Review

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Ethylene Receptors and Genetic Engineering of Ethylene Sensitivity in Plants

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Abstract

Ethylene is a gaseous plant hormone which gives cues to various developmental processes and stress responses in plants. Plants regulate their ethylene sensitivity for programmed development and responses to environmental stresses. After the successful isolation of the *Arabidopsis* ethylene receptor gene *ETR1*, ethylene receptor genes have been found in various plant species. Characterization of ethylene receptor genes provides clues to understanding how plants regulate their ethylene sensitivity. This knowledge also gives us potential methods for engineering the ethylene sensitivity of plants. Genetically-engineered plants with reduced ethylene sensitivity will be potential resources for improving crop performance. Moreover, those transgenic plants will provide further information to elucidate the mechanism of how plants regulate their ethylene sensitivity. The recent progress in genetic and protein analyses of ethylene receptors is summarized in this review. The possible strategies for altering the ethylene sensitivity of plants using the ethylene receptor genes are also discussed.

Introduction

Ethylene is implicated in a number of processes in plant development such as seed germination, senescence, abscission, sex determination and fruit ripening and in the response to a wide variety of stresses including pathogen attack, flooding and drought (Abeles *et al.*, 1992). In addition, the involvement of ethylene in the growth transition from vegetative to reproductive phase in *Arabidopsis* (Ogawara *et al.*, 1999) and ovule development in tobacco (Martinis and Mariani, 1999) have been observed. These processes are important for the determination of crop performance. Therefore, an alteration of ethylene action is a valuable target for the genetic engineering of crops.

The action of ethylene is based on two types of responses: the response to a change in the concentration of cellular ethylene, such as an increase in ethylene production, and the response to a change in the sensitivity of tissue to ethylene. Since these responses are closely related to each other and are involved in the intricate mechanism of ethylene action, we need to examine the biosynthesis of ethylene and its perception in order to identify the regulatory role of ethylene. However, our knowledge of the mechanism of how plants control their ethylene biosynthesis and sensitivity is still limited.

The pathway of ethylene biosynthesis has been elucidated during the last few decades, and the basis for subsequent biochemical and molecular genetic analysis of this pathway has been provided (Zarembinski and Theologis, 1994; Imaseki, 1999). Many studies have been performed to clarify the physiological and molecular mechanisms of the various events regulated by changes in ethylene production (Barry *et al.*, 1996; Blume and Grierson, 1997; Bouquin *et al.*, 1997; Clark *et al.*, 1997; Heidstra *et al.*, 1997; ten Have and Woltering, 1997; Trebitsh, *et al.*, 1997; Zarembinski and Theologis, 1997; Bui and O'Neill, 1998). Based on these results, transgenic plants altered their ethylene biosynthesis in tomato and melon by sense/antisense technology have been produced as reviewed by Fray and Grierson (1993) and Ezura (1999), respectively. Produced transgenic plants have become a tool for elucidating the roles of ethylene on plant devel-

opment (John, *et al.*, 1995) and stress response (Lund *et al.*, 1998) as well as for crop improvement.

The molecular mechanism underlying ethylene sensitivity in plants has been studied by using a genetic approach with *Arabidopsis* (Bleecker and Schaller, 1996). Ethylene-response mutants can be used to perform such a genetic approach. The ethylene-insensitive mutant *etr1* has been identified using the triple response screen of mutagenized seeds (Bleecker *et al.*, 1988). To date, various types of ethylene response mutants have been identified in *Arabidopsis* (Kieber and Ecker, 1993). The *ETR1* gene, which encodes an ethylene receptor, has been isolated and characterized (Chang *et al.*, 1993), and other *ETR1*-like genes (Hua *et al.*, 1995, 1998; Hua and Meyerowitz, 1998; Sakai *et al.*, 1998) have also been identified in *Arabidopsis*. Based on sequence similarity and structural features of the proteins, *ETR1*-like genes have been isolated from several other plants including tomato (Wilkinson *et al.*, 1995, Lashbrook *et al.*, 1998, Tieman and Klee, 1999), *Rumex palustris* (Vriezen *et al.*, 1997) and melon (Sato-Nara, *et al.*, 1997, 1999a). Using similar molecular genetic approach, genes related to the ethylene signaling pathway have also been isolated and characterized (Guzman and Ecker, 1990; Kieber *et al.*, 1993; Roman *et al.*, 1995; Chao *et al.*, 1997; Alonso *et al.*, 1999). Based on the analysis of these mutants and genes, a model of the ethylene perception and signal transduction pathway has been proposed (Bleecker *et al.*, 1998; Johnson and Ecker, 1998; Solano and Ecker, 1998; Bleecker, 1999).

Recent progress in understanding the mechanism underlying the ethylene perception and signal transduction pathway has stimulated the production of transgenic plants with altered ethylene sensitivity. Transgenic plants can be a powerful tool for elucidating the regulatory role of ethylene in plant development and stress responses as well as those with altered ethylene biosynthesis. In addition, transgenic plants that confer reduced sensitivity and insensitivity to ethylene are expected to have extended postharvest life for commercial use.

In this review, current progress in the analysis of ethylene receptor genes and their function, including our results, are summarized, and the potential strategies to regulate the ethylene sensitivity of plants using the ethylene receptor genes are discussed.

Isolation and characterization of ethylene receptor genes

The genetic approach with *Arabidopsis* has been used to identify the genes coding for early components in ethylene signal transduction (Bleecker *et al.* 1988; Guzman and Ecker 1993; Chang *et al.* 1993; Kieber *et al.* 1993; Roman *et al.* 1995; Alonso *et al.*, 1999). Ethylene inhibits the elongation growth of etiolated (dark-grown) seedlings. Many ethylene insensitive or constitutive triple response mutants have been identified by screening mutagenized populations of *Arabidopsis* seedlings grown in the dark and in the presence or absence of applied ethylene (Bleecker *et al.* 1988; Guzman and Ecker 1993; Kieber *et al.* 1993; Roman *et al.* 1995; Chao *et al.* 1997). One of the ethylene insensitive mutants, *etr1*, was first identified by Bleecker *et al.* (1988), and the *ETR1* gene has been isolated and characterized (Chang *et al.*, 1993).

