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Review

Production of male sterile transgenic plants

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Abstract Ethylene controls many physiological and developmental processes in plants, including fruit and flower development, reproductive physiology, and responses to environmental stimuli. Ethylene exerts its effects through the ethylene receptor of plants. Ethylene receptor genes have been isolated in a variety of plant species, and in early studies, these genes were used to genetically engineer fruit ripening in tomato and flower senescence in petunia and carnation. Recently, we demonstrated that the over-expression of mutated melon ethylene receptor genes affected pollen development and induced a male sterile phenotype in transgenic plants. One major concern regarding genetically modified plants is the transgene flow through pollen dispersal, which may pose a potential impact to the environment, especially on genetic diversity. Therefore, the inducible male sterility system using mutated ethylene receptor genes could be a possible strategy for preventing pollen dispersal from these plants, thereby reducing the potential impact associated with transgenic plants. This review summarizes the studies on the inducible male sterility system that uses ethylene receptor genes.

Key words: Ethylene receptor gene, pollen development, transgene flow, transgenic plant.

The gaseous phytohormone ethylene controls many physiological and developmental processes in higher plants, including fruit ripening, seed germination, leaf abscission, organ senescence, pathogen responses, growth transitions, and root hair development (Abeles et al. 1992; Ogawara et al. 2003; Tanimoto et al. 1995; Theologis, 1992; Wang et al. 2002). The role of this hormone in reproductive physiology has been elucidated for early pistil development (De Martinis and Mariani 1999), pollen tube-style interaction (De Martinis et al. 2002; Wang et al. 1996), pollination-induced floral senescence (Llop-Tous et al. 2000; Tang et al. 1994), and fruit ripening (Oeller et al. 1991). The involvement of ethylene in the very early stages of female sporogenesis and fertilization has been reported in transgenic tobacco with pistil-specific suppression of the gene for the ethylene-forming enzyme, 1-aminocyclopropane-1-carboxylate oxidase (ACO; De Martinis and Mariani 1999). However, in the inflorescences of immature carnation plants, ethylene promotes the elongation of floral organs such as styles (Camprubi and Nichols 1979), and the growth of *Gaillardia* filaments can be promoted by ethylene (Koning 1983). It is likely that ethylene sensitivity in filaments differs among plant species. However, little is known about the role of ethylene in male plant function.

In *Arabidopsis*, ethylene response mutants have been identified by screening for alterations of the so-called triple response, which refers to the morphological changes in dark-grown seedlings in response to ethylene, i.e., larger radius and shorter length of hypocotyls, exaggeration of the curvature of the apical hook, and horizontal growth (Bleecker et al. 1988). The ethylene insensitive mutants, e.g., *etr1-1*, do not exhibit a triple response. The *ETR1* gene encodes a protein that is similar, at the amino acid level, to a protein of the bacterial two-component sensor response-regulator system, which acts as an ethylene receptor early in the signal transduction pathway by using the phosphotransfer mechanism established for bacterial sensors and receivers (Chang 1996; Stepanova and Ecker 2000). The *ETR1* protein has three main domains: a sensor domain with putative membrane-spanning regions, a His kinase domain, and a receiver domain.

Molecular genetic analyses of *Arabidopsis* ethylene perception mutants have resulted in the isolation of five ethylene receptor genes: *ETR1*, *ETR2*, *EIN4*, *ERS1*, and *ERS2* (Chang et al. 1993; Hua and Meyerowitz 1998; Hua et al. 1995; Sakai et al. 1998). Ethylene responses can also be suppressed by particular dominant mutations in the ethylene receptor genes. *Arabidopsis ETR1*, the first cloned gene coding an ethylene receptor (Chang et al. 1993), was identified in *etr* mutants with ethylene-insensitive phenotypes and genetic dominance (Bleecker et al. 1988; Hall et al. 1999). The genetic dominance of ethylene insensitivity was also observed in transgenic *Arabidopsis* (Chang et al. 1993), tomato and petunia (Wilkinson et al. 1997), and tobacco (Knoester et al. 1998) that expressed *etr1-1*, a mutant form of *Arabidopsis ETR1*. These results provide evidence that

an ethylene receptor gene can induce low ethylene responsiveness in both the original and heterologous plants.

