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Towards the Development of Long-Life Crops by Genetic Engineering of Ethylene Sensitivity

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ABSTRACT Food production is a major role of agriculture. It has been projected that the world population continues to increase by the middle of the 21st century, and the population growth results in raising a serious problem of food shortage. Thus we have to increase food as possible. A considerable amount of crops have been abandoned due to short-life after postharvest. Ethylene is a factor responsible for the postharvest loss in crops, especially horticultural crops. If we can reduce ethylene production or sensitivity by genetic engineering, we can develop, so called, "long-life crop" conferring long postharvest lives. During last two decades, intensive research for molecular dissection of ethylene biosynthesis has been carried out, and the researchers have succeeded in engineering ethylene productivity in some crops. On the other hand, after the successful isolation of *Arabidopsis* ethylene receptor gene *ETR1*, the homolog genes have been isolated in various plant species. Currently the characterization of these genes and alteration of ethylene sensitivity using the genes are in progress. This review summarizes current progress in the analysis of these genes, and discusses genetic engineering of ethylene sensitivity using these genes.

Key words: Crop improvement, ethylene receptor, ethylene sensitivity, genetic engineering

Introduction

World population is rapidly increasing so far. According to 2000 World Population Data Sheet, over the next half century, world population will be increased by 9,039 millions. Food shortage is also predicted, accompanied with this population growth. Therefore we have to increase the amount of food production which corresponds to the projected population. How can we increase the amount of food production? There are two possible strategies: to actually increase the crop yield by conventional methods or to increase the amount of consumable crops by reducing the postharvest loss of produced crops (Figure. 1). The latter strategies allow increase available foods without an actual increase in crop production.

Ethylene regulates senescence and ripening process that accounts for the postharvest loss of crop (Abeles et al. 1992). Therefore, an alteration of ethylene biosynthesis and response is a valuable target for the genetic engineering of crops. Ethylene biosynthetic pathway was first established in apple fruit (Adam and Yang 1979). After this, genes related to this pathway have been isolated and characterized (reviewed by Imaseki 1999). Using these genes, genetic manipulation of ethylene biosynthesis was achieved in tomato (Hamilton et al. 1990) and melon (Ayub et al. 1996). The resultant fruits have longer shelf lives and have been expected to reducing postharvest loss of these crops.

The molecular mechanism underlying ethylene sensitivity in plants has been studied by using the genetic approach with *Arabidopsis* (Bleecker and Schaller 1996). Through the analysis of ethylene-response mutants in *Arabidopsis* (Bleecker et al. 1988; Kieber et al. 1993), five genes involved in ethylene response have been isolated and characterized (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). Together with these results, a model of ethylene perception and signal transduction pathway has been proposed (Bleecker et al.

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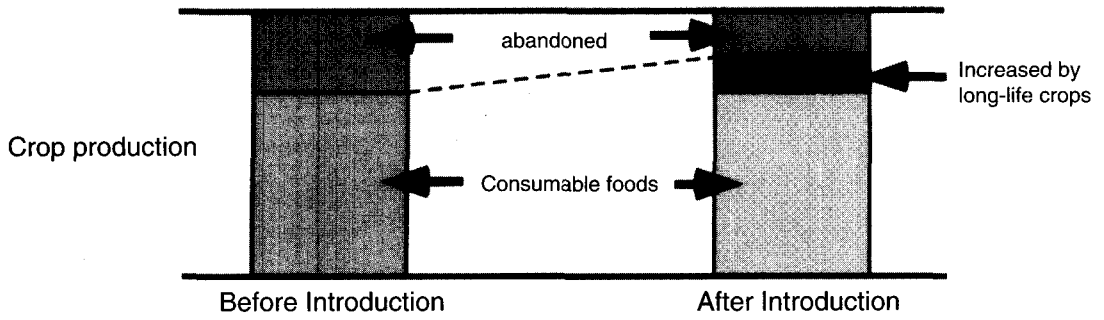

Figure 1. Effect of long life crops on increasing the consumable foods.