The ETR1 protein has three N-terminal hydrophobic domains; phytochrome-related T2L and R2L domains, which are homologous domains with the chromophore attachment domains of phytochrome photoreceptors; a GAF domain, which is a homologous domain found in phototransducing proteins; and two domains which are homologous to a histidine kinase and a receiver of the bacterial two-component system (**Fig. 1**; Kehoe and Grossman 1996; Aravind and Ponting 1997; Bleecker *et al.* 1998). Mutant alleles of *ETR1*, designated as *etr1-1*, *etr1-2*, *etr1-3*, *etr1-4*, cause ethylene insensitivity in the plant (Chang *et al.*, 1993; **Fig. 1**). All of these mutations cause single amino acid replacements in the three hydrophobic domains, which are putative membrane spanning domains of the protein (Chang *et al.*, 1993; Ala^{31} to Val in *etr1-3*, Ile^{62} to Phe in *etr1-4*, Cys^{65} to Tyr in *etr1-1*, and Ala^{102} to Thr in *etr1-2*; **Fig. 1**). When *etr1-1* was transformed into wild-type *Arabidopsis*, the transgenic plants lost ethylene sensitivity (Chang *et al.*, 1993). Wilkinson *et al.* (1997) recently reported that transgenic tomato and petunia with *etr1-1* also became insensitive to ethylene, showing that the dominant *etr1* mutation generally eliminates ethylene sensitivity in higher plants.

Analysis of *ETR1* expressed in yeast cells showed that the hydrophobic regions of the ETR1 protein are capable of reversibly binding ethylene (Schaller and Bleecker, 1995). This is a strong evidence that *ETR1* encodes an ethylene receptor. Recently, Rodríguez *et al.* (1999) reported that a copper ion associated with the ethylene-binding domain of

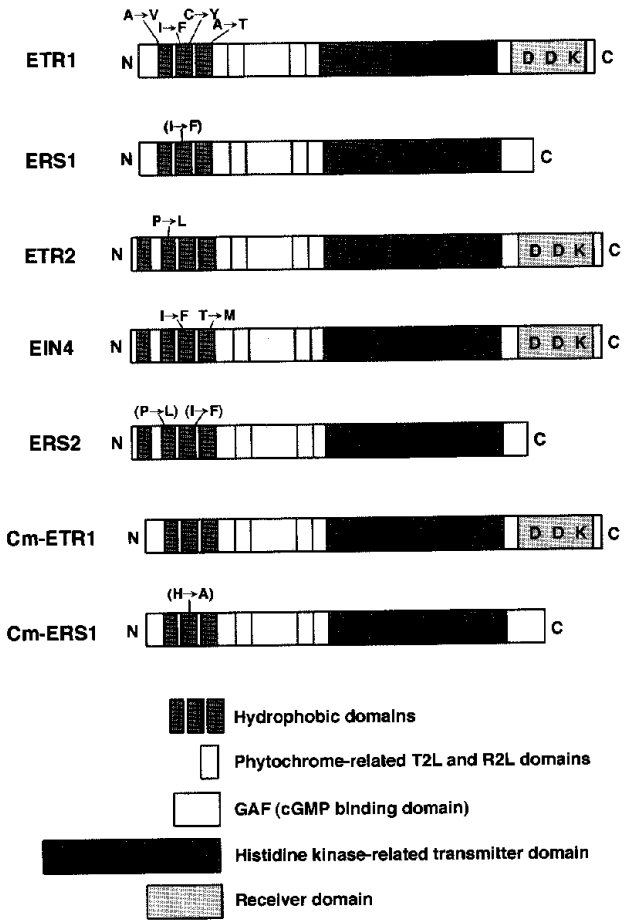

Fig. 1 Structural features of the *ETR1*-like gene family in *Arabidopsis* and *Cm-ETR1* and *Cm-ERS1* in melon. Three hydrophobic domains at N-terminus are three putative membrane-spanning domains which compose the ethylene binding domain. The *ETR2*-like subfamily are characterized by a hydrophobic N-terminal extension. The hydrophobic domains are followed by phytochrome-related T2L and R2L domains (Kehoe and Grossman 1996) containing a GAF domain (Aravind and Ponting 1997). The C-terminal shaded boxes represent the sequences homologous to the histidine kinase domain and the receiver domain of bacterial two-component environmental sensor systems. The histidine kinase domains of ETR1, ERS1, Cm-ETR1 and Cm-ERS1 contain all the consensus motifs of bacterial histidine kinase (Parkinson and Kofoid, 1992; Hua *et al.*, 1998; Sato-Nara *et al.*, unpublished results). The *ETR2*-like family lack some of these motifs (Hua *et al.* 1998). Receiver domains of ETR1, Cm-ETR1, ETR2 and EIN4 contain all the sequences thought to be essential for phosphotransfer (Hua *et al.* 1998; Sato-Nara *et al.*, unpublished results). Amino acid conversions shown in the mutant alleles of ETR1, ETR2 and EIN4, and the mutated sequences of ERS1, ERS2 and Cm-ERS1 leading to ethylene insensitivity by transfer into wild-type plants are indicated (Chang *et al.*, 1993; Hua *et al.* 1995; Sakai *et al.* 1998; Hua *et al.* 1998; Ezura *et al.*, unpublished results).

ETR1 is required for high-affinity ethylene binding activity, and that Cys^{65} is an essential residue for both copper association and ethylene binding to the receptor. Hirayama *et al.* (1999) isolated *RAN1* and demonstrated that RAN1 acts by delivering copper to create a functional hormone ethylene receptor. ETR1 forms a disulfide-linked dimer joined by the two cysteine residues (Cys-4 and Cys-6), which are located in the amino-terminal domain (Schaller *et al.* 1995), and ethylene may interact with a Cu(I) cofactor in an electron-rich hydrophobic pocket formed by membrane-spanning helices of the ETR1 dimer (Rodríguez *et al.* 1999).

To date, five *ETR1*-like genes, *ETR1* (Chang *et al.*, 1993), *ERS1* (Hua *et al.*, 1995; Hua and Meyerowitz, 1998), *ETR2* (Sakai *et al.*, 1998), *EIN4* and *ERS2* (Hua *et al.*, 1998) have been identified in *Arabidopsis* (**Fig. 1**). Based on sequence similarity and structural features of the proteins, Hua *et al.* (1998) classified the former two into the *ETR1*-like subfamily and the latter three into the *ETR2*-like subfamily. ETR1 and ERS1 have three hydrophobic domains at the N-terminus and five consensus motifs found in bacterial histidine kinase, while ETR2, EIN4 and ERS2 have four hydrophobic domains at the N-terminus and lack most of the motifs in histidine kinase (**Fig. 1**, Parkinson and Kofoid 1992; Hua *et al.* 1998). Although it has not yet been reported whether other members can bind ethylene, all of the residues thought to be essential for ethylene binding in ETR1 are conserved in the ETR2-like subfamily (Bleecker *et al.* 1998).