We have previously isolated and characterized the melon ethylene receptor genes *Cm-ETR1* and *Cm-ERS1* (Sato-Nara et al. 1999; Figure 1), which are homologous to *Arabidopsis ETR1* and *ERS1*, respectively. It was expected that a missense mutation in *Cm-ETR1* would produce a disruption of the ethylene-binding function and reduce ethylene sensitivity in transgenic plants. As has been shown with *Arabidopsis etr1-1* (Rodriguez et al. 1999), the missense mutation changing His-69 to Ala was introduced into *Cm-ETR1*, and this mutant gene was designated as *Cm-ETR1*/*H69A* (Ezura et al. unpublished data). As anticipated, the *Cm-ETR1*/*H69A* gene conferred reduced ethylene sensitivity to the heterologous transgenic plant *Nemesia strumosa* (Cui et al. 2004). Ezura et al. (unpublished data) also introduced a point mutation into the melon ethylene receptor gene *Cm-ERS1* by changing His-70 to Ala (*Cm-ERS1*/*H70A*), which abolished the ethylene binding ability. The transgenic *Lotus japonicus* plants expressing the *Cm-ERS1*/*H70A* gene produced no fruit and showed reduced ethylene sensitivity (Nukui et al. 2004). Therefore, it was suggested that the two mutated ethylene receptor genes conferred reduced ethylene sensitivity according to the so-called dominant-negative mechanism (Rodriguez et al. 1999).

The dominant mutant *etr1-1* allele also caused major delays in fruit ripening and flower senescence when expressed in some plants such as tomato, petunia, and carnation (Bovy et al. 1999; Wilkinson et al. 1997). These studies suggest that the manipulation of ethylene receptor genes can be a useful tool for extending the shelf life of plant tissues by delaying their senescence. In transgenic tomato plants, the antisense transgene to *LeETR4*, an ethylene receptor gene of tomato, produced low receptor levels and increased sensitivity to ethylene (Tieman et al. 2000). This report suggests that an ethylene receptor can function as a negative regulator, suppressing ethylene responses in plants. Further supporting this idea, transgenic tomato plants over-expressing the ethylene receptor gene *NR* showed low ethylene responsiveness (Ciardi et al. 2000). Thus, the addition of wild-type ethylene receptor genes should reduce ethylene responsiveness. Petunia plants transformed with a mutated allele of *ERS* from *Brassica* showed extended flower longevity (Shaw et al. 2002), and broccoli plants transformed with the *boers* gene driven by the CaMV 35S promoter showed delayed senescence in leaves and floral heads (Chen et al. 2004). However, the transformants also showed pleiotropic phenotypes, such as slower plant growth, shorter plant height, and late bolting. The heterologous expression of a mutated *Arabidopsis ERS1* (Hua et al. 1995) caused delayed senescence in coriander (Wang and Kumar 2004). Thus, although mutated ethylene receptor genes have been introduced into a variety of plants, it was thought in many cases that these genes were only useful for extending the life span of transformants.

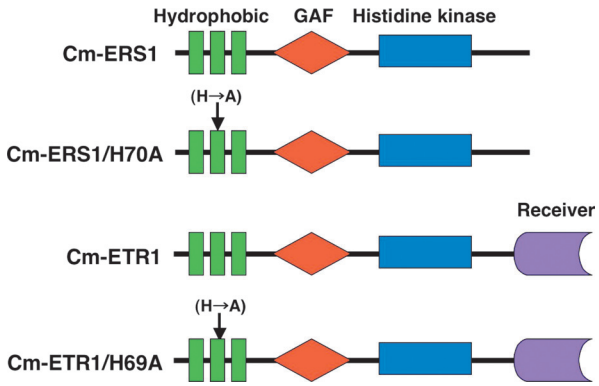

Figure 1. Structural features of the melon ethylene receptors *Cm-ETR1* and *Cm-ERS1* and of the mutants *Cm-ETR1*/*H69A* and *Cm-ERS1*/*H70A*. A missense mutation was introduced into the transmembrane domain, affecting the binding of ethylene to these proteins.

In this review, we describe the production of genetically modified plants with inducible male sterility by using mutated melon ethylene receptor genes, and we discuss the usefulness of this approach in preventing transgene flow through pollen dispersal, based on our recent studies.

