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Modified Ethylene Signaling as an Example of Engineering for Complex Traits: Secondary Effects and Implications for Environmental Risk Assessment

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Abstract. **The next wave of genetically engineered crops will use genes that modify gene regulation, plant metabolism, or signal transduction. The potential for these genes to have cascading effects on metabolism, physiology, and development increases the possibility for unintended effects that influence crop performance or environmental impact. This review examines altered ethylene signaling as an example of a complex trait with many horticultural applications. Genes for modified ethylene production or perception intended to regulate ripening, senescence, or stress or disease resistance have been observed to cause a broad range of secondary effects, including modified growth and development and increased severity to biotic and abiotic stresses. Successful use of complex traits in crop varieties will frequently require methods to reduce secondary effects, including the use of targeted gene expression. Risk assessment will need to consider observed pleiotropic effects on fitness within the context of potential environmental impacts.**

The commercialization and widespread cultivation of genetically engineered crops has grown rapidly in the 10 years since their initial introduction. More than 114 million ha were planted worldwide in 2007 (James, 2007). The vast majority of these crops (greater than 99%) are engineered for two traits, herbicide resistance and/or insect resistance using *Bacillus thuringiensis* (*Bt*) *cry* genes. In these crops, the engineered genes encode protein products that directly confer the trait of interest. Herbicide resistance genes either encode an herbicide-insensitive target molecule or an enzyme that degrades the herbicide (Sahora et al., 1998). Insect resistance is conferred by *Bt cry* genes that encode proteins toxic to certain species of insects (Carpenter et al., 2002). Despite expression throughout the plant, the *Bt* and herbicide resistance genes are not involved in modifying or regulating plant growth, development, or response to the environment and therefore do not generally cause pleiotropic (i.e., secondary) effects. Phenotypic changes other than direct effects of these traits have been primarily associated with the site of gene insertion, and those affecting performance are selected against by plant breeders in the early stages of crop development (Mihaliak, 2002).

A much broader range of traits and variety of genes are under currently under development by public researchers and private companies throughout the world. These include genes engineered to confer increased stress resistance, enhanced disease resistance, modified metabolism, and altered growth and development (Nickson, 2008; Wolfenbarger and Grumet, 2002; USDA-APHIS records). Such modifications could allow for cultivation of crops under adverse or marginal environmental conditions, reduce chemical inputs, or tailor plants for nutritional or industrial needs. Unlike genes used in the first wave of transgenic crops, these types of genes are intended to modify gene regulation or metabolic or signaling pathways of the plant. Multiple steps can occur between the protein product (e.g., transcription factor) and the ultimate desired phenotype (e.g., stress resistance). As a result, the activities of many additional gene products can be affected, increasing the possibility for pleiotropic effects that could cause ecologically relevant changes in phenotype (Wolfenbarger and Grumet, 2002).

In this review, we use modified ethylene production, perception, or response, collectively referred to as ethylene signaling, as an example of an engineered trait that can result in multiple effects, both intended and unintended. There is an extensive body of literature describing a diverse range of roles for ethylene throughout plant growth and development, and ethylene-related genes have been used to introduce a variety of traits in numerous species (Table 1). Plant processes influenced by ethylene include seedling development, root and shoot growth, flower development, senescence, fruit ripening, and responses to abiotic and biotic stresses (Abeles et al., 1992). Thus, altering ethylene signaling for a characteristic such as increased flower longevity may, in turn, result in secondary changes in abiotic stress responses or disease resistance. We discuss approaches used to modify ethylene production or perception, intended phenotypic effects, and examine the complexity of pleiotropic effects with respect to potential ecological impact.