Autophosphorylation of the putative histidine kinase domain of ETR1 expressed in yeast is detected by incubation with radiolabeled ATP (Gamble *et al.*1998). Autophosphorylation is abolished by mutations that eliminate either the presumptive site of phosphorylation (His-353) or putative catalytic residues within the kinase domain. It seems to be impossible for each member in the ETR2-like subfamily to function as a histidine kinase because they lack most of the consensus motifs of histidine kinase (**Fig. 1**). ETR1, ETR2 and EIN4 have the receiver domain that receives phosphate from the histidine kinase (transmitter) domain, while ERS1 and ERS2 lack the receiver domain (**Fig. 1**). The receiver domains of ETR1, ETR2 and EIN4 contain three residues (D, D, K) which are important for phospholylation (**Fig. 1**). Point mutations in the hydrophobic domains of ERS1 (Hua *et al.* 1995), ETR2 (Sakai *et al.* 1998), EIN4 and ERS2 (Hua *et al.* 1998) also cause insensitivity to ethylene in *Arabidopsis*, indicating that these homologs share a common function with ETR1.

ETR1 homologs have also been isolated from other plants: the *NR* gene (Wilkinson *et al.*, 1995), *LeETR1* and *LeETR2* cDNAs (Lashbrook *et al.*, 1998), *LeETR4* and *LeETR5* cDNAs (Tieman and Klee, 1999) from *Lycopersicon esculentum*, the *RP-ERS1* cDNA from *Rumex palustris* (Vriezen *et al.*, 1997), *Cm-ETR1* and *Cm-ERS1* cDNAs from *Cucumis melo* (Sato-Nara *et al.*, 1997, 1999a) and the *PE-ETR1* and *PE-ERS1* cDNAs from *Passiflora edulis* (Mita *et al.*, 1998). In addition, the sequences of putative ethylene receptor genes and cDNAs from several plants have been registered in databanks and the plant gene register of Plant Physiology (**Table 1**). *LeETR1*, *LeETR2*, *LeETR4* and *LeETR5*, *PE-ETR1* and *Cm-ETR1* have a receiver domain, while *NR*, *RP-ERS1*, *PE-ERS1* and *Cm-ERS1* have no receiver domain (Bleecker and Schaller, 1996; Vriezen *et al.*, 1997; Lashbrook *et al.*, 1998, Mita *et al.*, 1998, Sato-Nara *et al.*, 1999a, **Fig. 1**).

According to the protein features of Cm-ETR1 and Cm-ERS1, shown in **Fig. 1**, these belong to the *ETR1* subfamily. Both proteins have three hydrophobic domains, phytochrome-related T2L and R2L domain, GAF, and the histidine kinase-related transmitter domain. The hydrophobic domains have

Table 1. Genes or cDNAs for ethylene receptors and its homologs in various plants.

Species	Names of the genes	Type	Organs and Tissues expressing mRNA at relatively high level	Regulation of gene expression by ethylene (or ACC; organs and tissues, time for treatment)	References
Arabidopsis thaliana	*ETR1*	ETR1	Procambium cells in the stem, locules of the anthers and developing carpels etc.	Not affected (leaves, 12h)	Chang *et al.* 1993
A. thaliana	*ETR2*	ETR2	Developing carpels.	Up-regulated (leaves, 12h)	Sakai *et al.* 1998
A. thaliana	*EIN4*	EIN4	The locules of stamens.	Not affected (leaves, 12h)	Hua *et al.* 1998
A. thaliana	*ERS1*	ERS1	Embryos, etiolated seedlings including cotyledons, hypocotyls and roots, procambiun cells in stem, young floral primordia locules of the anthers etc.	Up-regulated (leaves, 12h)	Hua *et al.* 1995, 1998
A. thaliana	*ERS2*	ERS2	Flowers (the tapetum cells, developing pollen cells and carpels etc.).	Up-regulated (leaves, 12h)	Hua *et al.* 1998
Brassica oleracea	*ETR1*[c]	ETR1			Chen *et al.* 1998a
B. oleracea	*ERS*[c]	ERS1			Chen *et al.* 1998b
Citrus sp.	*CERS*[n]	ERS1	Fruit during colour changing, Abscission zone.	Up-regulated	Cubells *et al.* 1998
C. sinensis L. *Osbeck*	*ERS*[c]	ERS1			Li *et al.* 1998
Cucumis melo L. *reticulatus*	*Cm-ETR1*[c]	ETR1	Hypocotyls of seedlings, young leaves, seeds during matuaration, pericarp and placenta during fruit ripening.	Up-regulated (ACC; hypocotyls of seedlings and flesh of early-ripe fruit, 1h)	Sato-Nara *et al.* 1997, 1999a, b
C. melo L. *reticulatus*	*Cm-ERS1*[c]	ERS1	Hypocotyls of deedlings, young leaves, pericarp during fruit enlargement.	Down-regulated (ACC; hypocotyls of seedlings, 1h)	Sato-Nara *et al.* 1997, 1999a, b
Dianthus caryophyllus Clove pink	*DCERS*[c]	ERS1			Charng *et al.* 1997
D. caryophyllus	*DC-ERS2*[c]	ERS1			Shibuya *et al.* 1998
Lycopersicon esculentum	*LeETR1*[c] *(eTAE1)*	ETR1	Constitutive in all developmental stages.		Lashbrook *et al.* 1998 (Zhou *et al.* 1996a)

