Functional Analysis of Melon Ethylene Receptor Genes and its Application Possibility to the Development of Sterile Plants

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Agricultural Science

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Contents

Abbreviation

CMS: cytoplasmic male-sterile

DW: distilled water

GM: genetically modified

NFA: normal filament architecture

NR: Never ripe

PCD: programmed cell death

PCR: polymerase chain reaction

Rf: fertility restorer

SFA: short filament architecture

STS: silver thiosulfate

WT: wild-type

Chapter I: Introduction

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). 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 an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), and promoted by ethylene antagonists, cobalt ions, L-α-(2-aminoethoxyvinyl) glycine (AVG), and silver thiosulfate (STS). 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.

Sato-Nara et al. (1999) have previously isolated and characterized the melon ethylene receptor genes *Cm-ETR1* and *Cm-ERS1* (Fig. 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* (Cui et al., 2004). As anticipated, the *Cm-ETR1/H69A* gene conferred reduced ethylene sensitivity to the heterologous transgenic plant *Nemesia strumosa* (Cui et al., 2004). Nukui et al. (2004) 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 species 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 lifetime of plant tissues by delaying their senescence. In transgenic tomato plants, the antisense transgene

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* (*Never ripe*) 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* (broccoli *ers*) 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. However, heterologous plants introduced mutated melon ethylene receptor genes, *Cm-ETR1/H69A* and *Cm-ERS1/H70A* showed not only a extending these life spans but also a reduction of fertility (Cui et al., 2004, Nukui et al., 2004). It was thought that sterile transgenic plants could be produced using these mutated receptor genes.

On the other hand, after the first commercial introduction of a genetically modified (GM) crop in 1994, the cultivation of GM crops has increased worldwide, with production reaching 90 million hectares of commercially grown GM crops in 21 countries by 2005 (James, 2005). 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. Several molecular strategies for controlling transgene flow via pollen have been developed; these include maternal inheritance and male sterility (reviewed by Daniell, 2002).

Male-sterility is also of particular significance in improving crop yields because of its usefulness in hybrid seed production. Cytoplasmic maile-sterility (CMS) is a wide spread phenomenon observed in over 150 flowering plant species and is widely used for F_1 hybrid production (Laser and Lersten, 1972; Mackenzie et al., 1994;

Schnabel and Wise, 1998). CMS is a maternally inherited trait and is often associated with unusual open reading frames (ORFs) found in mitochondrial genomes, and in many instances, male fertility can be restored specifically by nuclear-encoded, fertility restorer (*Rf*) genes (Schnable and Wise, 1998). Therefore, CMS/*Rf* systems are ideal models for studying the genetic interaction and cooperative function of mitochondrial and nuclear genomes in plants. To date, a number of genetic loci for CMS and fertility restoration have been mapped in various plant species. Recently, *Rf* gene have been cloned from maize, petunia, radish and rice (Cui et al., 1996; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003, Komori et al., 2004), but very few CMS candidate genes have been functionally tested, and the molecular mechanisums of the CMS/*Rf* systems generally remain unclear (Schnable and Wise, 1998; Wise and Pring, 2002; Hanson and Bentolila, 2004). Therefore, engineering cytoplasmic male sterility has not yet been achieved because of the lack of efficient methods for transforming either mitochondria or chloroplasts.

On the other hand, biotechnology has added new possibilities to obtain male-sterile plants. Since the first transgenic male sterility system was described (Mariani et al., 1990), many strategies to produce male-sterile plants have been reported. The first system that relies the selective destruction of tapetum needed for the production of functional pollen by the tapetum-specific expression of *barnase* gene, the extracellular RNase of *Bacillus amyloliquefaciens* (Mariani et al., 1990). Additionally, inducible sterility can be obtained by expressing a gene encoding a protein that catalyzes the conversion of a pro-herbicide into a herbicide only in male reproductive tissues (O'Keefe et al., 1994; Kriete et al., 1996; Dotson et al., 1996). A different approach to engineer male sterility is to introduce a gene, or set of genes, able to alter the levels of metabolites needed for the production of viable pollen, such as sugar (Goetz et al., 2001), flavonols (Derksen et al., 1999), jasmonic acid (McConn and Browse 1996, Sanders et al., 2000) or auxins (Spena, 1992). Inducible fertility can be achieved for some of these systems by application of the missing metabolite (McConn and Browse, 1996; Sanders et al., 2000). However, most of these systems induced not only male sterility but also modification of floral architectures. Although GM crops produced by these systems for induction of male sterility will have no pollen grain, the modification of floral architecture will become a fault in the cut flowers for appreciation. It was not known whether transgenic plants produced by these systems could adjust to the change

of environment.

