cDNAs showed differential expression under at least two conditions. From this survey we can conclude that SAR enrolls at least hundreds of genes and is a very costly process.

To study SAR, my laboratory performed a mutant screen using a reporter gene. This reporter has a *PR* gene promoter that is responsive to SAR induction. We transformed this reporter into *Arabidopsis* and mutagenized the transformant. Through analysis of the reporter gene expression, we identified two classes of mutants: one called *cpr* mutants and the other called *npr* mutants (Bowling *et al.*, 1994; Cao *et al.*, 1994). *cprs* showed constitutive reporter without an inducer present, while the *npr* mutants showed the opposite phenotype, i.e., in the presence of an SAR inducer there was no reporter gene expression. We found many different *cpr* mutant loci but only one *npr* locus, *npr1*. Since then, twelve *npr1* alleles have been collected by different investigators.

The *cpr* mutants not only have constitutive reporter gene expression but also show significantly enhanced disease resistance against pathogens such as *Pseudomonas syringae* pv. *maculicola,* a bacterial pathogen that causes leaf spot disease on wild-type *Arabidopsis*, and *Peronospora parasitica*, an oomycete pathogen that leads to downy mildew on wild-type plants. Besides the resistance phenotype, all *cpr* mutants are smaller in size compared with the wild-type plants.

The effect of a *cpr* mutation on resistance may be direct or indirect. It is not uncommon to obtain the *cpr* phenotype when a physiological process, not necessarily related to defense, is perturbed. Among the *cpr* mutants, we believe that *cpr1* and *cpr6* are probably mutants in the defense pathways. When the *cpr1* mutant was crossed into loss-of-resistance mutants, *pad4* and *eds1*, the *cpr1* phenotype was completely suppressed (Clarke *et al.*, 2001; Jirage *et al.*, 2001), indicating that *cpr1* is upstream and *pad4* and *eds1* are downstream in a linear pathway.

The *npr1* mutant has the opposite phenotype compared to the *cpr* mutants. In the *npr1* mutant, induction of all the SAR-related genes is blocked, indicating that the wild-type NPR1 protein is a master regulator of gene expression in SAR. However, the NPR1 protein itself is not a transcription factor; instead, it interacts with the TGA subclass of transcription factors to regulate their transcriptional activity (Zhang *et al.*, 1999; Fan and Dong, 2002).

Mutation in the NPR1 gene affects not only SA-mediated SAR but also another inducible resistance response known as induced systemic resistance (ISR; Pieterse *et al.*, 1998), which is mediated by ethylene and JA. ISR is induced by non-pathogenic, root-colonizing bacteria. In addition to SAR and ISR, NPR1 is also involved in cross-talk between the SA and JA pathways. In wild-type plants, when SA is applied at the same time as JA, JA induced gene expression is inhibited. However, in the *npr1* mutant, this inhibitory effect of SA on JA-mediated gene expression is compromised.

Putting all the genetic data together, we could derive a simplified pathway, with *CPR1* (and possibly other *CPR* genes) at the top as a negative regulator of SAR; mutation in the *CPR1* gene results in SA accumulation and constitutive SAR. NPR1 is placed downstream of SA as an essential component for SA signal transduction. At the same time, NPR1 is also required for ethylene- and JA-mediated ISR and cross-talk between SA and JA pathways.

The essential role of NPR1 in the various defense responses makes it a favorite target for genetic engineering of disease resistance in plants. Overexpression of *NPR1* in *Arabidopsis* led to significantly enhanced resistance to *Pseudomonas syringae maculicola* and *Peronospora parasitica* (Cao *et al.,* 1998). Excitingly, overexpression of the *Arabidopsis NPR1* gene in rice conferred resistance to rice blight (Chern *et al.,* 2001).

Unlike all the *cpr* mutants, which show stunted growth, the *NPR1*-overexpressing *Arabidopsis* (*35S::NPR1H*) and rice have wild-type morphology. Examination of SAR status under uninduced conditions in the NPR1-overexpressing lines showed that they did not express SAR constitutively. Induction was still required to activate the overexpressed NPR1 protein. This active-only-upon-induction characteristic of NPR1 is critical for genetic engineering of disease resistance because enhanced resistance can be achieved with no obvious fitness penalty since NPR1 is only activated, i.e., SAR induced, upon pathogen challenge.