Modification of Ethylene Signaling

Modification of the production or response to ethylene to manipulate plant growth and development is not a new concept. Ethylene-releasing compounds and inhibitors of ethylene synthesis and perception have been broadly used in agriculture to control multiple facets of crop production. The ethylene-releasing compound, ethephon (2-chloroethylphosphonic acid), has been used to retard growth and prevent lodging in wheat and barley, initiate uniform flowering in pineapple, induce female sex expression in cucurbits, increase latex flow in rubber, increase bud hardiness and bloom delay in sweet cherry and plum, reduce curing time in tobacco, promote ripening in banana and tomato, and promote fruit abscission in cotton and defoliation in tree species (Abeles et al., 1992; Arshad and Frankenberger, 2002; Hedden and Phillips, 2000; Yang and Oetiker, 1998). Ethylene production can be inhibited by treatment with aminoethyloxvinylglycine or aminooxyacetic acid; ethylene response is inhibited by silver-containing compounds, 2,5-norbornidiene, or 1-methylcyclopropene (Abeles et al., 1992; Sisler and Serek, 1997). Applications of these compounds include delay of fruit ripening, prevention of flower senescence or fruit abscission, and increased shelf life of leafy vegetables (Arshad and Frankenberger, 2002). Given the range of applications, it is not surprising that modified ethylene production or response has been a subject of numerous genetic engineering efforts (Czarny et al., 2006; Stearns and Glick, 2003).

The ability to engineer altered ethylene synthesis or signaling requires knowledge of underlying molecular mechanisms and cloning of key ethylene-related genes. Ethylene is synthesized from the widely used methyl donor, S-adenosyl methionine (S-AdoMet), in two steps (Yang and Hoffman, 1984). The first committed step in the pathway is

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Table 1. Examples of genetically modified ethylene signaling for agronomic or horticultural performance.

Gene	Species (promoter)[z]	Intended trait	Other phenotypes	Reference
Reduced ethylene				
ACC synthase (ACS)[y]	Tomato	Delayed ripening		Oeller et al., 1991
			Starch granules in fruit	Sozzi et al., 2001
	Apple	Fruit quality	Volatile suppression	DeFilippi et al., 2004, 2005
	Pineapple	Synchronize flowering		Trusov and Botella, 2006
	Tobacco	Ozone tolerance		Nakajima et al., 2002
	Potato	Ozone tolerance	Phenolic regulation	Sinn et al., 2004
			Slower growth in field	
	Citrus	Chilling tolerance		Wong et al., 2001
ACC oxidase (ACO)[y]	Tomato	Delayed ripening	Delayed leaf senescence	Picton et al., 1993
				Xiong et al., 2005
	Melon	Delayed ripening		Ayub et al., 1996
			Delayed fruit abscision	Guis et al., 1997
			Chilling resistance	Ben-Amor et al., 1999
				Flores et al., 2001
			ABA, polyamines	Martinex-Madrid et al., 2002
				Silva et al., 2004
	Apple	Fruit quality	Volatile suppression	Dandekar et al., 2004
		Modified aroma		DeFilippi et al., 2004, 2005
		Ripening, aroma		Schaffer et al., 2007
	Torenia fournieri	Floral longevity		Aida et al., 1998
	Broccoli	Post harvest quality		Henzi et al., 2000
			Increased protein	Gapper et al., 2005
	Tomato	Compacted soil tolerance		Hussain et al., 1999
ACC deaminase (ACD)	Tomato	Fruit ripening		Klee, 1993
				Reed et al., 1995
	Tomato	Metal tolerance		Grichko et al., 2000
	Canola (root)	Metal tolerance		Stearns et al., 2005
	Tomato (root)	Flood tolerance		Grichko and Glick, 2001
	Tobacco (PR, root)	Ozone tolerance		Nakajima et al., 2002
	Tomato (root)	Fungal resistance		Robison et al., 2001
SAM hydrolase	Tomato (fruit)	Delayed ripening		Good et al., 1994
SAM decarboxylase	Potato	Altered polyamines	Increased branching	Kumar et al., 1996
Increased ethylene				
ACS	Melon	Increase femaleness	Sequential fruit set	Papadopoulou et al., 2005
Ethylene response				
Mutant *ers*	Petunia	Flower longevity	Larger flowers	Shaw et al., 2002
			Reduced disease resistance	
			Decreased fertility	
	Chrysanthemum	Flower longevity		Narumi et al., 2005
	Broccoli	Postharvest quality	Shorter, late bolting	Chen et al., 2004
			Increased branching	
			Increased insect damage	
	Lotus japonica	Increase nodule formation	Reduced flower size	Nukui et al., 2004
			Increased flower longevity	
Mutant *etr1*	Tomato	Fruit ripening		Wilkinson et al., 1997
	Carnation	Flower longevity		Bovy et al., 1999
	Petunia	Flower longevity		Wilkinson et al., 1997
			Reduced rooting	Clevenger et al., 2004
			Reduced pollen viability	Shibuya et al., 2004
			Reduced seed size, viability	Gubrium et al., 2000
			Increased stress sensitivity	
			Delayed fruit ripening	
	Nemesia strumosa	Flower longevity		Cui et al., 2004
	Campanula carpatica	Flower longevity		Sriskandarajah et al., 2007
	Melon	Modify sex expression	Decreased rooting	Little et al., 2007
	Potato	Reduce sprouting		Haines et al., 2003
	Birch	Ozone tolerance		Vahala et al., 2003
Mutant *EIN2*	Petunia	Flower longevity	Decreased rooting	Shibuya et al., 2004
			Increased stress sensitivity	
			Delayed fruit ripening	
Le-etr4	Tomato	Accelerate HR response		Ciardi et al., 2001
CaERF	Hot pepper	Pathogen resistance		Lee et al., 2004
TERF1	Tobacco	Pathogen resistance		Zhang et al., 2004
	Tomato	Salt tolerance	Altered triple response	Huang et al., 2004
	Tobacco	Drought stress resistance	ABA hypersensitivity	Zhang et al., 2005
JERF3	Tobacco	Salt tolerance	PR gene induction	Wang et al., 2004