1998; Johnson and Ecker, 1998; Solano and Ecker 1998; Bleecker 1999). The model has stimulated the production of transgenic plants with altered ethylene sensitivity. It is likely that these plants show extended-postharvest life.

We are studying how melon regulates their sensitivity to ethylene, and try to apply those knowledge to crop improvement. This review summarizes current progress in the analysis of ethylene receptor genes and discusses the potential strategies to regulate ethylene sensitivity using these genes.

Cloning and characterization of ethylene receptor genes

Ethylene-response mutants of *Arabidopsis* and their corresponding genes coding for ethylene receptors have been identified and characterized (Bleecker et al. 1988; Chang et al. 1993; Guzman and Ecker 1990; Kieber et al. 1993; Roman et al. 1995; Alonso et al. 1999), including *ETR1* (Chang et al. 1993), *ERS1* (Hua et al. 1995; Hua and Meyerowitz 1998; Hall et al. 1999, 2000), *ETR2* (Sakai et al. 1998), *EIN4* and *ERS2* (Hua et al. 1998). 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. 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 or putative catalytic residues within the kinase domain. It seems however unlikely that members of the *ETR2*-like subfamily function as a histidine kinase since they lack most of the consensus motifs of histidine kinase. ETR1, ETR2 and EIN4 have a domain that receives phosphate from the histidine kinase (transmitter) domain, while ERS1 and ERS2 lack the receiver domain. The receiver domains of ETR1, ETR2 and EIN4 contain three residues which are important for phospholylation. 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 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. 1999a), the *PE-ETR1* and *PE-ERS1* cDNAs from *Passiflora edulis* (Mita et al, 1998) and *CS-ETR1*, *CS-ETR2* and CS-ERS cDNAs from *C. sativus* (Yamasaki et al. 2000). In addition, sequences of putative ethylene receptor genes and cDNAs from several plants have been registered in databanks (reviewed by Sato-Nara et al. 1999b).

Ethylene signal transduction pathway in plants

A model of ethylene signal transduction pathway in *Arabidopsis* has been proposed (Figure. 2, modified from Hua and Meyerowitz 1998). CTR1 acts at or downstream of ETR1, ERS1, ETR2, ERS2 and EIN4, and that

EIN2 and EIN3 act after CTR1. 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. 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*. Based on deletion analysis, the portion of CTR1 that interacts with ETR1 roughly aligns with the regulatory region of Raf kinase. Through the phenotypic analysis of multiple mutants regarding ethylene receptor genes, Hua and Meyerowitz (Hua et al. 1998) have suggested that ethylene receptors positively regulate CTR1 in the absence of ethylene, and that ethylene binding cancels this interaction. 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.

ein3 mutants show a loss of ethylene-mediated effects including gene expression, 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. *EIN3* is able to complement *ein3*, and overexpression of *EIN3* in wild-type or *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 most likely acts after EIN2. EIN3 is both necessary and sufficient for activation of all known responses mediated by the ethylene pathway. EIN3 might activate the target genes directly or indirectly via transcription factors such as *AtEBPs* (ethylene-responsive element binding protein of *Arabidopsis*) (Buttner et al. 1997).