Species	Names of the genes	Type	Organs and Tissues expressing mRNA at relatively high level	Regulation of gene expression by ethylene (or ACC; organs and tissues, time for treatment)	References
L. esculentum	*LeETR2*[c] *(TFE27)*	ETR1	Present in all tissues at low level.		Lashbrook *et al.* 1998 (Zhou *et al.* 1996b)
L. esculentum	*LeETR3 (NR)*	ERS1	Present in all tissues. Relatively high abundances in ripening fruit, ovaries, leaf petioles and so on.	Up-regulated (maturating fruit, 24h)	Wikinson *et al.* 1995, Klee *et al.* 1998, Lashbrook *et al.* 1998
L. esculentum	*LeETR4*[n]	ETR2	Reproductive tissues.		Tieman *et al.* 1999
L. esculentum	*LeETR5*[n]	ETR2	Reproductive tissues.		Tieman *et al.* 1999
Malus domestica L. Borkh cv Granny Smith	*ETR1*[c]	ETR1			Lee *et al.* 1998
Nicotiana tabacum	*NT-ETR1*[c]	ETR1			gb: AF0222727
N. tabacum	*NT-ERS*[p]	ERS1			gb: U87239
N. tabacum	*ETS*[p]	ERS1			gb:AF039921
Oryza sativa	*OSERS*[c]	ERS1			gb:AF013979
Passiflora edulis Sims	*PE-ETR1*[c]	ETR1	Arils in fruit.	Not markedly affected (fruit, >10h)	Mita *et al.* 1998
P. edulis Sims	*PE-ERS1*[c]	ERS1	Arils in fruit.	Not markedly affected (fruit, >10h)	Mita *et al.* 1998
Phalaenopsis sp. 'KCbutterfly'	*ERS*[c]	ERS1			gb:AF113541
Pisum sativum	*ERS1*[c]	ERS1			gb:AF039746
P. sativum cv Alaska	*ERS*[c]	ERS1			emb:AJ005829
Rumex parstris	*RP-ERS1*[c]	ERS1	Shoots more than 4 h after submergence.	Up-regulated (leaf, 24h)	Vriezen *et al.* 1997, Voesenek *et al.* 1997
Solanumly copersicum	*Never-ripe*	ERS1	See "Le-ETR3".		Wilkinson *et al.* 1995
Vigna radiata	*ERS1*[c]	ERS1			gb:AF098272

c: The sequence of only mRNA (complete cds) is published or available in GenBank.
p: The sequence of mRNA (partial cds) is available in GenBank.
n: The sequence is not available in GenBank.

an essential residue, Cys^{70}, for both copper association and ethylene binding to the receptor. Two cysteine residues (Cys-5 and Cys-7), which are necessary for forming the disulfide-linked dimer, exist in the amino-terminal domain of both proteins. In addition, Cm-ETR1 has a receiver domain. The other genes from *L. esculentum, R. palustris*, and *P. edulis* also seem to belong to the *ETR1* subfamily due to the high identities between their sequences and that of ETR1. Point mutations in the hydrophobic domains of NR (Lanahan *et al.*, 1994; Wilkinson *et al.*, 1995), and LeETR4 and LeETR5 (Tieman and Klee, 1999), and Cm-ERS1 (Yuhashi *et al.* unpublished results) also cause insensitivity to ethylene in *L. esculentum* and *Arabidopsis*, respectively. Furthermore, NR (A.B. Bleecker personal communication) and Cm-ERS1 (Yuhashi *et al.* unpublished results) bind ethylene when expressed in yeast, indicating that these homologs share a common function with ETR1.

Ethylene perception and signal transduction

Ethylene perception is most likely carried out by the receptors encoded by the *ETR1*-like family. The genes encoding other components in ethylene signal transduction have also been identified and characterized using a genetic approach with ethylene-related mutants in *Arabidopsis* (Guzman and Ecker, 1990; Kieber *et al.*, 1993; Roman *et al.*, 1995; Chao *et al.*, 1997). Based on double mutant analysis, it is thought that CTR1 acts at or downstream from ETR1, ERS1 and EIN4, and that EIN2, EIN3, EIN5, EIN6 and EIN7 act after CTR1 (Hua *et al.*, 1995;

Roman *et al.*, 1995; **Fig. 2**).

CTR1 is a negative regulator of the ethylene response pathway because *ctr1* null mutants exhibit constitutive ethylene responses even in the absence of ethylene (Kieber *et al.*, 1993). The deduced CTR1 protein sequences are most similar to the Raf family of serine/threonine protein kinase, suggesting that CTR1 may act as a mitogen-activated protein (MAP) kinase cascade (Kieber *et al.*, 1993). By using the yeast two-hybrid assay, Clark *et al.* (1998) detected a specific interaction between the CTR1 amino-terminal domain and the predicted histidine kinase domain of ETR1 and ERS1. In addition, the amino-terminal domain of CTR1 can be associated with the predicted receiver domain of ETR1 in vitro (Clark *et al.*, 1998). Based on deletion analysis, the portion of CTR1 that interacts with ETR1 roughly aligns with the regulatory region of Raf kinase (Clark *et al.*, 1998). CTR1 acts in the pathway of ETR1 and ERS1 (**Fig. 2**) and suggests that these interactions could be involved in the regulation of CTR1.

ein3 mutants show a loss of ethylene-mediated effects including gene expression ethylene regulated-genes, the triple response, cell growth inhibition, and accelerated senescence (Chao *et al.* 1997). The *EIN3* gene encodes a novel nuclear-localized protein that contains a highly acidic domain at N-terminus, five small clusters of basic amino acids throughout the EIN3 polypeptide, a proline-rich domain, and an asparagine-rich domain at C-terminus (Chao *et al.*, 1997). They have also reported that three EIN3-LIKE (EIL) proteins share sequence similarity, structural features, and genetic function with EIN3. *EIN3, EIL1* and *EIL2* are able to complement ein3, and overexpression of *EIN3* or *EIL1* in wild-type or *ethylene-insensitive 2* (*ein2*) plants confers constitutive ethylene responsive phenotypes, indicating that they are members of the ethylene signaling pathway, and activate the pathway in the absence of ethylene and/or in the absence of a functional EIN2 protein. Thus, EIN3 and EIL1 most likely act after EIN2 (Chao *et al.*, 1997; **Fig. 2**). EIN3 is both necessary and sufficient for activation of all known responses mediated by the ethylene pathway. EIN3 and EILs might activate the target genes directly or indirectly via transcription factors such as ERFs (ethylene responsive transcription factor, Suzuki *et al.* 1998; former name EREBP, ethylene-responsive element binding