[z]Unless otherwise noted in parentheses, expression is driven by a constitutive promoter.
[y]Reduced ethylene synthesis was achieved with ACS and ACO genes by antisense, sense suppression, or siRNA gene silencing.

conversion of S-AdoMet to 1-aminocyclopropane carboxylate (ACC) by the enzyme ACC synthase (ACS); in the second step, ACC is oxidized to ethylene by ACC oxidase (ACO). The *ACS* and *ACO* genes have been cloned from multiple species and are generally encoded by multigene families whose members are differentially regulated by a variety of developmental, hormonal, or environmental signals (Bleecker and Kende, 2000).

The path of ethylene perception and signaling also is well characterized (Wang

et al., 2002; Fig. 1). A family of receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) homologous to two component histidine kinases are responsible for binding ethylene (Bleecker and Kende, 2000; Johnson and Ecker, 1998). Mutations that eliminate ethylene binding result in dominant, ethylene-insensitive phenotypes. In the absence of ethylene, the receptors activate CTR1 (constitutive ethylene response), a Ser/Thr protein kinase, which negatively regulates downstream ethylene responses (and when mutated results in constitutive ethylene response). In the presence of ethylene, CTR1 is deactivated, allowing the positive regulator EIN2 (ethylene-insensitive) to promote expression of members of the EIN3 transcription factor family. EIN3 induces expression of the ethylene-responsive element binding factor ERF1 (and other members of the ERF or ethylene response element binding protein family), which, in turn, regulate expression of genes responsible for various ethylene responses (Bleecker and Kende, 2000; Wang et al., 2002). The ERF family includes both transcriptional activators (e.g., ERF1) and repressors (ERF3, ERF4), which when overexpressed, confer constitutive ethylene response or insensitivity, respectively (Fujimoto et al., 2000; Yang et al., 2005).

Approaches to modulate ethylene production or activity have included up- or down-regulation of ethylene biosynthetic genes *ACS* and *ACO*; introduction of genes encoding enzymes to reduce ethylene production (e.g., ACC deaminase, S-AdoMet hydrolase); introduction of mutant ethylene receptor genes (e.g., *etr1-1*); and introduction of the *ERF* family transcription factor genes (Table 1).

Four plants engineered for modified ethylene signaling have received regulatory approval for environmental release, which is a necessary prerequisite to commercial production. These include: a carnation engineered for increased cut flower life through sense suppression of *ACS* in Australia and the European Union (http://www.agbios.com); and tomato lines engineered for delayed ripening either by a truncated *ACS* gene or by introduction of SAM hydrolase or ACC deaminase (*ACD*) in the United States (http://www.agbios.com). A wider range of crops engineered with ethylene-related genes have been tested in field trials, including apple, broccoli, coffee, melon, papaya, pineapple, potato, plum, and strawberry (USDA-APHIS records). In almost all cases, the plants were engineered for decreased ethylene production to facilitate long-distance transport and extended product shelf life.

