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Genetic Engineering of Melon (*Cucumis melo* L.)

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Abstract

Melon is an important fruit in the Japanese food market. Accordingly, to be a leading variety in Japan, the fruit must meet demands from growers, markets, and consumers. Melon breeders have made great efforts to incorporate such demands into breeding programs. We have developed methods for improving two important traits, shelf-life and fruit size, by genetic engineering. First, we isolated and characterized genes related to these characteristics and subsequently used the genes to develop methods for altering shelf-life and fruit size. We isolated two ethylene receptor genes, *Cm-ERS1* and *Cm-ETR1*, which we expected to be related to shelf-life, and developed methods for altering the ethylene sensitivity of the plants. We also isolated a gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, *Cm-HMGR*, which we expected to be related to fruit enlargement, and developed methods for altering fruit size. These two methods will provide basic technologies for the molecular breeding of melon.

Introduction

Melon fruit is produced year-round in Japan and is one of the most popular fruits sold, along with Japanese pea, apple, citrus fruits, and strawberries. Unlike many other fruits sold in Japan, most of the melons consumed are produced domestically. Therefore, melon breeders are striving to improve commercially beneficial aspects of the fruit.

In Japan, melon production is hindered by fungal infection and by cold growing seasons. Fusarium wilt and powdery mildew are two major fungal diseases that infect melon plants. Fusarium wilt is caused by the fungus *Fusarium oxysporum*, and several physiological races have been identified in Japan (Zitter, 1999). The main cause of powdery mildew is the fungus *Sphaerotheca fuliginea*, and a variety of physiological races have been identified in Japan (Hosoya *et al.*, 1999; 2000). Melon breeders already have lines available that are resistant to these diseases and can introduce the resistance to their lines by conventional breeding methods. In contrast, it is both difficult and time-consuming to use such methods to improve fruit size, since the current leading variety of melon has many characteristics that must be maintained. Alternative strategies include somaclonal variation and genetic transformation. In fact, somaclonal variants that produce larger fruits than the original lines during a cold growing season have been identified in melon (Ezura *et al.*, 1995).

Shelf-life is a characteristic that is of major interest to melon consumers and producers. *C. melo* is comprised of six subvarieties (Robinson and Decker-Walters, 1997), one of which, *C. melo* L. *inodorus*, also called winter melon, has fruits with an extremely long shelf-life. However, the appearance and quality of the fruit differ completely from the leading variety in Japan, *C. melo* L. *reticulatus*. In addition, genetic analyses indicate that long shelf-life is a recessive trait (Ezura, unpublished results). Therefore, the F_1 breeding program commonly used for commercial melon production in Japan would not be a practical method to enhance shelf-life.

To address these difficulties, we are studying technologies that may be used to improve melon shelf-life and fruit size. Here, we describe our current progress, as well as practical applications and further perspectives.

Genetic engineering of fruit ripening

Ethylene is essential for the ripening of climacteric fruits in several plants, such as tomato, banana,