With the mutants and transgenic lines described above in hand, we performed both growth chamber and field experiments to measure the fitness costs of SAR. The lines used were: *cpr1*, *cpr5*, and *cpr6* with SAR turned on constitutively, *npr1* with compromised SAR, and *35S::NPR1H* with higher-than-wild-type level of NPR1. To ensure that all the lines used in the study were isogenic, seven rounds of backcrosses were carried out.

For the growth chamber experiment, we grew the mutants and transgenic lines under high and low nutrient conditions and measured seed yield to determine fitness. Under high nutrient conditions, we noticed that the *npr1* mutant showed slightly lower seed yield than wild type, while the NPR1-overexpressing line showed slightly higher seed yield than wild type. In all the *cpr* mutants, seed yield was significantly lower than the wild-type plants, indicating that constitutive activation of SAR indeed has fitness costs. Under low nutrient conditions, the overall seed yield for all the genotypes dropped 20 fold, but the trend was the same as in high nutrient conditions, with the *cpr* mutants showing lower seed yield than wild type. These results indicate that turning on SAR constitutively in *cpr* mutants clearly has fitness costs, and resource availability does not significantly change these costs.

To determine the potential benefit of constitutive SAR, we performed the second growth chamber experiment in the presence of a pathogen. We inoculated 400 plants with *Peronospora parasitica*, which causes downy mildew on wild-type *Arabidopsis*, and left another 400 uninfected. Our disease rating measurement showed that while *npr1*, wild-type, and NPR1- overexpressing plants showed different degrees of infection (in a decreasing order), all the *cpr* mutants were clearly resistance to this pathogen. The fitness of these plants under pathogen challenge was then determined by seed yield. We found that infection significantly reduced fitness of all genotypes tested. Interestingly, the *cpr* mutants that were resistant to the pathogen still had lower seed yield compared with wild type, indicating that fitness costs outweighed the benefit of enhanced resistance in the *cpr* mutants.

We then carried out field experiments to further determine the fitness of each SAR-related mutant. Since the experiment involved transgenic plants, we used rosette diameter, instead of seed yield, as a measure of fitness in compliance with USDA regulations on transgenic plants. Growth chamber test results showed that *Arabidopsis* rosette diameter correlates well with seed yield.

We first germinated seeds in the greenhouse and transplanted 2000 seedlings to the field and allowed them to grow under natural conditions. We found that wild-type and NPR1-overexpressing plants had the largest rosettes among all the lines tested. Interestingly, the *npr1* plants had significantly smaller rosettes than wild type, indicating the importance of the wild-type NPR1 in plant fitness. Perhaps NPR1-mediated SAR is involved in conferring basal level resistance against pathogens present in the natural environment. In laboratory tests, *npr1* mutant also shows enhanced disease susceptibility phenotype, but the effect of this phenotype on seed yield is less dramatic than under the field conditions. All the *cpr* mutants were also smaller in size when compared with the wild-type plants. Among all the plants used in this study, *cpr1* plants were the smallest, which is consistent with the growth chamber experiment. This

data showed once again that constitutive activation of SAR results in significant fitness loss.

We also looked for leaf damages caused by flea beetles present in the field to determine whether constitutive activation of SAR in *cpr* mutants would have any effect on herbivory resistance, since cross-talk has been observed between these two responses. For comparison, some blocks of plants were treated with insecticides, whereas other blocks were left untreated. We found that, with the exception of *cpr5*, the degree of insect damage found in plants was directly proportional to the size of the plant. In other words, constitutive activation SAR in *cpr* mutants had no effect on herbivory resistance. In the case of *cpr5*, the increased damage observed in this mutant was probably due to the effect of poor trichome development caused by the mutation. It has been demonstrated that in North Carolina, *Arabidopsis* trichome density is negatively correlated to herbivory damage [Mauricio, 1998].

In summary, our study showed that NPR1-mediated SAR is important for basal level pathogen resistance in wild-grown plants. However, constitutive activation of SAR has a substantial fitness cost that is relatively insensitive to nutrient availability. The cost of constitutive SAR outweighs the benefit of enhanced resistance in *cpr* mutants. Since overexpression of NPR1 results in no significant fitness loss, it is a viable strategy for engineering disease resistance in plants.