Phenotypes Associated with Modified Ethylene Signaling

Plant growth and development. Genes for altered ethylene production or perception have been introduced to modify numerous growth and development-related traits, including root growth, nodule formation, floral development and sex expression, fruit development, ripening and quality characters, senescence, and abscission (Table 1). Based on the demonstrated effectiveness of external applications of ethylene-releasing or inhibitory compounds to regulate postharvest performance, the traits receiving the most interest for genetic engineering have been modified ripening and senescence. Fruit ripening is characterized by biochemical and physical changes in color, texture, flavor, aroma, and nutritional content (Adams-Phillips et al., 2004; Giovannoni, 2004; Gray et al., 1994). For many fruits, termed climacteric such as tomato, banana, apple, and peach and pear, ripening is initiated by a burst in ethylene production. The burst in ethylene is associated with numerous changes in gene expression, including changes in signal transduction and transcription factors as well as genes associated with processes such as softening, aroma, and pigment production (e.g., Alba et al., 2005; Schaffer et al., 2007).

Reduced expression of the ethylene biosynthetic enzymes ACS and ACO, or the introduction of ACD or SAM hydrolase, has been used to retard ripening in tomato and melon (Good et al., 1994; Guis et al., 1997; Klee, 1993; Reed et al., 1995; Silva et al., 2004; Sozzi et al., 2001) and modify aroma and improve fruit quality in apple (Dandekar et al., 2004; DeFilippi et al., 2004, 2005; Schaffer et al., 2007). Changes observed in the transgenic fruits include reduced ester, alcohol, and volatile production; higher titratable acidity; reduced malic acid degradation; modified sugar content; increased soluble solids; increased firmness; reduced cell wall degradation; and reduced chlorophyll degradation (Dandekar et al., 2004; DeFilippi et al., 2004, 2005; Flores et al., 2001; Guis et al., 1997; Schaffer et al., 2007; Silva et al., 2004; Sozzi et al., 2001).

Senescence and abscission involve complex biochemical processes whereby catabolism, mobilization of metabolites, and physical disintegration lead to programmed cell death (Chandlee, 2001). Mutant ethylene receptor or regulator *etr1*, *ERS*, and *EIN2* genes have been used to delay senescence in petunia, chrysanthemum, *Nemesia strumosa*, and *Campanula carpatica* flowers (Aida et al., 1998; Bovy et al., 1999; Clevenger et al., 2004; Cui et al., 2004; Narumi et al., 2005; Shaw et al., 2002; Shibuya et al., 2004; Sriskandarajah et al., 2007; Wilkinson et al., 1997) and delay abscission in tomato fruit (Whitelaw et al., 2002). *ACD*, antisense *ACO*, and mutant *ers* genes have been used to retain postharvest quality (reduce chlorophyll loss) in broccoli (Chen et al., 2004; Gapper et al., 2005; Henzi et al., 2000). The inhibited ethylene signaling was associated with downregulation of cysteine protease, metallothionein-like protein, hexokinase, invertase, and sucrose transporters (Gapper et al., 2005). These examples indicate how modification of a single gene can cause a host of changes in gene expression, metabolism, and physiology necessary to produce the intended phenotype.

Interactions of ethylene with other plant hormones has been extensively demonstrated, leading to a complex network of overlapping signals and responses (Gazzarrini and McCourt, 2003; Rashotte et al., 2005; Swarup et al., 2002; Visser and Voesenek, 2005). One of the most frequently observed examples of interaction involves auxin and ethylene, which coordinately regulate several processes, including apical hook formation, root growth, and hypocotyl phototropism (Swarup et al., 2002). Individual members of *ACS* gene families exhibit increased expression in response to auxin and, in several cases, possess upstream auxin response element sequences (Johnson and Ecker, 1998; Swarup et al., 2002; Woeste et al., 1999). Auxin can also influence stability of the ACS and ACO enzymes (Chae et al., 2000; Chae and Keiber, 2005).