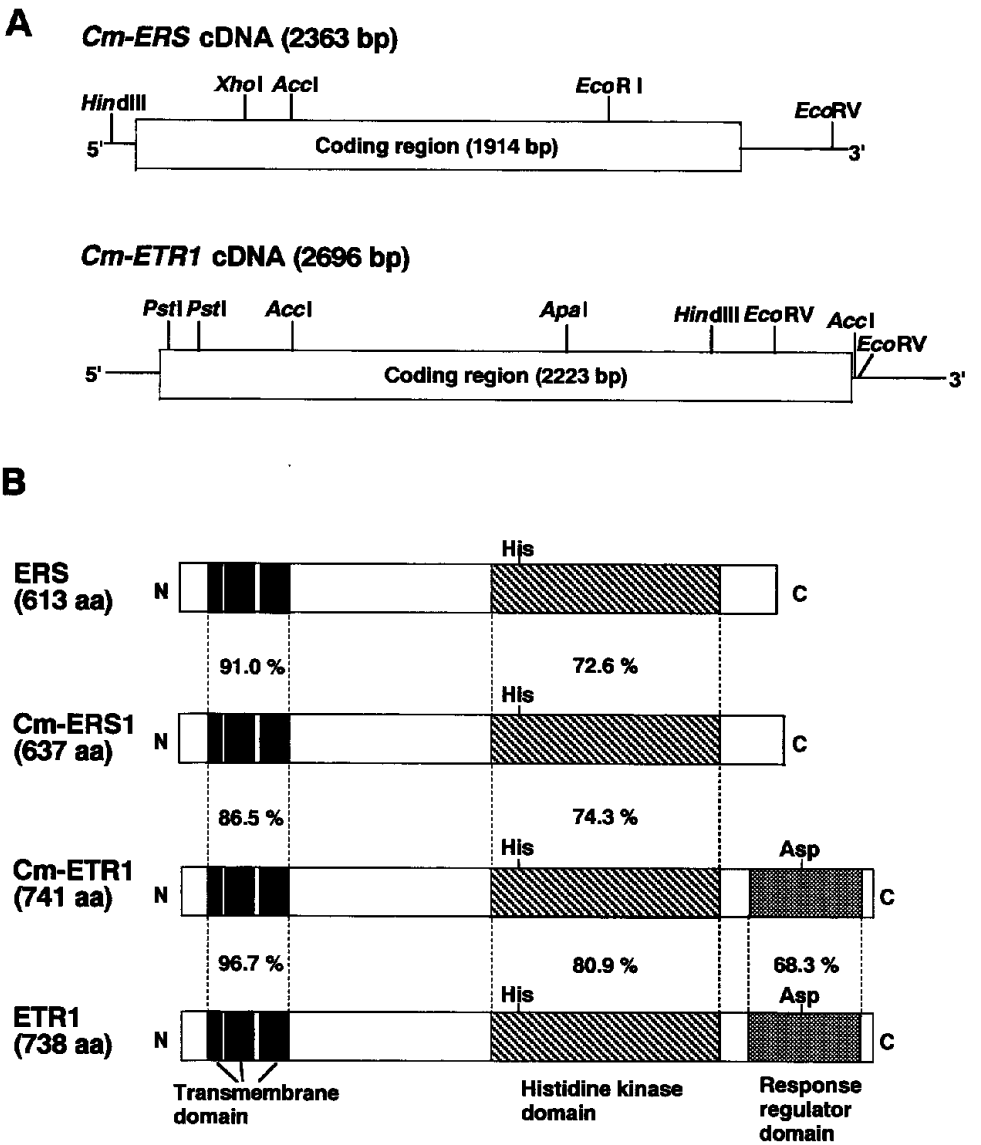

Fig. 1 A, Schematic diagram of the *Cm-ERS1* and *Cm-ETR1* cDNAs. The open boxes and the lines represent the open reading frames and flanking regions, respectively. B, Structural drawing comparing the deduced amino acid (aa) sequences of melon Cm-ERS1 and Cm-ETR1 and *Arabidopsis* ERS1 and ETR1. The shaded boxes (N-termini) represent three hydrophobic domains that are putative transmembrane domains. The shaded boxes (C termini) represent the sequences homologus to the His kinase domain and the receiver domain of bacterial two-component environmental sensor systems. The positions of the conserved His and Asp residues that may be phosphorylated *in vivo* are shown. The amino acid identity between the domains of neighboring proteins is indicated.

and melon. The sharp increase in ethylene production at the onset of ripening promotes rapid changes in sweetness, color, firmness, and aroma, and causes the fruit to change from hard and immature to soft and ripe (Seymour and McGlasson, 1993; Tucker, 1993). Two genetic engineering strategies are currently used to regulate fruit ripening. The first is designed to reduce ethylene production by altering the expression levels of genes related to ethylene biosynthesis, such as 1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS*) and ACC oxidase (*ACO*). Indeed, expression of antisense *ACO* confers improved shelf-life in melon (Ayub *et al.*, 1996). The second strategy involves reducing the ethylene sensitivity of the fruit by expressing genes related to the ethylene signal transduction pathway (Sato-Nara *et al.*, 1999a).