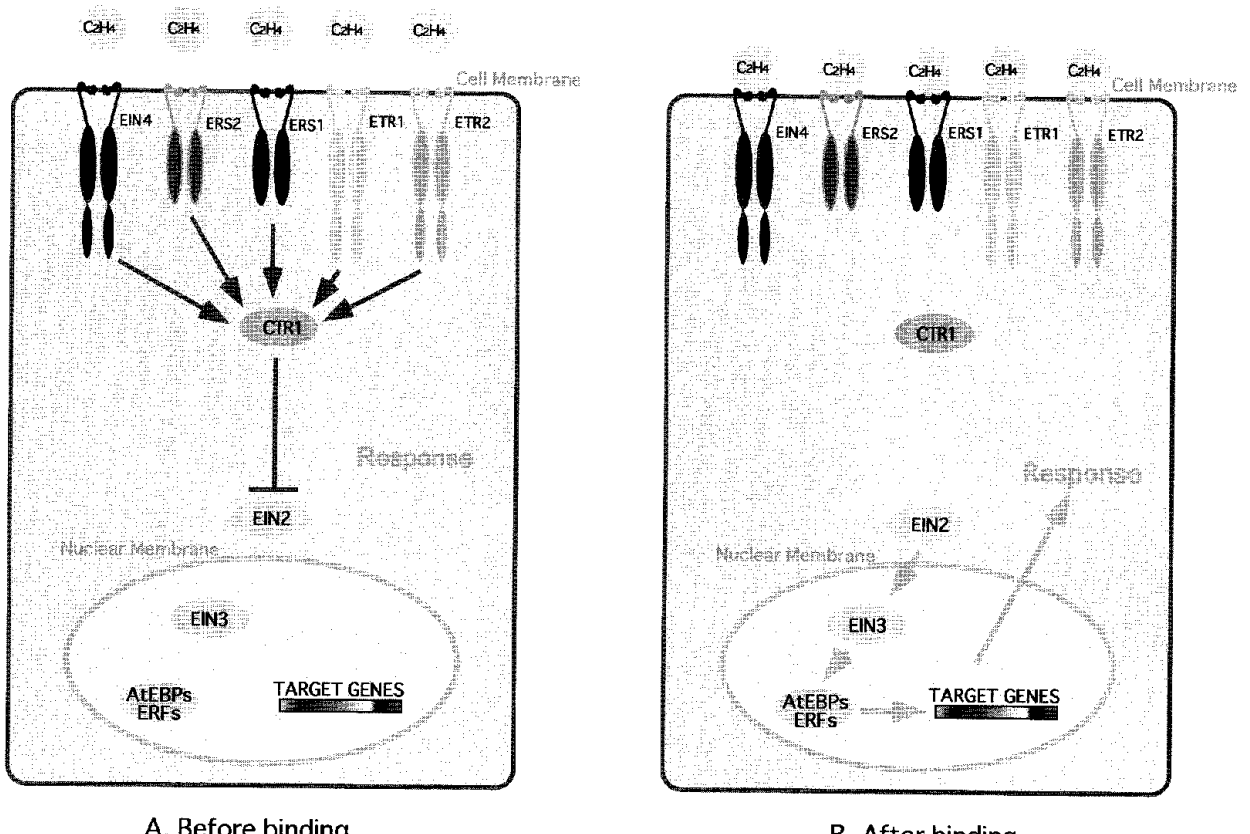

Fig. 2 The schematic diagram of the pathway of ethylene signal transduction in *Arabidopsis*. In the receptor inhibition model, receptor isoforms signal to CTR1 in the absence of ethylene. Ethylene inhibits this signaling and CTR1 is inactivated. Then, EIN2, EIN3 and EILs are activated and ethylene responses occur. Mutants which lost the ethylene-binding ability would continue to activate CTR1 in the presence of ethylene and thus suppress response pathways downstream from CTR1.

protein, Ohme-Takagi and Shinshi, 1995), which mediate ethylene response gene activation in tobacco, and related gene products, AtEBPs, (EBP, ethylene-responsive element binding protein) of *Arabidopsis* (Buttner and Singh 1997; H. Shinshi, personal communication; **Fig. 2**) (Solano *et al.*, 1998a, b). However, the orders of action and functions for other downstream ethylene response genes, such as *EIN5*, *EIN6* and *EIN7*, are still unknown.

Through the phenotypic analysis of multiple recessive mutants in regarding ethylene receptor genes, Hua and Meyerowitz (1998) have suggested that the ethylene receptors positively regulate CTR1 in the absence of ethylene, and that ethylene binding cancels this interaction (**Fig. 2**). In the absence of ethylene, therefore, an active form of CTR1 inhibits downstream components and ethylene responses. CTR1 is inactive in the presence of ethylene, and then downstream components are activated and ethylene responses occur.

Plant development and receptor genes

In general, each ethylene receptor gene in plants has its particular expression pattern in each tissue. Five ethylene receptor genes were expressed ubiquitously in *Arabidopsis* plants, but each gene was strongly expressed in its particular tissues and stages (Hua and Meyerowit, 1998, **Table 1**). Five genes in tomato and two genes in melon were also differentially regulated throughout plant development (Lashbrook *et al.*, 1998, Klee *et al.* 1998, Sato-Nara *et al.* 1999a, Tieman and Klee, 1999). Regulation of each ethylene receptor gene by ethylene is different among distinct members in the same species (e.g. between *ETR1* and *ETR2* in *Arabidopsis*), or among the homologs in different species (e.g. *ETR1* in *Arabidopsis* and *Cm-ETR1* in melon). The expression of *ETR2*, *ERS1* and *ERS2* (Hua *et al.*, 1998), *NR* (Wilkinson *et al.*, 1995; Payton *et al.*, 1996) and *RP-ERS1* (Vriezen *et al.*, 1997), *CERS* (Cubelles *et al.* 1998), and *Cm-ETR1* (Sato-Nara *et al.* 1999b) are up-regulated by ethylene, while those of *ETR1* and *EIN4* (Hua *et al.* 1998), *eTAE1* (Zhou *et al.*, 1996a, b; corresponding to *LeETR1*), *PE-ETR1* and *PE-ERS1* (Mita *et al.* 1998), and *Cm-ERS1* (Sato-Nara *et al.* 1999b) are not markedly affected by ethylene treatment. In addition to ethylene, expression of *RP-ERS1* is regulated by environmental factors such as flooding, O_2 and CO_2 concentrations (Voesenek *et al.*, 1997; Vriezen *et al.*, 1997). Thus, expression of each ethylene receptor gene is regulated by many developmental and environmental factors, and its pattern is characteristic of each gene in spite of its redundant function. Here, we focus on the expression of the ETR1-like subfamily, especially expression of those in melon, and discuss common and distinct characteristics between melon and other species.