Expression of a mutated ethylene receptor gene affects floral development in tobacco

In order to elucidate the functions of the melon ethylene receptor genes *Cm-ERS1* and *Cm-ETR1* in plant development, we introduced the mutated ethylene receptor genes into tobacco plants. Transgenic tobacco plants expressing the mutated melon ethylene receptor genes, *Cm-ERS1*/*H70A* and *Cm-ETR1*/*H69A*, under the control of the constitutive CaMV 35S promoter showed increased flower longevity (Figure 2), as had previously been observed in transgenic *Nemesia* plants expressing *Cm-ETR1*/*H69A* (Cui et al. 2004) and transgenic *Lotus japonicus* plants expressing *Cm-ERS1*/*H70A* (Nukui et al. 2004). Additionally, the yellowing of leaves during senescence in these transgenic tobacco plants was delayed, in comparison to the wild type. These results suggest that both mutant genes conferred reduced ethylene sensitivity to transgenic plants, just as the expression of ethylene receptor genes in heterologous plants conferred insensitivity or reduced sensitivity to ethylene in several plant species (Bovy et al. 1999; Chen et al. 2004; Knoester et al. 1998; Shaw et al. 2002; Wang and Kumar, 2004; Wilkinson et al. 1997).

In addition to reduced ethylene sensitivity, the transgenic tobacco plants expressing either *Cm-*

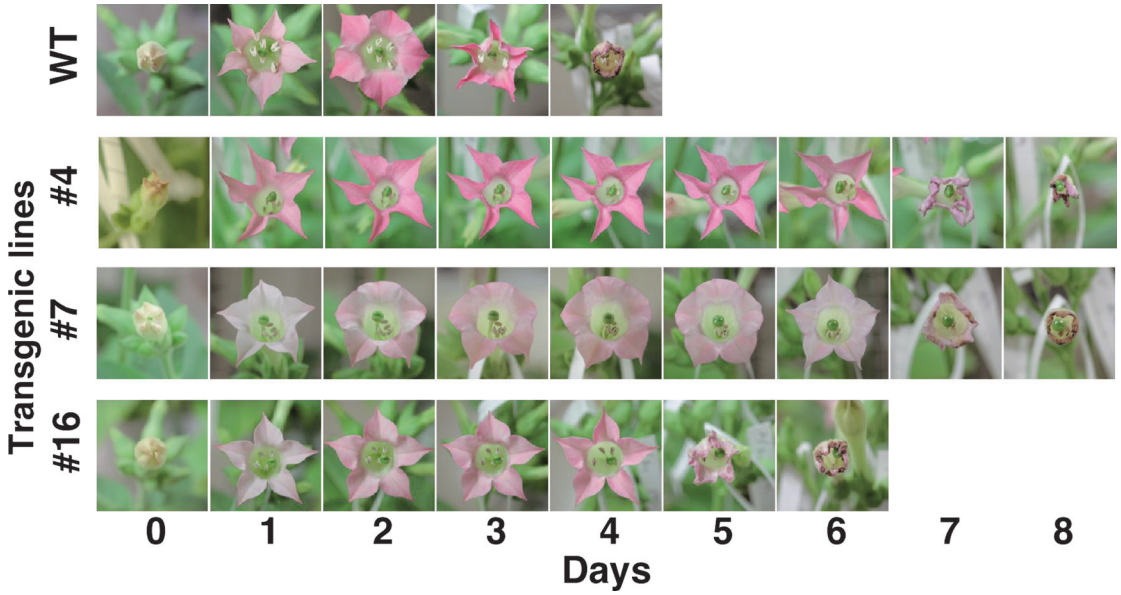

Figure 2. Flower longevity of transgenic tobacco plants carrying *Cm-ETR1*/*H69A*. Flower longevity in the transgenic lines is two to three times that of the wild type.

ERS1/*H70A* or *Cm-ETR1*/*H69A* displayed pleiotropic phenotypes that included the heterostyly type of floral architecture, a reduction in seed yields and normal pollen grains, a decrease in plant height, a higher level of anthocyanin accumulation in transgenic petals, and an infrequent delay of anther dehiscence. In tobacco plants, four ethylene receptor genes, *NtETR1*, *NtERS1*, *NTHK1*, and *NTHK2*, have been identified (Knoester et al. 1996; Terajima et al. 2001; Zhang et al. 1999, 2001a, b). It is thought that the pleiotropic phenotypes of those tobacco transformants result from coordinated action between each mutated melon ethylene receptor gene and the four tobacco ethylene receptor genes. The mRNA expression levels were not correlated with the phenotypes in these transformants. It is likely that this inconsistency is attributable to the interactions among the five ethylene receptor genes, i.e., one mutated melon ethylene receptor gene and the four endogenous genes, expressed in the transgenic plant. The phenotypes of these tobacco transformants may provide clues to elucidate this inconsistency.