In this study, we developed a method to reduce ethylene sensitivity. We first isolated and characterized cDNAs for two putative ethylene receptor genes in melon (*Cucumis melo* L. *reticulatus*). These genes are homologs of the *Arabidopsis* ethylene receptor genes *ETR1* and *ERS1*. We designated them *Cm-ETR1* and *Cm-ERS1*, respectively (Sato-Nara *et al.*, 1999b). *Cm-ERS1* (GenBank accession number AF037368) is 2,363 nucleotides long, and

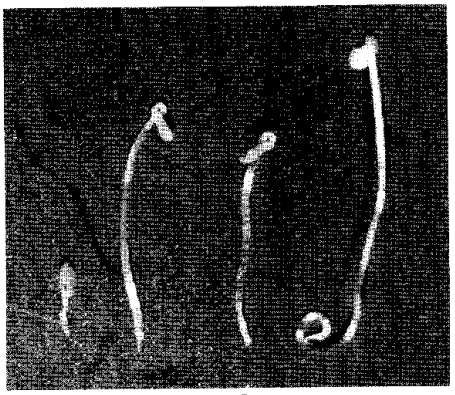

Fig. 2 Comparison of triple response to ethylene among wild type of Arabidopsis, the transgenic lines with melon ethylene receptor gene *Cm-ERS1* or a mutant version *Cm-ERS1/H70A* and the ethylene-insensitive mutant *etr1*. *Cm-ERS1/H70A* can not bind ethylene when expressed in yeast (Ezura, unpublished result). 1, Wild type; 2, *Cm-ERS1*; 3, *Cm-ERS1/H70A*; 4, *etr1*.

its open reading frame encodes a predicted protein of 637 amino acids (**Fig. 1A**). The deduced amino acid sequence contains the hydrophobic sequences and histidine kinase domain common to the ethylene receptors, but lacks the receiver domain found in ETR1, indicating that it is a homolog of ERS1 (**Fig. 1B**). *Cm-ETR1* (GenBank accession number AF054806) is 2,696 nucleotides in length, and its open reading frame encodes a predicted protein of 741 amino acids (**Fig. 1A**). The deduced amino acid sequence has the receiver domain in addition to the kinase and transmembrane domains, indicating that it is a homolog of ETR1 (**Fig. 1B**). Genomic Southern analysis revealed the existence of two ethylene receptor genes in melon, which correspond to *Cm-ETR1* and *Cm-ERS1*, and evidence for a third gene that was not isolated in the cDNA screen. Northern blot analysis revealed that the level of *Cm-ERS1* mRNA in the pericarp increased in correlation with an increase in fruit size and then decreased markedly as fruit growth slowed. In fully enlarged fruit, the level of *Cm-ERS1* mRNA was low in all tissues, while that of *Cm-ETR1* mRNA was high in the seeds and placenta. During ripening, *Cm-ERS1* mRNA again increased slightly in the pericarp of fruit before a strong increase in *Cm-ETR1*, which correlated with climacteric ethylene production. These results indicate that both *Cm-ETR1* and *Cm-ERS1* may play specific roles in ripening as well as in the early development of melon fruit and have distinct roles in particular fruit tissues at specific developmental stages. We prepared antibodies against Cm-ERS1 and Cm-ETR1 proteins and examined their temporal and spatial expression during fruit development. Western blot analysis revealed that the *Cm-ERS1* and *Cm-ETR1* genes are differentially expressed at the protein level, and that their expression is post-transcriptionally regulated (Ezura, unpublished results).