Other hormones that interact with ethylene include gibberellins with respect to seed dormancy and growth responses (Brady and McCourt, 2003) and brassionosteroids, which can cause increased ethylene production and enhance ethylene-associated responses such as ripening, apical hook formation, and female sex expression in cucurbits (Arteca et al., 1988; Papadopoulou and Grumet, 2005; Sasse, 2003). In contrast, jasmonic acid and abscisic acid can antagonize ethylene signaling (Beaudoin et al., 2000; Ellis and Turner, 2001; Vardhini and Rao, 2002; Woeste et al., 1999). In many cases, interactions with other hormones were uncovered when genetic screens intended to identify genes associated with one hormone identified genes in other hormone pathways (Brady and McCourt, 2003). These observations demonstrate the interrelatedness among hormone effects and potential for modified ethylene production or perception to influence a broad range of responses.

Response to abiotic and biotic stresses. Ethylene also plays important roles in plant

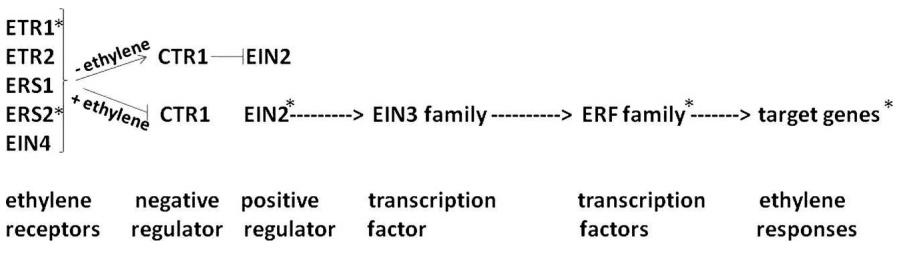

Fig. 1. Schematic representation of key components of the ethylene signaling pathway. Asterisks indicate genes targeted for modification of ethylene perception or response by genetic engineering.

responses to abiotic and biotic stresses. Increased ethylene production has been observed in response to temperature extremes, salinity, shade, drought, flooding, ozone, and heavy metal contamination (Morgan and Drew, 1997). In some species, stress-induced ethylene production is associated with increased growth or modified development to allow for stress avoidance. Elevated ethylene can cause more acute leaf angles to optimize light capture under shade conditions or petiole or internode elongation in response to submergence (Cox et al., 2004; Kende et al., 1998; Pierek et al., 2003, 2004; Voesenek et al., 2003). Ethylene-related root responses include development of aerenchyma to facilitate oxygen transport in response in response to flooding and development of adventitious roots (Bragina et al., 2001, 2003; McDonald and Visser, 2003; Visser and Voesenek, 2005). Overexpression of the ethylene and the osmotic stress-inducible responsive factor from tomato, TERF1, which binds to both ethylene and osmotic stress response elements, increases salt and drought tolerance in tobacco (Huang et al., 2004; Zhang et al., 2005). Similarly, the ethylene, osmotic, and jasmonic acid response factor from tomato confers increased salt tolerance to tobacco (Wang et al., 2004).

In contrast to adaptive ethylene-mediated responses, in flood-sensitive species such as tomato, flood-induced ethylene production is associated with deleterious consequences, including epinasty, chlorosis, and necrosis (Grichko and Glick, 2001). Introduction of a root-specific *ACD* gene to reduce ethylene production caused increased flood tolerance in tomato as measured by increased growth, leaf chlorophyll content, and reduced epinasty (Grichko and Glick, 2001). Similarly, decreased tomato leaf and shoot growth caused by compacted soil was reversed by reduced ethylene production through expression of antisense *ACO* (Hussain et al., 1999). Constitutive or root-specific expression of *ACD* in canola conferred increased growth in nickel-contaminated soil (Stearns et al., 2005). Transgenic tobacco expressing ACD acquired higher levels of metals (cadmium, cobalt, copper, nickel, lead, zinc) in their tissues but was less subject to the deleterious effects than its nontransgenic counterparts (Grichko et al., 2000). Transgenic tobacco, potato, tomato, or birch with suppressed expression of an ozone-inducible *ACS* gene or an introduced *ACD* or mutant *etr1-1* gene was more ozone-tolerant (Nakajima et al., 2002; Sinn et al., 2004; Vahala et al., 2003).