As shown in **Fig. 3**, the level of *Cm-ERS1* mRNA in flesh dynamically increased during fruit enlargement, and decreased at the end of enlargement (Sato-Nara *et al.* 1999a). Such an increase of mRNA for tomato ethylene receptor genes is not observed in early-developing fruit (Lashbrook *et al.* 1998). When melon fruits enlarge, the pericarp cells mainly divide in the early developmental stage, and mainly expand during the following stage (Higashi *et al.*, 1999), and high accumulation of *Cm-ERS1* mRNA is observed during the stage of cell expansion in the pericarp (Sato-Nara *et al.* 1999a). A high level of *Cm-ERS1* mRNA in hypocotyls of etiolated seedlings and younger leaves in melon was also observed (Sato-Nara and Ezura, unpublished results). In RNA in situ hybridization with various *Arabidopsis* tissues, the signals of *ERS1* appeared higher in younger and smaller cells like leaves, etiolated seedlings, and roots than they did in older and more expanded cells (Hua *et al.*, 1998). The high level of *ERS1* mRNA in younger and smaller cells of *Arabidopsis* is consistent with a higher level of *Cm-ERS1* mRNA in the pericarp of the young and expanding fruit, hypocotyl, and expanding leaves in melon. An increase of receptors may reduce the sensitivity (Hua and Meyerowitz, 1998), and the increase of ERS1 and Cm-ERS1 might be related to the regulation of cell expansion through changing ethylene sensitivity. There is a possibility that the different distribution of the ethylene receptor within tissues (or a cell) causes the different sensitivity to ethylene and different responses among tissues (or parts of a cell).

The expression of *Cm-ETR1* differed from those of *LeETR1* and *LeETR2* (**Fig. 3**; Lashbrook *et al.*, 1998; Sato-Nara *et al.*, 1999a). *LeETR1* is expressed constitutively in all plant tissues, and *LeETR2* is expressed at low levels throughout the plant except for high levels in imbibing tomato seeds prior to germination (Lashbrook *et al.*, 1998). In melon, however, the level of *Cm-ETR1* mRNA changes more dynamically in the fruit tissues during the developmental stage, and *Cm-ETR1* has an expression pattern different from that of *Cm-ERS1* (**Fig. 3**; Sato-Nara *et al.*, 1999a). The level of *Cm-ETR1* mRNA was high in the seed and placenta of developing and fully enlarged fruit, while the increase of the mRNA level in the pericarp of fruit was concurrent with the beginning of ethylene production during ripening (**Fig. 3**). Kato *et al.* (1997) reported that the levels of mRNAs for ACC

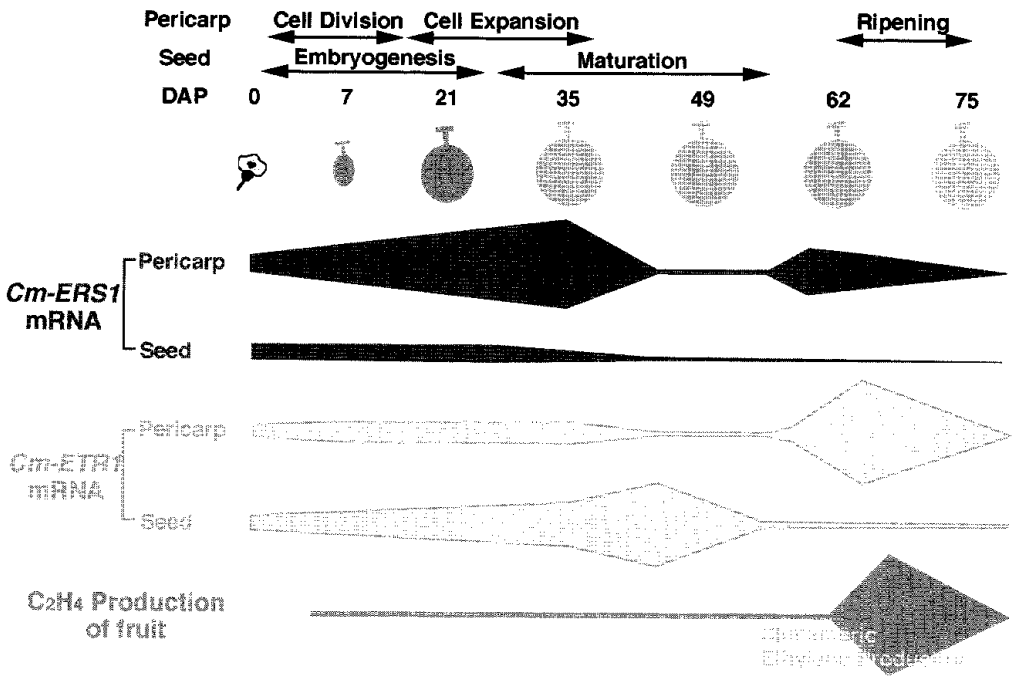

Fig. 3 The schematic diagram of expression of *Cm-ERS1* and *Cm-ETR1* during fruit development in melon. An increase of the *Cm-ERS1* mRNA paralleled that of the fruit size is observed at the middle stage of fruit enlargement, and a dramatic decrease at the end of fruit enlargement. Similarly, the *Cm-ETR1* mRNA is increased 1 day after pollination and is accumulated at a constant level during fruit enlargement. In the pre-climacteric fruit, the *Cm-ERS1* mRNA level in the pericarp is slightly increased, while the *Cm-ETR1* mRNA level is still low. The *Cm-ETR1* mRNA level in the pericarp is markedly increased in the climacteric fruit. The *Cm-ERS1* mRNA level in the pericarp in the climacteric fruit is the same as that in the pre-climacteric fruit, and decreases as fruit ripens. In the developing fruit, the level of *Cm-ERS1* mRNA is much higher in the pericarp than in the immature seeds, while that *Cm-ETR1* mRNA is lower in the pericarp than in the other. In fully enlarged fruit, the level of *Cm-ERS1* mRNA is lower in all tissues and that of *Cm-ETR1* mRNA is lower in the pericarp, while the level of *Cm-ETR1* mRNA is high in the seeds.

oxidase and auxin-responsive ACC synthases (ME-ACS2, ME-ACS3) were increased in seeds and placenta of immature fruit, and that the level for wound ACC synthase (ME-ACS1) was increased in the flesh and placenta during ripening. The stages and tissues showing expression of mRNAs for ACC synthases and ACC oxidase are similar to those showing expression of *Cm-ETR1* mRNA (**Fig. 3**; Sato-Nara *et al.* 1999a), suggesting that the expression of *Cm-ETR1* and the genes for enzymes of ethylene biosynthesis were closely related to each other, and that the mechanism for regulation of fruit development involves the reception and biosynthesis of ethylene.