To study possible practical applications of these genes, we expressed one of the melon ethylene receptor genes and a mutant form of the same gene in *Arabidopsis* and in tobacco. Transgenic *Arabidopsis* expressing Cm-ERS1 or a mutant version, Cm-ERS1/H70A, exhibited reduced sensitivity to ethylene (**Fig. 2**), indicating that both genes are functional in regulating ethylene sensitivity in *Arabidopsis*. This result also suggests that expression of either gene confers reduced ethylene sensitivity to the transgenic plants. Transgenic tobacco plants expressing either protein also showed reduced sensitivity to ethylene, as in *Arabidopsis*. These transgenic plants will be useful for studying the molecular mechanisms by which plants regulate ethylene sensitivity. Additionally, the reduction in ethylene sensitivity should be useful for extending the postharvest shelf-life of horticultural crops. For altering the postharvest performance of several useful plants, we are introducing these ethylene receptor genes to horticultural crops, such as tomato, lettuce, and various flowers, as well as melon.

Genetic engineering of fruit size

In order to alter the size of melon fruit using genetic engineering methods, we first need to elucidate how various melon cultivars set different sizes of fruit. In order to understand this mechanism, we observed cells of two different melon cultivars (*Cucumis melo* L. *reticulatus*) during fruit development. Histological observation of the two genotypes, Fuyu A and Natsu 4 (Higashi *et al.*, 1999), revealed that although they have nearly identical genetic backgrounds, when grown under identical conditions, the fruit of Fuyu A is larger than the Natsu 4 fruit. Microscopic observation of pericarp cells at several developmental stages revealed differences in cell size between the genotypes. Despite its smaller fruit size, Natsu 4 had a slightly larger cell size than Fuyu A. The cell proliferation period of Fuyu A was longer than that of Natsu 4, which may account for the difference in cell number of the genotypes. The number of cells also defined a difference in fruit size when the two genotypes were cultivated at different temperatures. These results suggest that fruit size is determined by the level of cell proliferation in the early stage of fruit development, and that factors that regulate cell prolif-

eration are affected by temperature.

Next, we isolated genes related to cell division during the early stages of fruit development. During the screening for ethylene receptor homologs in melon, we serendipitously isolated a cDNA clone with high similarity to plant 3-hydroxy-3-methylglutaryl coenzyme A reductases (HMGRs) (Kato-Emori *et al.*, 2001) and denoted it *Cm-HMGR* (*Cucumis melo* L. *reticulatus*; GenBank accession no. AB021862). *Cm-HMGR* encodes a putative polypeptide of 588 amino acids with two transmembrane domains and a catalytic domain. Database searches revealed that Cm-HMGR is 63.7 and 70.3% similar to HMG1 and HMG2 of tomato, respectively, 77.2 and 69.4% similar to HMG1 and HMG2 of *Arabidopsis thaliana*, respectively, and 72.6% similar to tobacco *HMGR*. Functional expression in an HMGR-deficient mutant yeast demonstrated that protein products of the *Cm-HMGR* gene mediate the synthesis of mevalonate. Northern analysis revealed that the level of *Cm-HMGR* mRNA in the fruit increased after pollination and decreased strongly as enlargement concluded. During ripening, *Cm-HMGR* mRNA increased markedly in the fruit. In parallel with mRNA expression, Cm-HMGR activity also increased after pollination, whereas no Cm-HMGR activity was detected during fruit ripening. Our results suggest that *Cm-HMGR* is important during early post-pollination fruit development in melon.

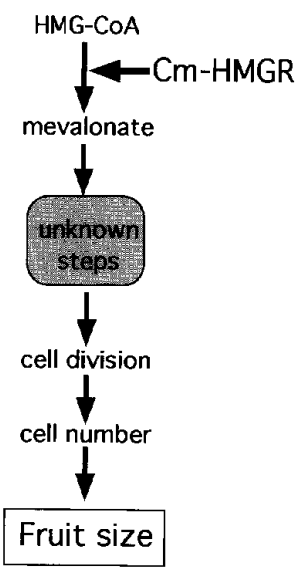

Fig. 3 Schematic model showing how the fruit size of melon is regulated by Cm-HMGR. Cm-HMGR expression enhances mevalonate biosynthesis, and the mevalonate promotes cell division through unknown step(s). Thereby, cell number in pericarp, which accounts for final fruit size, is determined.