These studies demonstrate that the effects of ethylene on plant stress responses vary depending on the species and environment. In each case, ethylene production was induced in response to the stress. However, the nature of the downstream events, and whether they cause beneficial or injurious effects, appears to be dependent on the range of adaptive responses of individual species and thus the introduction of ethylene-related transgenes can have differential stress-related responses depending on the species.

Ethylene also plays important roles in plant defense, including induction of the hypersensitive response, pathogenesis-related (PR) genes, and other defense genes (Okubara and Paulitz, 2005; Rojo et al., 2003). Increased ethylene signaling can confer increased disease resistance and decreased ethylene can cause decreased resistance. Overexpression of the TERF1-positive regulator in tobacco and tomato led to constitutive *PR* gene expression and enhanced resistance to *Ralstonia solanacearum* (Zhang et al., 2004). Increased sensitivity to ethylene in antisense *LeETR4* tomato plants resulted in stronger and more rapid *PR* gene expression and more rapid hypersensitive response after inoculation with avirulent populations of *Xanthomonas campestris* (Ciardi et al., 2001). Loss of ethylene sensitivity in tobacco conferred by mutant ethylene receptor *etr1* caused loss of nonhost resistance against normally nonpathogenic fungi and decreased resistance to several soilborne or necrotropic pathogens (Geraats et al., 2003; Knoester et al., 1998).

The results of modified ethylene signaling can be variable depending on the host–pathogen combination. Reduced ethylene sensitivity has been associated with reduced severity of bacterial spot symptoms for transgenic tomatoes expressing ACD despite equivalent pathogen levels, presumably as a result of reduction of ethylene-related necrosis responses (Lund et al., 1998). Similarly, transformation of tomato with ACD led to reduced ethylene production and reduced symptom severity in response to *Verticillium* infection despite the presence of the pathogen (Robison et al., 2001). Ethylene insensitivity caused increased resistance to *Peronospora tabacina* in tobacco but did not affect the hypersensitive response to Tobacco mosaic virus (Geraats et al., 2003; Knoester et al., 1998). This range of responses, including both increased and decreased resistance, indicates the complexity of predicting the effect of modulating ethylene responses on specific diseases and further indicates that the same gene in the same species may cause different responses, depending on the pathogen.

As was noted for plant development, ethylene does not act alone. Plant defense responses involve a complex network of signaling involving salicylic acid (SA), jasmonic acid (JA), and ethylene. Deciphering this network is the subject of current research in many laboratories and several recent reviews (e.g., Bostock, 2005; Glazebrook, 2005; Jalali et al., 2006; Pozo et al., 2004; Rojo et al., 2003). JA and ethylene frequently act synergistically to confer tolerance to necotrophic root pathogens such as *Pythium*, *Rhizoctonia*, and *Phytophthora*. It was suggested that the effects of JA and ethylene on root development may lead to altered balance between root growth and defense gene expression (Glazebrook, 2005; Okubara and Paulitz, 2005). The outcome of interaction with SA signaling has been contradictory. Depending on the pathogen, expression of the tomato *ERF* in Arabidopsis was either positively regulated by JA and ethylene or by SA, indicating antagonism between the pathways. It has been suggested that responses to the different hormones can be antagonistic, cooperative, or synergistic to balance the metabolic costs of defense with the ability to deter opportunistic agents that may be able to invade should initial infection become successful. (Bostock, 2005; Rojo et al., 2003). Transcriptional elements for JA, SA, and ethylene often reside with the same defense gene promoter, presumably allowing fine-tuned responses to various pathogens (Jalali et al., 2006)

Pleiotropic Effects of Modified Ethylene Signaling

Given the wide variety of ethylene-mediated developmental processes, disease and stress responses, and interaction with other hormone responses, it is not surprising that alteration of ethylene for one purpose results in secondary effects on other phenotypes, especially if the introduced genes are expressed at all times throughout the plant as is the case with genes driven by a constitutive promoter. Studies cited in Table 1 show numerous cases in which multiple effects were observed in addition to the specific intended trait. Accompanying changes included modified volatile production, phenolic regulation, effects on ABA and polyamine levels, modified fruit set patterns, fruit ripening and abscission, modified branch and root structure, reduced plant and flower size, increased sensitivity to abiotic stresses, and increased susceptibility to insects and diseases (Table 1).