It was thought that the reduced seed yields in transgenic tobacco plants with *Cm-ERS1*/*H70A* and *Cm-ETR1*/*H69A* was caused by both the modification of floral architecture and the reduction of normal pollen production. These transgenic tobacco plants displayed the heterostyly type of floral architecture, in which the anthers are located below the stigma, probably as a result of their reduced ethylene sensitivity, given that the chemical reduction of ethylene sensitivity by treatment in non-transgenic tobacco plants also resulted in a heterostyly phenotype. However, it has been shown that filament elongation in *Fuchsia hybrida* (Jones and Koning 1986) and *Ipomoea nil* (Koning and Raab 1987) can be inhibited by 1-aminocyclopropane-1-carboxylic acid (ACC) and promoted by cobalt ions, L-α-(2-aminoethoxyvinyl) glycine (AVG) and STS. These reports conflict with our results in tobacco and with the findings that ethylene promotes the elongation of floral organs such as styles in carnation plants (Camprubi and Nichols 1979) and the growth of filaments in *Gaillardia* (Koning 1983). Thus, it is likely that the ethylene sensitivity of filaments differs among plant species.

The reduction of normal pollen production was thought to be caused by delayed tapetal degeneration during anther development. Tapetal degeneration is a process of programmed cell death (PCD; Wang et al. 1999). The inhibition of ethylene synthesis or perception blocked camptothecin-induced PCD in tomato suspension cells (De Jong et al. 2002). In pea carpels, the appearance of both DNA ladders and condensed nuclei was prevented by ethylene inhibitors, and ethylene treatment accelerated DNA fragmentation (Orzáez and Granell 1997). Delayed tapetal degeneration was found in the anthers of these transformants. It may be that reduced ethylene sensitivity in these plants causes a delay of PCD in the tapetum.

Male sterility that results from tapetal cell abnormalities is divided into two categories. In the first category, earlier degeneration or destruction of the tapetum cells occurs, as in *TAZ1*-silenced plants (Kapoor et al. 2002) and in plants using the TA29 promoter (Cho et al. 2001; Mariani et al. 1990). In the second category, the tapetal cells show vacuolation during pollen development, as in the *ms1* mutant (Wilson et al. 2001) and A9(tl)PR-transformed tobacco (Worrall et al. 1992).

The H69A and H70A transgenic plants expressing *Cm-ETR1*/*H69A* and *Cm-ERS1*/*H70A*, respectively, do not fit either of these categories. However, it has not been confirmed that delayed tapetum degeneration was the cause of reduced pollen production in these plants, and this possibility requires further investigation.

The studies described above have demonstrated that mutated ethylene receptor genes are involved in stamen development, a major step in plant reproductive physiology.

Tapetum-specific expression of mutated ethylene receptor gene reduces normal pollen production in tobacco

The tapetum is the innermost of the four sporophytic layers of the anther wall, and it comes in direct contact with the developing gametophyte. It has, therefore, for a long time been considered to play a crucial role in the maturation of microspores (Shivanna et al. 1997). Tapetal tissue has a secretory role, providing nutrients for pollen development and enzymes for the release of microspores from tetrads (Goldberg et al. 1993). Several articles have reported male sterility associated with the selective destruction of the tapetum (Goldberg et al. 1993), induced by a tapetum-specific promoter (Goetz et al. 2001; Kriete et al. 1996) and gene (Kapoor et al. 2002), during anther development, demonstrating the association between mutant male sterility and abnormal tapetal development (Wilson et al. 2001).