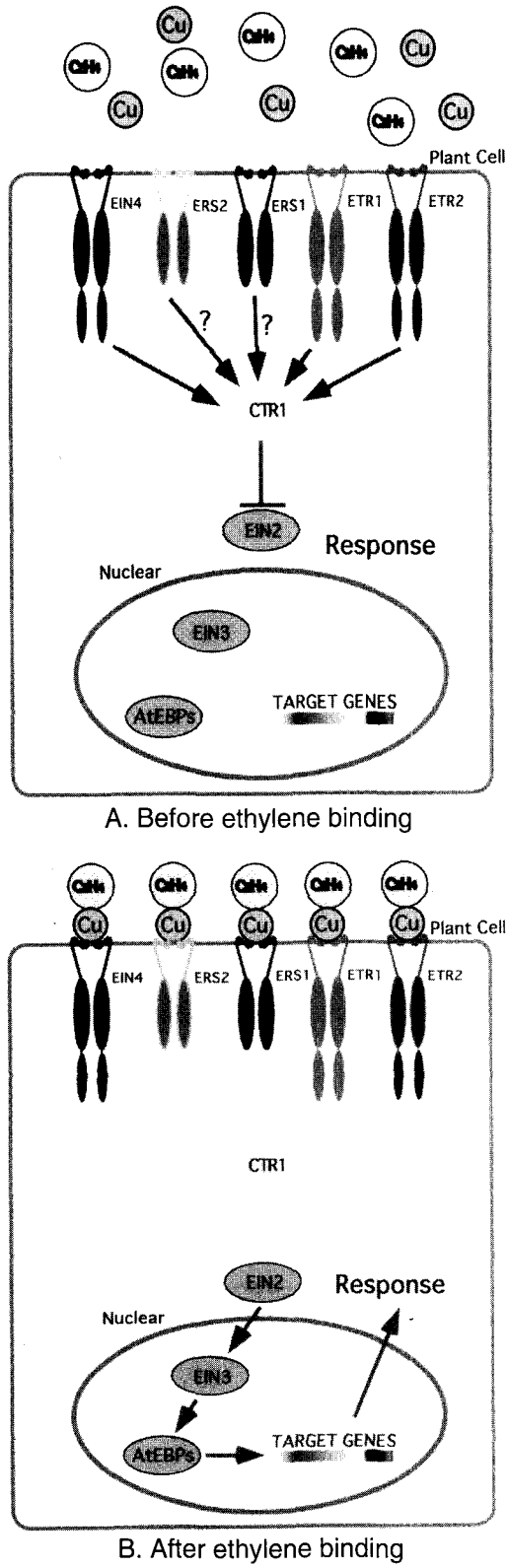

Figure 2. The schematic diagram of ethylene signal transduction pathway in *Arabidopsis*. In the receptor inhibition model, receptor isoforms signal to CTR1 in the absence of ethylene. Ethylene inhibits this signal and CTR1 is inactivated. Then, EIN2 and EIN3 are activated and ethylene responses occur.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

Regulation of ethylene receptor gene expression during plant development

The 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 of melon, *Cm-ETR1* and *Cm-ERS1*, during fruit development (Figure. 3, modified from Sato-Nara et al. 1999b).

The level of *Cm-ERS1* mRNA in flesh dynamically increased during fruit enlargement, and decreased at the end of enlargement. Such an increase of mRNA for tomato ethylene receptor genes is not observed in early-developing fruit (Lashbrook et al. 1998). When melon fruit enlarge, the pericarp cells mainly divide in the early developmental stage, and 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. In RNA *in situ* hybridization with various *Arabidopsis* tissues, the signals of *ERS1* appeared higher in younger and smaller cells of leaves, etiolated seedlings and roots than 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 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.