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ENGINEERING NEW PHENOTYPES FOR ABIOTIC STRESS TOLERANCE BY EXPRESSION OF TRANSCRIPTION FACTORS

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INTRODUCTION

Environmental stresses have major negative impacts on crop production. They limit the geographical locations where crops can be grown and cause significant losses in yield on an annual basis. A number of years ago, John Boyer (Boyer, 1982) estimated that on average, the major row crops in the United States only yield about 20% of their genetic potential. His analysis indicated that the "missing" 80% in yield was largely due to environmental stresses, with drought and low temperature being the major players. One can argue about the exact percentages, and these studies certainly need to be updated, but without question, abiotic environmental stresses have major negative impacts on crop productivity in the U.S. and worldwide.

Given this situation, it is not surprising that plant breeders have been trying to improve the abiotic stress tolerance of crops. Progress has been made, but success has been limited. For instance, despite considerable effort, there has been little improvement in wheat freezing tolerance over varieties developed some 80 years ago (Sarhan and Danyluk, 1998). The difficulty in breeding for increased stress tolerance is due to a number of reasons, including the fact that the trait is complex both physiologically and genetically. Thus, the notion that has emerged is that if we understood more about the underlying molecular responses to environmental stresses, that information would not only increase our basic understanding of plant biology, but it might also suggest new approaches for improving stress tolerance in plants. Indeed, recent progress in understanding the molecular responses of plants to environmental stresses has led to the hypothesis that tolerance might be improved through "regulon engineering"; that is, using transcription factors to optimize the expression of gene regulons with roles in stress tolerance. Here, I will discuss one such potential opportunity to improve freezing and drought tolerance.

PLANT COLD ACCLIMATION

Plants vary greatly in their freezing tolerance (Levitt, 1980). Tomatoes, for instance, are killed upon the slightest freeze, whereas rye can survive freezing down to about -25°C. Importantly, this difference in freezing tolerance is not a constant property of the plants. If tomatoes or rye are grown at warm temperature, there is not much difference in freezing tolerance between them. What rye can do that tomato cannot is sense a lowering of the temperature and activate mechanisms that cause an increase in the freezing tolerance. This process is known as "cold acclimation" (Thomashow, 1999).

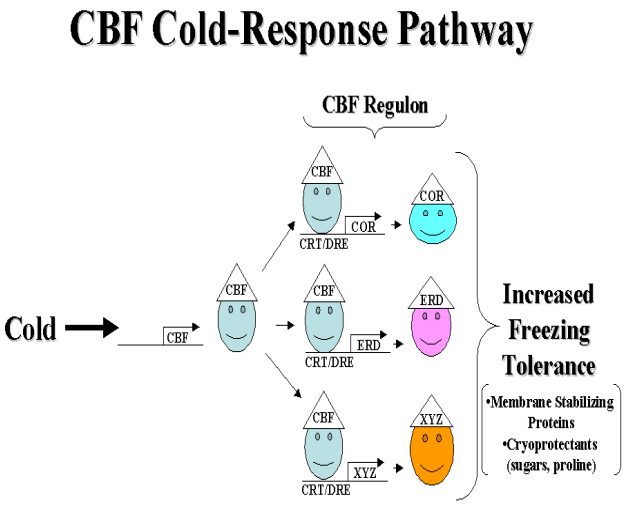
We and others have been using *Arabidopsis* (*Arabidopsis thaliana*) to try to understand the molecular basis of cold acclimation. The central objective is to determine what occurs in response to low temperature that increases freezing tolerance. Toward this end, we have been testing the simple hypothesis that low temperature-induced changes in gene expression contribute to an increase in freezing tolerance. As summarized below, our findings over the past few years validate this hypothesis.

COLD-INDUCED CHANGES IN GENE EXPRESSION

To what extent does gene expression change in response to low temperature? We have addressed this question using Affymetrix gene chips to monitor the expression levels of about 8,000 *Arabidopsis* genes. As the complete *Arabidopsis* genome encodes about 25,000 genes, the gene chips allow one to survey expression of about 1/3 of the genome. What we have found is that 218 genes are up-regulated and 88 are down-regulated at least 3-fold during cold acclimation (Fowler and Thomashow, 2002). Assuming that the genes on the gene chips are generally representative of

the entire genome, it would mean that about 4% of the total genome, or as many as 1000 genes, are cold-responsive. Ultimately, we want to develop a low temperature "wiring diagram" that indicates how all of these genes are regulated and which have roles in cold tolerance. At this point, we have discovered an important component of the diagram, the CBF cold-response pathway (Figure 1) (Thomashow, 2001).