One major concern related to transgenic plants is the possibility of transgene flow via pollen dispersal into the environment. To address this concern, a reliable method for preventing pollen dispersal is needed. Several studies have described systems for inducing male-sterile plants, e.g., the well-known system using the TA29 promoter in tobacco (Mariani et al. 1990, 1992), in which the TA29 gene is expressed specifically in anther tapetal cells (Koltunow et al. 1990). This system induces male sterility with *TA29::barnase*, which expresses a natural ribonuclease gene from *Bacillus amyloliquefaciens* under the control of the tapetal-specific TA29 promotor. There are many other systems that use the TA29 promoter (Cho et al. 2001; Huang et al. 2003; Kriete et al. 1996; Sa et al. 2002), but the phenotype of transformants carrying the TA29::*ipt* gene, which expresses isopentenyl transferase, the rate limiting enzyme in cytokinin biosynthesis, shows various alterations in floral architecture (Sa et al. 2002). As the reports on the use of the TA29 promoter for inducing male sterility showed phenotypes only in the upper part of the flower (Huang et al. 2003; Kriete et al. 1996; Mariani et al. 1990), the reason for the pleiotropic effects on the whole plant was not clear.

To create transgenic plants that produce less pollen without altering the floral architecture, we constructed mutated melon ethylene receptor genes that would be expressed specifically in the tapetum, because it was suggested that the constitutive expression of mutated receptor genes resulted in delayed tapetum degeneration and reduced pollen production. However, it was thought that a promoter with higher tapetum specificity was needed in the new system for induction of male sterility. We chose the *Nin88* promoter (Goetz et al. 2001). The Nin88 protein is present only in the tapetal cell layer during the early stage of anther development; when the tapetum begins to degrade, Nin88 protein can be detected in both the tetrad and the microspores. It was predicted that the tapetum-specific expression of *Cm-ERS1*/*H70A* would induce the delay of tapetum degeneration and result in pollen abortion without altering the floral architecture. Therefore, the coding region of *Cm-ERS1*/*H70A* was fused to *pNin88*, and the construct *pNin88::Cm-ERS1*/*H70A* was used to transform tobacco plants (NH70A transgenic plants).

As expected, delayed tapetum degeneration was observed in the anthers of transgenic plants with the Nin88 promoter and 35S promoter-driven *Cm-ERS1*/*H70A* (Takada et al. unpublished results), which was similar to the result in transformants expressing *Cm-ETR1*/*H69A* (Takada et al. 2005). This finding indicates that the constitutive and specific expression of *Cm-ERS1*/*H70A* resulted in delayed tapetum degeneration. As tapetal degeneration during anther development is known to be a process of PCD (Wang et al. 1999), PCD of the tapetum was thought to be delayed by the expression of the mutated ethylene receptor gene in the tapetum. It had not been clear previously whether there was a correlation among reduced normal pollen production, delayed tapetal degeneration, and the constitutive expression of ethylene receptor genes. Given that the specific expression of *Cm-ERS1*/*H70A* induced these phenomena, it is suggested that the expression of the mutated gene is involved in the delay of tapetal degeneration. There was no modification of the floral architecture in these transformants with abnormal male gametes (Figure 3), and seed yield was reduced in NH70A transformants under open and artificial pollination with pollen from the same transgenic plants. Additionally, under cross-pollination with pollen from the wild type, the seed yield was also reduced. Thus, the expression of the transgene reduced both male and female fertility, possibly because *Nin88* gene expression normally occurs only in male gametes. To develop a better system for the induction of male sterility, a promoter with greater specificity for the tapetum layer must be identified.