For example, transgenic ethylene-insensitive tomato, petunia, and melon plants engineered for flowering and ripening traits exhibited reduced root mass and reduced ability to be propagated by cuttings (Clark et al., 1999; Clevenger et al., 2004; Gubrium et al., 2000; Klee, 2002; Little et al., 2007; Shibuya et al., 2004). The ethylene-insensitive plants also exhibited difficulty in penetrating heavy soils and increased sensitivity to water stress, possibly as a result of reduced root mass and/or generally increased sensitivity to abiotic stresses (Clark et al., 1999; Little et al., 2007; Shibuya et al., 2004). In at least two cases, when subjected to stressful environments, decreased ethylene production or perception was associated with plant death. Transgenic *etr1-1* and *EIN2* petunias exhibited premature plant death that was more frequent at stress points such as transfer to soil when exposed to high temperature conditions or when grown in the field (Shibuya et al., 2004). A large portion of the petunia plants carrying a mutant *ERS* gene did not survive; the loss was attributed to increased disease susceptibility (Shaw et al., 2002).

Secondary effects were also observed for traits influencing reproductive development.

Expression of a mutated ethylene receptor gene in tobacco led to an alteration in floral structure; the ethylene-insensitive lines exhibited heterostyly with the stigma located above the anthers (Takada et al., 2005). Because heterostyly is viewed as a mechanism to promote outcrossing, changes of this sort could influence expected levels of gene flow. Indeed, the heterostylus transgenic tobacco lines exhibited greatly reduced numbers of seeds unless they were artificially pollinated (Takada et al., 2005). In the most severely affected lines, however, even artificially pollinated fruit produced less seed, suggesting effects on both mechanism of pollination and fertility. Pollen grains in the most severe lines failed to develop properly. For petunias, the effects of ethylene insensitivity were primarily observed on reduced seed size and viability rather than reduced seed set and pollen viability (Clevenger et al., 2004). It was suggested that the effects on seed size, which were maternally controlled, resulted from altered ripening processes that might influence seed development processes. The seed viability was directly correlated to seed size.

Modified ethylene signaling also had unexpected effects on carpel-bearing flower development in transgenic melons (Papadopoulou et al., 2005). The observed increase in initiation of carpel-bearing buds on *ACS*-overexpressing lines was predicted based on prior studies demonstrating the promotive effect of exogenous ethylene on female sex expression. Analysis of flower development, however, indicated that ethylene has a second role and is necessary for completed maturation of the carpel-bearing bud to anthesis (Papadopoulou et al., 2005). Transgenic melons expressing mutant *etr1* under a carpel-directed promoter further demonstrated the requirement of ethylene for maturation of carpel-bearing flowers to anthesis (Little et al., 2007). Fruit set patterns also were affected. Typically, melons will not set fruit at closely spaced nodes. However, ethylene overproducing lines exhibited a fivefold increase in closely spaced fruits (Grumet et al., 2007; Papadopoulou et al., 2005), suggesting that increased ethylene modified internal signaling normally involved in regulating resource allocation.

These results also show that the observed secondary effects can vary, depending on the physiology of the species, and also provide valuable new insights into plant developmental processes. Reduced expression of the *LeETR1* transcript in tomato affected fruit abscission and internode length, but not ripening characteristics, suggesting specificity among ethylene receptors with respect to different ethylene-mediated processes (Whitelaw et al., 2002). Inhibited ethylene perception caused different phenotypes in species in the same family, heterostyly and reduced pollen viability in tobacco, and reduced seed size and viability in petunia (Clevenger et al., 2004; Takada et al., 2005).