Finally, we performed a detailed analysis of the correlation between the expression of the Cm-HMGR protein and cell division in the pericarp (Ezura, unpublished results). The results strongly support the hypothesis that the expression of Cm-HMGR is involved in determining the size of the melon fruit by regulating cell division during early fruit development. In addition, we are currently generating transgenic melon and tomato that overexpress *Cm-HMGR*, and we have evidence to support the prediction that overexpression of Cm-HMGR enhances fruit enlargement. Detailed results of these analyses will be presented elsewhere.

As a summary of our results, a model of how *Cm-HMGR* affects the fruit size of melon is shown in **Fig. 3**. Overexpression of Cm-HMGR enhances mevalonate synthesis because it catalyzes a limiting step in this metabolic pathway. Mevalonate is an essential compound in isoprenoid synthesis in plants. The end products of isoprenoid synthesis are important in numerous biological activities in plants, such as the synthesis of membrane sterols and plant growth hormones (cytokinin, abscisic acid, gibberellins, and brassinosteroids) (Chappell, 1995). Increases in these compounds enhance cell division, thereby leading to an increase in total cell count in the pericarp. Consequently, the transgenic plants set larger fruit than their non-transgenic counterparts.

Conclusions and Perspectives

In this study, we analyzed the molecular basis of ethylene sensitivity and fruit enlargement in melon. Subsequently, we developed genetic technologies to alter ethylene sensitivity and cell division in pericarp, thereby improving postharvest performance and fruit size in melon.

Ethylene is important in a number of plant developmental processes, such as seed germination, senescence, abscission, sex determination, and fruit ripening, as well as responses to a wide variety of stress conditions, including pathogen attack, flooding, and drought (Abeles *et al.*, 1992). Ethylene receptors are key proteins in the signal transduction of these responses. Mevalonate is an essential compound for isoprenoid synthesis in plants and the end products are important in many events, such as the synthesis of membrane sterols and growth hormones, electron transport, isoprenylation of proteins, and pathogen resistance mediated by sesquiterpenoid phytoalexins (Chappell, 1995; McGarvey and Croteau 1995; Weissenborn *et al.*, 1995; Westwood *et al.*, 1998). HMGR is a key enzyme in the synthesis of isoprenoids; it catalyzes

the production of mevalonate from HMG-CoA. Consequently, when such genes are expressed under the control of a constitutive promoter (such as CaMV-35S), they are likely to affect various traits of the plants. Therefore, further studies are required to express the genes specifically during fruit development. Such studies will require a molecular dissection of fruit development.

An efficient protocol for the transformation of melon is still required. Genetic transformation of melon was first reported in 1990 (Fang and Grumet); subsequently, several reports were published (reviewed by Ezura, 1999). However, commercially useful transgenic melon is still not available, for two possible reasons: genes that are useful for melon breeding have not been isolated, and transformation efficiency is low. In practical breeding programs, it is currently necessary to produce a huge number of transgenic lines expressing the gene of interest. Since we are unable to express the exogenous genes at the desired levels, we must instead select transgenic lines with the correct expression levels. Recently, we found that inhibition of ethylene biosynthesis during the co-cultivation of melon cotyledonary explants with *Agrobacterium* improved the transfer of genes from *Agrobacterium* to explants (Ezura *et al.*, 2000a). This finding may be a breakthrough for improving the efficiency of transformation in melon.

Ethylene is responsible for the postharvest performance of many horticultural crops. 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 advanced by ethylene. Abscission in cut flowers, such as carnation, rose, snapdragon, and sweet pea, and potted plants, such as Christmas cactus, *Impatiens*, and *Pelargonium*, are also progressed by ethylene (Abeles *et al.*, 1992). It has been reported that the fruit size of other crops, such as apple (Fukuda and Moriyama, 1997) and avocado (Cowan *et al.*, 1997), is regulated at the level of cell division after pollination, as in melon and tomato. Therefore, the two technologies developed in this study may be useful for improving the performance of these crops. These genetically engineered crops could result in increased food production, and might be a potential choice for overcoming food shortage crises in the coming century (Ezura, 2000b).

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