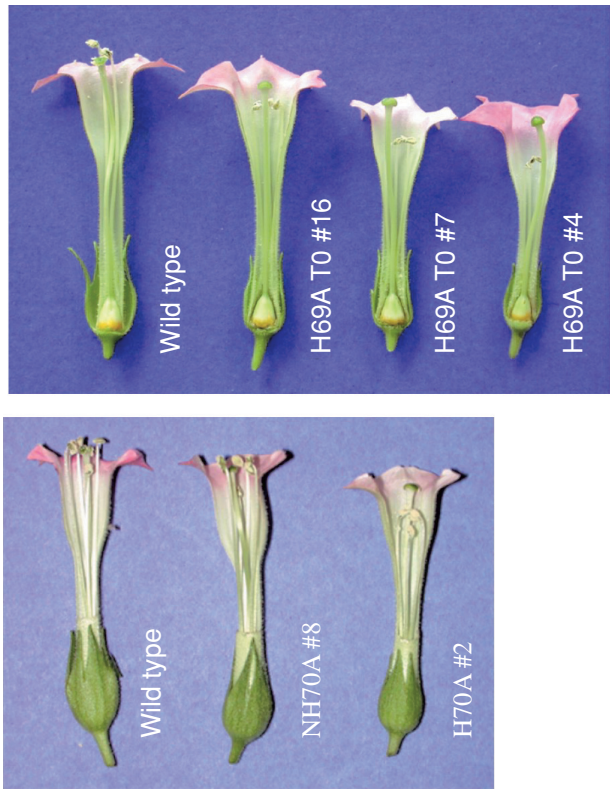

Figure 3. Alteration of floral architecture in the transgenic tobacco plants expressing mutated ethylene receptor genes, *Cm-ETR1/H69A* and *Cm-ERS1/H70A*. Two traits related to reproductive physiology, floral architecture, and pollen production, are altered in the transgenic lines. Both traits were altered when *Cm-ETR1/H69A* and *Cm-ERS1/H70A* were driven by a constitutive promoter as in lines H69A T0 #4, #7 and #16 and H70A#2. On the other hand, only pollen production was altered when *Cm-ERS1/H70A* was driven by a tapetum-specific promoter as in line NH70A #8.

Stability of male sterility induced by the expression of mutated ethylene receptor genes in tobacco

After the first commercial introduction of a genetically modified (GM) crop in 1994, the cultivation of GM crops has increased worldwide, with production reaching 81 million hectares of commercially grown GM crops in 17 countries by 2004 (James 2004). As GM crop production has increased, so too has interest in the potential environmental impacts of GM crops (Dale et al. 2002). One major concern is the potential for transgene flow through pollen dispersal, which could potentially result in the emergence of herbicide-resistant weeds or the introduction of undesirable plant traits. Several molecular strategies for controlling transgene flow via pollen have been developed; these include maternal inheritance and male sterility (reviewed by Daniell 2002).

The over-expression of mutated melon ethylene receptor genes conferred sterility or reduced fertility in transgenic tobacco plants because of the alteration of floral architecture and the abortion of pollen production due to delayed tapetum degeneration in the anther (Takada et al. 2005, unpublished results). We evaluated the traits of these transgenic plants in growth chambers under constant conditions; thus, the stability of these traits outdoors under naturally occurring variations in environmental conditions (e.g., light, temperature, wind, and rainfall) were not known. For the practical application of this phenomenon to transgene containment in GM plants, the stability of these traits under more variable environmental conditions would need to be determined.

Transformants displaying sterility or reduced fertility conferred by the expression of either *Cm-ERS1/H70A* or *Cm-ETR1/H69A* were grown in an open-air greenhouse in which environmental conditions varied (Ishimaru et al. unpublished results). The flowers of the transformants displayed a heterostyly phenotype as reported by Takada et al. (2005, unpublished results), whereas the anthers of wild-type flowers were all positioned higher than the stigma, a homostyly phenotype. The floral architecture of the transformants did not change greatly with the time of flowering; wild-type plants always showed a homostyly phenotype, and transgenic plants always showed a heterostyly phenotype. These observations indicate that the heterostyly phenotype conferred by the mutated melon ethylene receptor genes *Cm-ETR1/H69A* and *Cm-ERS1/H70A* was quite stable under varying environmental conditions, including temperature and light. The pollen-aborted phenotype and the reduced seed yields of transgenic plants with mutated melon ethylene receptors were also stable traits in the open-air greenhouse under variable environmental conditions. These observations demonstrate that the traits related to sterility or reduced fertility that are induced by the constitutive expression of mutated melon ethylene receptor genes in transgenic tobacco plants are stable under varying environmental conditions and suggest that a system using ethylene receptor genes to induce male sterile may be useful for preventing transgene flow via pollen dispersal.

Heterologous expression of a mutated ethylene receptor gene confers stable sterility in transgenic lettuce

In order to validate the adaptability of the inducible male sterile system using mutated melon ethylene receptor genes, *Cm-ERS1/H70A* that driven by CaMV 35S promoter was introduced into lettuce (*Lactuca sativa* L.) plants, and more than 50 transgenic lines were generated (Ezura et al. unpublished data). Among them, 13 randomly selected lines of transgenic lettuce plants were grown in growth chambers under constant temperature and light conditions. Six lines showed a sterile

phenotype, and two lines were nearly sterile. When the fertility of these lines grown in an open-air greenhouse was evaluated, the lines continued to show sterility or substantially reduced fertility in the variable environmental conditions. These findings indicate that the inducible male sterility system using mutated melon ethylene receptor genes is adaptable to other plant species. The detailed results of the transgenic lettuce plant experiments will be reported in another paper.