Figure 1. Cold-Response Pathway



ROLE OF CBF COLD-RESPONSE PATHWAY IN COLD ACCLIMATION

Central to the CBF cold-response pathway is a small family of cold-responsive transcriptional activators that we have designated CBF1, CBF2 and CBF3 (Gilmour *et al.,* 1998; Stockinger *et al.,* 1997). These same transcription factors have been independently described by Shinozaki and coworkers and are designated DREB1b, DREB1c and DREB1a, respectively (Kasuga *et al.,* 1999; Liu *et al.,* 1998). These transcription factors are members of the AP2/EREBP family of DNA-binding proteins (Riechmann and Meyerowitz, 1998). They bind to the cold- and dehydration-responsive DNA regulatory element designated the CRT (C-repeat)/DRE (dehydration responsive element) (Stockinger *et al.,* 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). CRT/DRE elements are present in the promoter regions of many cold and dehydration responsive genes of *Arabidopsis* including those designated *COR* (cold-regulated) (Thomashow, 1999). The *CBF* genes are induced within 15 min of plant exposure to low, nonfreezing temperatures, followed at about 2 h by induction of cold-regulated genes that contain the CRT/DRE-regulatory element; i.e., the "CBF regulon" (Gilmour *et al.,* 1998; Liu *et al.,* 1998). Over the next few days at low temperature, the plants increase in freezing tolerance reaching a maximum level within 1 to 2 weeks.

A role for the CBF regulon in the enhancement of freezing tolerance has been established by conducting *CBF* overexpression experiments. Constitutive expression of the *CBF* genes in transgenic *Arabidopsis* plants results in the induction of *COR* gene expression and an increase in freezing tolerance without a low temperature stimulus (Gilmour *et al.,* 2000; Kasuga *et al.,* 1999; Jaglo-Ottosen *et al.,* 1998; Liu *et al.,* 1998). Multiple biochemical changes that are associated with cold acclimation and that are thought to contribute to increased freezing tolerance, including the accumulation of cryoprotective molecules (sugars and proline) and cryoprotective proteins (Steponkus *et al.,* 1998; Artus *et al.,* 1996), occur in nonacclimated transgenic *Arabidopsis* plants that constitutively express CBF (Gilmour *et al.,* 2000). Thus, we have proposed that the *CBF* genes act to integrate the activation of multiple components of the cold acclimation response (Gilmour *et al.,* 2000).

Significantly, activation of the CBF cold-response pathway also results in enhancement of plant tolerance to drought and high salinity (Haake *et al.,* 2002; Kasuga *et al.,* 1999; Liu *et al.,* 1998). The mechanistic basis for this "cross-protection" lies in the fact that the injury caused by freezing is largely due to the severe cellular dehydration that occurs with freezing. Freezing tolerance must include tolerance to dehydration stress and thus, it would not be surprising to find an overlap in the genetics of freezing and drought tolerance. Are there, then, *CBF*-like genes that are induced in response to drought and bind to the CRT/DRE? Indeed, there are. The Shinozaki lab has described such a transcription factor, DREB2a (Liu *et al.,* 1998). In collaboration with James Zhang and Volker Haake (Mendel Biotechnology, Inc.), we have described another such factor, CBF4. Moreover, we have shown that constitutive overexpression of CBF4 in transgenic *Arabidopsis* plants induces expression of the CBF regulon and

increases both freezing and drought tolerance (Haake *et al.,* 2002). Interestingly, overexpression of DREB2a does not induce constitutive expression of the CBF regulon of genes (Liu *et al.,* 1998). Thus, it has been suggested that the DREB2a protein must be activated by a stress-inducible pathway to function as an effective transcriptional activator (Liu *et al.,* 1998).