Further assessment of the complexity of effects and interactions can be obtained from global gene expression studies. Gene expression profiles of ethylene mutants, and control and ethylene-treated wild-type Arabidopsis plants, showed that 3% to 7% of ≈6000 tested genes were regulated by ethylene (DePaepe et al., 2004; Van Zhong and Burns, 2003). Among the differentially expressed genes were ethylene biosynthetic and signaling components, transcription factors, components of other hormone pathways, primary metabolic proteins, disease and defense-related proteins, and many of unknown function. Overlaps were observed among responses to ethylene, ABA, auxin, and sugar (DePaepe et al., 2004). Analysis of the affected genes may provide new insight into possible secondary effects that may arise from altered ethylene signaling.

Implications for Environmental Risk Assessment

It is clear from the previously cited examples that introduction of genes for modified ethylene signaling can cause a broad range of effects, including both intended phenotypes and unintended secondary effects. From a risk assessment standpoint, key questions are: what is the ecological relevance of these changes and are there ways to minimize such changes? Possible ecological impacts would result if the engineered crop, or interfertile wild relatives who become recipients of the engineered gene, exhibit changes causing them to become weedy in agricultural fields, invasive in natural environments, or otherwise disrupt the surrounding ecosystem (Conner et al., 2003; Dale et al., 2002; Ellstrand, 2001; Snow et al., 2005). These environmental concerns need to be addressed whether we are dealing with a complex engineered trait, a simple engineered trait such as *Bt*-mediated insect resistance, or a conventionally bred trait (e.g., disease resistance). The aspect that is different with a more complex engineered trait such as altered ethylene signaling, relative to a simple engineered trait, is the range of phenotypes potentially affected. Although examples of secondary effects have been occasionally reported for the current commercialized transgenes [outcrossing rates in herbicide resistant Arabidopsis (Bergelson et al., 1998); increased lignin in *Bt* maize (Saxena and Stotzky, 2001)], these effects are much more limited than the broad range and frequency of occurrence of modified phenotypes summarized in Table 1.

Pleiotropic effects associated with complex traits could be potentially advantageous or detrimental. For a trait such as ethylene signaling, which modulates a multitude of developmental and environmental responses, constitutive modification often results in maladapted plants [e.g., loss of disease resistance or water stress tolerance (Clark et al., 1999; Shaw et al., 2002; Shibuya et al., 2004)] resulting from disruption of finely tuned, interdependent processes. Such plants will not be of use for commercial production and so are not of concern with respect to ecological risk assessment consideration.

Successful engineering of complex traits in crop varieties will frequently require methods to minimize secondary effects. One method to reduce secondary effects is to use specific promoters that mediate tissue-specific, developmentally specific, and/or environmentally responsive gene expression. Fruit- or root-targeted gene expression has been tested for some ethylene-related traits, including modified ripening and resistance to flooding or heavy metals (Good et al., 1994; Grichko and Glick, 2001; Stearns et al., 2005). Use of restricted promoters will also reduce the risk of potential ecological impacts of the transgenic crop.

Of critical importance in assessing the potential ecological impact of a GE crop is whether the introduced gene and its associated phenotype confers a fitness advantage, a prerequisite for increased weediness or invasiveness (Conner et al., 2003; Dale et al., 2002; Hancock, 2003). Direct comparisons of performance are routinely made in greenhouse and confined field trials between the engineered line and its equivalent nontransgenic counterpart of an array of factors related to fitness such as plant vigor, flowering time, reproductive capacity, and susceptibility to pathogens and insects (Mihaliak, 2002). Potential for gene exchange with compatible relatives should also be evaluated; where crosscompatible wild populations coexist with the crop, the fitness impact of the transgene should be assessed in the genetic background of the relative (Burke and Riesberg, 2003; Campbell et al., 2006; Snow et al., 2003). If the transgene can confer a fitness advantage, especially in native settings that may not be observed under cultivation (e.g., nutrient limited or routinely water-logged soils), studies should be performed in natural environments or conditions that simulate those environments. Nickson (2008) emphasizes the importance of focusing on situations in which there could be an adverse effect on native communities.

In summary, ethylene plays critical roles in coordinating plant growth and development and response to biotic and abiotic stresses. These roles make ethylene signaling an attractive target for genetic engineering, but also can result in numerous pleiotropic effects. Targeted gene expression can potentially minimize secondary effects that influence successful crop performance or ecological impact. Risk assessment will need to consider the full range of pleiotropic changes within the context of potential ecological impacts.

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