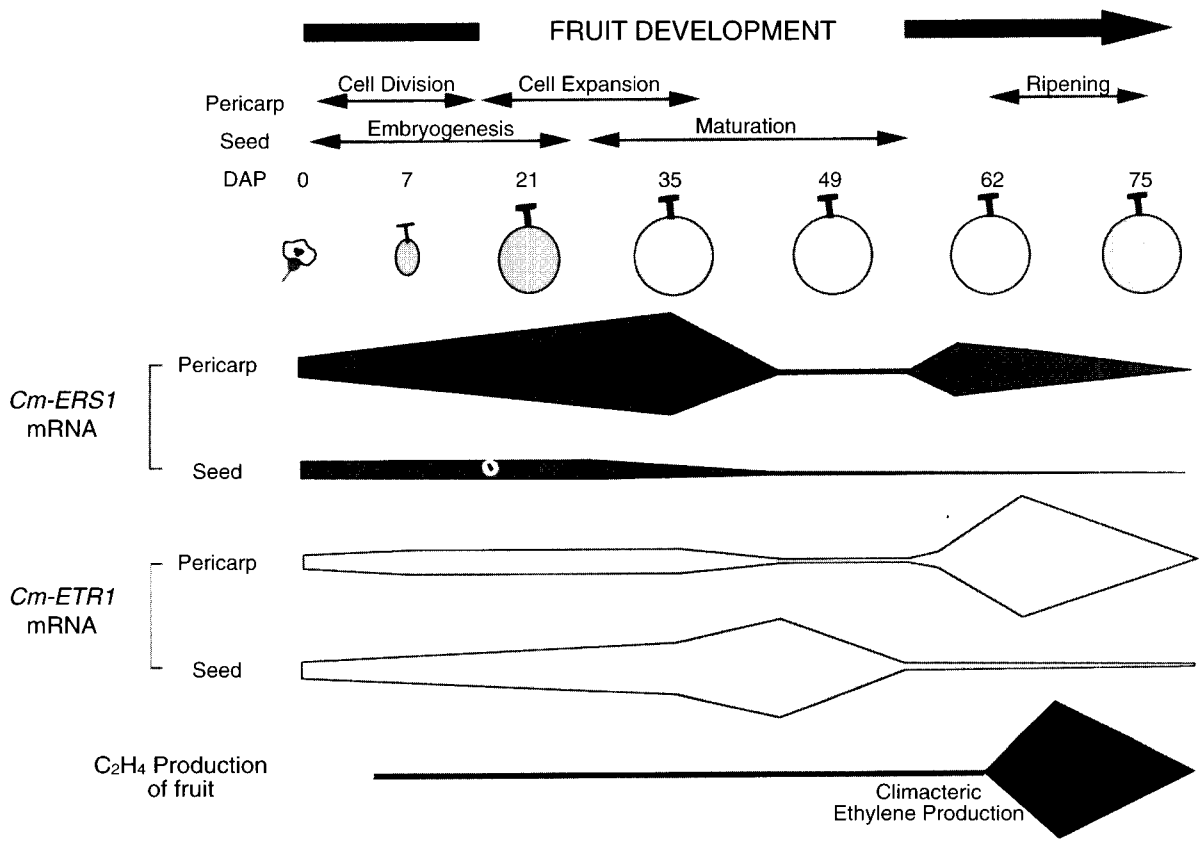

Figure 3. The schematic diagram of expression of melon *Cm-ERS1* and *Cm-ETR1* during fruit development. An increase of the *Cm-ERS1* mRNA paralleled that of the fruit size is observed at the middle stage of fruit enlargement, and a dramatic decrease at the end of fruit enlargement. Similarly, the *Cm-ETR1* 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.

The expression of *Cm-ERS1* differed from those of *LeETR1* and *LeETR2* (Lashbrook et al. 1998; Sato-Nara et al. 1999a). *LeETR1* is expressed constitutively in all plant tissues whereas *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 is changed more dynamically in fruit tissues during the developmental stage, and *Cm-ETR1* has an expression pattern different from that of *Cm-ERS1*. 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. Kato et al. (1997) reported that the levels of mRNAs for ACC oxidase and auxin-responsive ACC synthase (*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 synthase and ACC oxidase were similar to those showing expression of *Cm-ETR1* mRNA, 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 perception and biosynthesis of ethylene.

The tomato *NR* is expressed at low levels during the early stages of fruit development, but at higher levels 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 on 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 (Wilkinson et al. 1995; Lashbrook et al. 1998; Sato-Nara et al. 1999c). Furthermore, expression of both *NR* (Wilkinson et al. 1995) and *Cm-ETR1* (Sato-Nara et al. 1999c) 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. 1996) and *Cm-ERS1* (Sato-Nara et al. 1999c) 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. 1999c) 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).