The tomato *NR* was expressed at low levels during the early stage of fruit development, and increased during maturation (Lashbrook *et al.*, 1998). During fruit maturation and ripening, *NR* seems to be expressed in a manner more similar to *Cm-ETR1* than to *Cm-ERS1*, because the increase of both *NR* and *Cm-ETR1* mRNAs occurs simultaneously with the climacteric burst of ethylene production, while the increase of *Cm-ERS1* mRNA occurs preceding the burst (**Fig. 3**; Wilkinson *et al.*, 1995; Lashbrook *et al.*, 1998; Sato-Nara *et al.* 1999a). Furthermore, expression of both *NR* (Wilkinson *et al.*, 1995) and *Cm-ETR1* (Sato-Nara *et al.*, 1999b) are up-regulated by ethylene in particular stages (maturation stage in tomato; early ripening stage in melon) of fruit development, while those of *eTAE1* (Zhou *et al.*, 1996b) and *Cm-ERS1* (Sato-Nara *et al.*, 1999b) are not. It is yet unknown why the same types of ethylene receptor genes are regulated quite oppositely between tomato and melon by ethylene. The regulation of ethylene receptors by ethylene may be changed during fruit development, because expression of *NR* in immature fruits of tomato (Wilkinson *et al.*, 1995) and *Cm-ETR1* (Sato-Nara *et al.*, 1999b) in late-ripe fruit of melon is not markedly affected by ethylene treatment like *PE-ETR1* and *PE-ERS1* in passion fruit (Mita *et al.*, 1998). Further studies on each role of an isoform in fruit development in each plant are required to elucidate the cause of the different pattern of the ethylene receptor genes among the different species and/or different developmental

stages.

How do ethylene receptors work to alter ethylene responses? Hua and Meyerowitz (1998) proposed the model that the ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis*, but this cannot explain the phenomenon that receptors are increasing at a time when tissue sensitivity to ethylene is also increasing. Vriezen *et al.* (1997) reported that enhanced levels of ethylene and a low O_2 concentration both stimulate petiole elongation in *Rumex palstris*. They also reported that the treatment with low O_2 concentrations causes both the increase of *RP-ERS1* mRNA level and the enhancement of petiole extension by increasing the sensitivity to ethylene without changing the rate of ethylene production. The level of *NR* mRNA, the sensitivity to ethylene and ethylene production also increase during fruit maturation in tomato (Wilkinson *et al.*, 1995; Lashbrook *et al.*, 1998). Investigation of the relationship between changes of ethylene production, ethylene sensitivity and the levels of mRNAs and proteins of all members of ethylene receptor family is required to clarify how each type of ethylene receptor gene shares a common function and its particular role.

Genetic engineering of ethylene sensitivity

Current Progress in the understanding of ethylene perception and the signal transduction pathway at the molecular level provides potential methods for the genetic engineering of ethylene sensitivity in plants. Transgenic plants with altered ethylene sensitivity should promote the understanding of how plants regulate the sensitivity to ethylene. They are also useful for crop improvement.

On the basis of the model of ethylene perception and the signal transduction pathway in plants (**Fig. 2**), various strategies for altering the ethylene sensitivity have been provided. In the case of using the ethylene receptor genes, there are three potential methods and one proven, reliable method (**Table 2**). When one of the receptor genes is expressed in a sense direction, the amount of the ethylene receptor protein should increase in the transgenic plants. Consequently, the transgenic plants should show reduced sensitivity to ethylene because a large amount of ethylene is required to reject the inactivation of CTR1 by ethylene receptors, which result in the activation of EIN2 in the transgenic plant, compared to the wild type plant. We tried to obtain transgenic plants expressing the sense gene of melon *Cm-ERS1*. However, the number of transgenic plants with the sense gene is lower than those with the antisense gene or mutant gene, suggesting some function of the sense gene in the transgenic plant (Ezura *et al.*, unpublished results). Although we do not know why the frequency of production of transgenic plants with *Cm-ERS1* is so low, physiological analysis of the transgenic plants with *Cm-ERS1* may prove the clue to understant a function of the gene.

On the other hand, transgenic plants expressing the antisense gene of ethylene receptor should show increased sensitivity to ethylene, because the transgenic plants will require a small amount of ethylene

Table 2. Potential methods for altering the ethylene sensitivity using the components of ethylene signal-transduction pathway in transgenic plants.

Genes used for transformation	Methods	Expected sensitivity to ethylene	Related references
<Receptor>			
ETR1, ERS2, ETR2, ERS2, EIN4, and the homologs	Expression of sense gene	Reduced sensitivity	
	Expression of antisense gene	Increased sensitivity	
	Expression of gene mutated in the metal binding residue	Insensitivity	Chang *et al.* (1993) Wilkinson *et al.* (1997)
	Expression of gene mutated in the residue responsible for autophosphlrylation	Reduced sensitivity	
<Other components>			
CTR1, and the homologs	Expression of sense gene	Reduced sensitivity	
	Expression of antisense gene	Constitutive sensitivity	Kieber *et al.* (1993)
EIN2, EIN3, and the homologs	Expression of sense gene	Constitutive sensitivity	Chao *et al.* (1997) Alonso *et al.* (1999)
	Expression of antisense gene	Reduced sensitivity	

for rejecting the downregulation of EIN2, compared to the wild type plants. However, through the screening of transgenic *Arabidopsis* plants with the antisense gene of the melon ethylene receptor gene, *Cm-ERS1*, we have obtained transgenic plants showing a phenotype identical to *etr1* mutant, the ethylene insensitive *Arabidopsis* mutant (Ezura *et al.*, unpublished results). Further analysis of the transgenic plants is required.