CONSERVATION OF THE CBF COLD-RESPONSE PATHWAY

Is the *Arabidopsis* CBF cold-response pathway highly conserved in plants? Recent results indicate that it is present in canola (*Brassica napus*), an important oilseed crop that, like *Arabidopsis*, is a member of the Cruciferae family (Jaglo *et al.,* 2001). We have shown that canola encodes *CBF*-like genes and that transcripts for these genes accumulate rapidly in response to low temperature followed closely by expression of the cold-regulated *Bn115* gene, an ortholog of the *Arabidopsis* CBF-regulated *COR15a* gene. Moreover, we have shown that constitutive overexpression of the *Arabidopsis CBF* genes in transgenic canola plants induces expression of *Bn115* and orthologs of other *Arabidopsis* CBF-regulated genes and increases the freezing tolerance of both nonacclimated and cold-acclimated plants. Additional experiments have established that transcripts encoding CBF-like proteins accumulate rapidly in response to low temperature in wheat and rye, plants that cold acclimate, as well as in tomato, a freezing sensitive plant that does not cold acclimate (Jaglo *et al.,* 2001). From these studies, we conclude that components of the CBF cold-response pathway are highly conserved in flowering plants and are not limited to those that cold acclimate. Whether the differences in freezing tolerance between *Arabidopsis* and tomato is due, in part, to differences in the CBF cold-response pathway remains to be determined.

USE OF *CBF* GENES TO IMPROVE PLANT STRESS TOLERANCE

The results presented above raise the possibility of using the CBF transcriptional activators to improve the freezing and drought tolerance of crop species. However, results indicate that this will probably not be accomplished by simply overexpressing the CBF proteins at high level using a strong constitutive promoter. This is because high level overexpression of the CBF transcriptional activators can have negative effects on plant growth and development. In *Arabidopsis*, these include a delay in flowering, reduced seed production and formation of stunted plants, traits that are not generally consistent with optimal agronomic performance (Gilmour *et al.,* 2000; Liu *et al.,* 1998). The occurrence of these negative growth characteristics is not surprising, however, given that the CBF pathway is normally "turned off" in non-stressed plants. As previously discussed, induction of the pathway causes a range of physiological changes including the accumulation of proline and sugars such as sucrose and raffinose, which is not likely to be conducive to optimal plant growth under non-stress conditions.

So what can one do? The general idea is to optimize expression of the pathway; that is, activate it to higher levels, but only under stressful conditions or at specific times during development. There are indications that this can work. Results from the Shinozaki laboratory indicate that placing CBF3/DREB1a under control of a stress-inducible promoter results in minimal negative growth effects in *Arabidopsis*, but imparts improved tolerance to freezing, drought, and high salinity stress (Kasuga *et al.,* 1999). As Dr. Klee pointed out in his talk on plant genetic engineering, "It all comes down to promoters, promoters, promoters."

OTHER POTENTIAL TRANSCRIPTION FACTORS FOR IMPROVING ABIOTIC STRESS TOLERANCE

The CBF proteins are not the only transcription factors that have potential for improving stress tolerance in plants. Kang *et al.,* (2002) have reported that constitutive overexpression of the *Arabidopsis* ABF3 or ABF4 transcription factors, zinc finger proteins that bind to abscisic acid (ABA) responsive elements, increases drought tolerance. Overexpression of these genes, however, also has negative effects: seeds from ABF3-expressing plants display a delay in germination, and seedlings of ABF4-expressing plants exhibit severe growth retardation. Park at al. (2001) have demonstrated that overexpression of the tobacco AP2 domain transcription factor Tsi1 enhances salt stress tolerance in transgenic tobacco. Finally,

Tamminen *et al.,* (2001) have shown that expression of the *Arabidopsis* ABI3 transcription factor results in a small, but detectable increase in freezing tolerance in *Arabidopsis*.

CONCLUDING REMARKS

Abiotic stress tolerance mechanisms are complex, involving the action of multiple genes. In some cases, however, genes with roles in stress tolerance are organized into regulons that are coordinately controlled through the activities of specific transcription factors. It is now apparent that in some cases, the stress tolerance of plants can be enhanced by modifying expression of transcription factors that control stress-tolerance regulons. This approach of "regulon engineering" has great potential to improve the stress tolerance of agriculturally important plants. However, constitutive high-level overexpression of transcription factors can also have undesirable effects on plant growth and development. Thus, regulon engineering will likely involve the use of conditional promoters to optimize expression of key transcription factors that control stress-tolerance regulons.

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