Genetic engineering of ethylene sensitivity

On the basis of the model of ethylene signal transduction pathway (Figure. 2), three strategies for reducing the ethylene sensitivity have been proposed. Firstly, 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. Actually we have obtained the transgenic *Arabidopsis* expressing melon Cm-ERS1 protein, and some of those transgenic lines showed reduced sensitivity to ethylene (Ezura H. et al. unpublished results). Recently, it has been reported that transgenic tomato expressing the ethylene receptor *NR* showed reduced sensitivity to ethylene (Ciardi et al. 2000), and that expressing antisense *LeETR4* showed increased sensitivity to ethylene (Tieman et al. 2000), indicating these ethylene receptors are negative regulator of ethylene response. These results support that this first strategy is equaly useful for reducing ethylene sensitivity both in homologous and heterologous systems in plants.

Secondly, from the analysis of ethylene receptor proteins and mutant plants, ethylene receptor proteins have two residues important for their function, responsible for ethylene binding and histidine kinase activity. Therefore, if we introduce mutations in these residues, the transgenic plants should show an 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. 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, petunia and carnation, these transgenic plants showed insensitivity to ethylene (Wilkinson et al. 1997; Bovy et al. 1999). We have

obtained the same result using melon ethylene receptor gene (Ezura, unpublished results). We introduced a point mutation in the ethylene binding residue of melon receptor, Cm-ERS1, and transformed the mutant gene into *Arabidopsis*. The transgenic *Arabidopsis* plants showed reduced sensitivity to ethylene.

Finally, 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. We have obtained transgenic *Arabidopsis* with such mutation gene of *Cm-ERS1*, and the evaluation of ethylene sensitivity is in progress.

Perspective

Transgenic plants conferring reduced-sensitivity to ethylene have been obtained by introducing mutated ethylene receptor gene *etr1-1* of *Arabidopsis*, where the transgenic tomato, petunia and carnation showed long postharvest lives of fruits and flowers (Wikinson et al. 1997; Bovy et al. 1999). However, it is still unclear how the reduced-sensitivity to ethylene affects the development of transgenic plants including their commercial traits. Although the reduction of ethylene sensitivity is seems to be useful for the improvement commercial crops, we have to carefully evaluate the total performance as a crop.

Ethylene regulates various processes in plant development including seed germination, senescence, abscission, sex determination and fruit ripening, and in response to a wide variety of stresses including pathogen attack, flooding and drought (Abeles et al. 1992). In the most experiments regarding ethylene receptor genes, *35S* promoter was used as a promoter to drive the genes in transgenic plants. Consequently transgenic plants with these genes should show altered development in other traits, in addition to target trait like long-life after postharvest. In order to effectively apply these genes for crop improvement, analysis of the native promoters and other tissue/organ specific promoters will be required.

It is obvious that each ethylene receptor has a specific role and response in plant development, based on the results obtained from the expression analysis of each gene. However, the specific role of each ethylene receptor is still unknown. Therefore, it is important to look at the precise role of each ethylene receptor in plant development and response, providing a significant goal of this field. 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.

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 ripening, senescence and abscission of these crops, a variety of storage systems like controlled atmosphere storage and hypobaric storage have been developed, but these systems are costly. Alternatively, crops have been harvested at the immature stage for extending the postharvest life. However, harvest in the immature stage results in problems like low quality of fruits and less volume of cult flowers. Since crops conferring the reduced sensitivity to ethylene could reduce the postharvest loss, it would allow their storage without further storage systems, and to harvest such crops at the mature stage. These result in reducing the cost and increasing the qualities. Finally these transgenic plants will be a potential choice for overcoming the problem in food shortage in the coming century.

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