From the analysis of ethylene receptor proteins and the mutant plants, ethylene receptor proteins have two important residues for their function, residues responsible for ethylene binding and histidine kinase activity. Therefore, if we introduce the mutation in those residues and transform those genes to plants, it is expected that the transgenic plants will show the altered sensitivity to ethylene. The receptor protein with a mutation in ethylene binding residue can not bind ethylene and constitutively activate CTR1 even in the presence of ethylene. The transgenic plants expressing the mutant receptor should confer insensitivity to ethylene. Actually *etr1* mutants of *Arabidopsis* have the mutation in such sites of ETR1 protein (Chang *et al.*, 1993). When the *etr1-1* gene was introduced into tomato and petunia, both transgenic plants showed insensitivity to ethylene (Wilkinson *et al.*, 1997). We have obtained the same results using melon ethylene receptor gene (Ezura and Yuhasi, Unpublished results). We introduced a point mutation in the ethylene binding residue of melon receptor, Cm-ERS1, and transformed the mutant gene to *Arabidopsis*. As expected, transgenic *Arabidopsis* plants showed reduced sensitivity to ethylene.

On the other hand, when the ethylene receptor protein with a mutation in the residue responsible for histidine kinase activity is overexpressed, competition of ethylene binding between mutants and wild type proteins could occur. Consequently, a large amount of ethylene is required for the cancellation of CTR1 activation. The transgenic plants should show reduced sensitivity to ethylene.

In the case of using the components of the ethylene signaling pathway, two strategies, expression of sense or antisense genes, will be effective in regulating ethylene sensitivity. The *ctr1* null mutant showed constitutive sensitivity to ethylene (Kieber *et al.*, 1993). Therefore, if we express *CTR1* and its homologs in a sense direction, the transgenic plants might show the reduced sensitivity. However, if we express the gene in an antisense direction, the transgenic plants should show increased sensitivity. *ein2* and *ein3* mutants showed insensitivity to ethylene (Guzuman and Ecker, 1990; Roman *et al.*, 1995). Therefore, if we overexpress *EIN2*, *EIN3* and the homologs in a sense direction, the transgenic plants should show the constitutive response to ethylene while, if we express those in an antisense direction, the transgenic plants should show the reduced response. Actually, overexpression of EIN3 in *Arabidopsis* conferred the constitutive ethylene response (Chao *et al.*, 1997).

Through the analysis of ethylene insensitive mutants and transgenic plants of *Arabidopsis*, tomato and petunia (Bleecker *et al.*, 1988; Lanahan *et al.*, 1994; Wilkinson *et al.*, 1997; Ogawara *et al.*, 1999), it is expected that transgenic plants conferring reduced sensitivity or insensitivity to ethylene show the altered phenotypes like long shelf-life of fruits, flowers and leaves, and delayed growth transition from vegetative to reproductive growth. These alterations will be useful for a variety of crops with commercial importance.

The ripening of climacteric fruits, including apple, avocado, melon, tomato, banana, peach and persimmon (Abeles *et al.*, 1992), and the senescence of lettuce (Rood, 1956) and broccoli (Tian *et al.*, 1994) are rapidly progressed by ethylene. Abscission in cut flowers like carnation, rose, snapdragon and sweet pea, and potted plants like Christmas cactus, *Impatiens* and *Pelargonium* are also progressed by ethylene (Abeles *et al.*, 1992). In order to prevent the ripening, senescence and abscission of these crops, a variety of storage systems like controlled atmosphere storage and hypobaric storage have been developed, and these systems sometimes result in the crops becoming more costly. Alternatively the crops have been harvested at the immature stage for extending the postharvest life. However, the harvest at the immature stage results in problems like low quality of fruits and less volume of cut flowers. Crops conferring the insensitivity or reduced sensitivity to ethylene could reduce the postharvest loss. They allow storage of the crops without specialized storage systems and such crops may be harvested at the mature stage. This results in reducing cost and increasing the quality. Additionally these transgenic plants will contribute to reduce postharvest losses in the countries that do not have the storage systems for crops. Bolting of chinese cabbage, lettuce and spinach limits the period of their production. If we can delay the bolting time of these crops, it will be possible to cultivate these crops for a longer period. Since the ethylene insensitive plants tend to delay bolting time (Bleecker *et al.*, 1988; Ogawara *et al.*, 1999), genetically engineered crops conferring the insensitivity or reduced sensitivity to ethylene could contribute to overcoming this problem as well.

Perspective

Ethylene receptor genes and the homologs from a variety of plant species have been isolated and characterized after the successful isolation of *Arabidopsis* ethylene receptor genes. Structural analysis of *Arabidopsis* ethylene receptor genes provides information about the function of each domain. Through this analysis, transgenic *Arabidopsis* plants with a mutated ethylene receptor gene have been produced and have demonstrated that they are insensitive to ethylene. However, we still do not know how plants regulate their sensitivity to ethylene. Understanding molecular events that lead to the alteration of ethylene sensitivity in transgenic plants with ethylene receptor genes may be a clue to elucidate the mechanism of how plants regulate the sensitivity to ethylene.

It is possible that each ethylene receptor has a specific role in plant development and responses based on the expression analysis of each gene.

However, we still do not know the specific role of each ethylene receptor, although we have some information about differential and specific mRNA expression of ethylene receptor genes (Hua and Meyerowitz, 1998, Lashbrook *et al.*, 1998, Tieman and Klee, 1999, Sato-Nara *et al.*, 1999a). Therefore, it is important to look for the precise role of each ethylene receptor in plant development and responses, providing a significant goal of this field.

In addition, since the differences in the expression of each ethylene receptor gene may account for the regulation of sensitivity to ethylene, it is necessary that the mechanisms of how plants regulate the expression of ethylene receptor genes be understood. Consequently, analysis of promoters of ethylene receptor genes and the *trans*-acting factors will be required. The knowledge obtained by such analysis will contribute not only to the explanation of how plants regulate their sensitivity to ethylene, but also to the application of genetic engineering of ethylene sensitivity to make crop improvements.

Ethylene is implicated in the development of adaptations and stress responses in plants. They include the shelf-life of climacteric fruits and flowers, the transition from vegetative growth to reproductive growth, and plant-microbe interaction. Therefore, if we can effectively alter the sensitivity to ethylene, the methods will be a significant tool for crop improvements.

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