Perspective

We have developed a unique system for the induction of male sterility in transgenic plants using mutated melon ethylene receptor genes *Cm-ERS1/H70A* and *Cm-ETR1/H69A* (Figure 4). The over-expression of these genes by a constitutive promoter induced male sterility in transgenic plants, but also conferred pleiotropic effects such as alterations in floral architecture and plant height. However, when these genes were driven by a tapetum-specific promoter, the transgenic plants showed male sterility without a noticeable alteration of floral architecture. When we used the *Nin88* gene promoter from tobacco as a tapetum-specific promoter, the transgenic plants showed inducible male sterility without noticeable pleiotropic effects on plant morphology, although the transgenic plants still showed female sterility, i.e., the cross-pollination of transformants with wild-type pollen resulted in reduced seed production. Therefore, to develop a better system for inducible male sterility, we need to identify a promoter with greater specificity for the tapetum layer. Using a more specific promoter may enable us to circumvent the complication of female sterility and to produce transgenic plants for cross breeding.

Although recent studies have reported various systems for inducible male sterility in transgenic plants, the systems were designed only to prevent transgene flow through pollen dispersal. To enhance the practical application of the inducible male sterility system, genes conferring useful traits to crops of interest could be introduced into the transgenic plants in addition to the mutated ethylene receptor genes. For example, by simultaneously introducing *Bacillus thuringiensis* (*Bt*) and a mutated melon ethylene receptor gene, it may be possible to produce transgenic plants with both insect resistance and male sterility. Such transgenic plants should reduce the concern about transgene flow through pollen dispersal while providing an advantage for cultivation. The development of a new vector system for the simultaneous expression of useful genes and mutated ethylene receptor genes would assist in promoting the incorporation of an inducible male sterility system in a wide variety of plants.

Our system for inducible male sterility can be applied in transgenic crops that are vegetatively propagated and from which neither fruits nor seeds are harvested. The development of fruits requires pollination, and therefore, fruit crops are not suitable for the application of this system. Similarly, cereal crops are also not suitable candidates for inducible male sterility, because sterile transgenic cereal plants could not set the seed that is harvested as product. However, our system may be advantageous for tuber crops (e.g., potato, sweet potato, and taro), trees, and perennial and bulbous plants from which cut flowers are harvested (e.g., carnation, chrysanthemum, lily, and gladiolus).

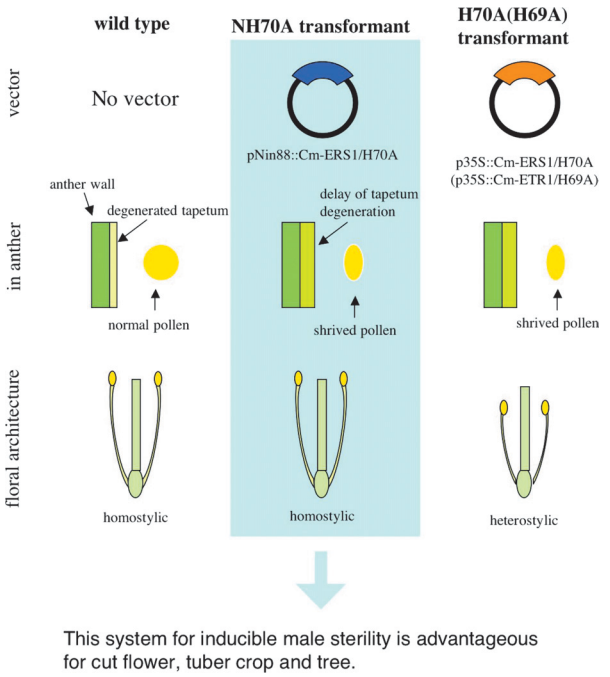

Figure 4. A schematic representation of the inducible male sterility system using mutated melon ethylene receptor genes. Pollen production and flower architecture are affected when the genes are driven by a constitutive promoter. While only pollen production is affected when the genes are driven by a tapetum-specific promoter.

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