In this study, I evaluated the performance of mutated melon ethylene receptor genes for inducing male sterility in transgenic tobacco plants and elucidated the mechanisms (Chapter II), tried to improve the inducible male sterility by expressing the genes in a tissue specific manner (Chapter III), evaluated the stability of induced sterility in transgenic tobacco plants under varing environmental conditions (Chapter IV), and tested the applicability of the genes for inducing stable sterility in other plant, lettuce (Chapter V). Finally, I discussed the usefulness of this approach in preventing transgene diffusion to environment through pollen dispersal (Chapter VI).

Chapter II: Expression of a mutated melon ethylene receptor gene *Cm-ETR1/H69A* affects stamen development in *Nicotiana tabacum*

Abstract

To investigate the role of an ethylene receptor gene in plant reproduction, I introduced a mutated melon ethylene receptor gene, *Cm-ETR1/H69A*, into tobacco plants and regenerated 11 independent transformants. In 5 of the 11 transformants, the flower longevities were longer than that of the wild type, indicating that the transformants had a reduced sensitivity to ethylene than the wild type. The seed yields of these transformants were lower than those of the wild-type plants. The reduced seed yields were mainly caused by abnormal stamen development, modification of floral architecture, and reduced pollen production. Filament elongation was reduced in the transformants, resulting in the short fillaments. Histological observation of anthers at several developmental stages showed abnormality of tapetum degeneration in the transgenic plants, and results in inducing pollen abortion. These results suggest that stamen development is related to ethylene sensitivity, and the mutated melon ethylene receptor genes could be useful for inducing pollen abortion in transgenic plants.

Introduction

Induction of mutated melon ethylene receptor genes, *Cm-ETR1/H69A* or *Cm-ERS1/H70A* conferred reduced ethylene sensitivity or ethylene insensitivity to heterologous plants, *Nemesia strumosa* (Cui et al., 2004) and *Lotus japonicus* (Nukui et al., 2004). These reports also described that the mutated genes affected fertilities of transgenic plants, suggesting that the mutated genes could be useful for generating sterile transgenic plants.

In this chapter, to further investigate the effect of the mutated melon ethylene receptor genes in plant reproduction, transgenic tobacco plants constitutively expressing *Cm-ETR1/H69A* were produced and analyzed for plant development and especially pollen development.

Materials and methods

Plant growth conditions and statistical analysis

For all experiments *Nicotiana tabacum* cv. Sumsun plants and the transgenic plants (T_0 generation) were used. Untransformed wild-type plants and the transgenic plants were grown on solid MS medium (Murashige and Skoog, 1962) at 23°C and a 16 hr light / 8 hr dark photoperiod, under sterile conditions. Then, the plants were transferred to soil and grown to flowering in a containment growth room at 25°C under a 16 hr light / 8 hr dark photoperiod with fluorescent light at an intensity of 60 μmol m^{-2} s^{-1}. All experiments were repeated at least two times. The data were subjected to Dunnet test by using the software 'Excel statistics 2002' (Social Survey Research Information Co., Ltd, Tokyo, Japan).

Production and confirmation of transgenic plants

Tobacco plants were transformed using the *Agrobacterium tumefaciens* strain C58C1Rif^R harboring the plasmid pBI Cm-ETR1/H69A, mutated *Cm-ETR1* (Fig. 1,3) (Cui et al., 2004) by the cocultivation method (Horsch et al., 1985). The transformation was performed by briefly immersing leaf discs from surface-sterilized young leaf into MS medium containing *Abrobacterium.* After the cocultivation period, shoots were regenerated from the explants in the shoot induction medium (MS medium with 1mg/l BAP and NAA), and kanamycin-resistant transformants were maintained in MS medium according to Maliga et al. (1995). To confirm the presence of the *Cm-*

ETR1/H69A transgene, PCR was performed with total DNA from the transgenic tobacco plants using the primers p35S-20f (5'-CGG GGG ACT CTA GAG GAT CC-3') and CT+735r (5'-TCC AGC CAC ATA TCT ACC AG-3'). The PCR conditions used were 95°C for 9 min; 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; and a final 8 min at 72°C. AmpliTaq Gold polymerase (Applied Biosystems) was used for PCR reaction according to the manufacture's instructions.

Isolation of mRNA and reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated from tobacco leaves using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. First-strand cDNA was then synthesized from the total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, CA) according to the manufacturer's instructions. The first-strand cDNA synthesis reaction was primed using oligo(dT) primer. The resulting first-strand cDNA was used as a template for amplification by PCR (30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; followed by 8 min at 72°C). To confirm the integrity of the cDNA, control PCR reactions were performed using actin primers (actin-f: 5'-CAA AAG GAT GCA TAT ATT GGT-3', actin-r: 5'-ATC TAC ATC ACA CTT CAT GAT-3').

Evaluating flower longevity

The flower longevity of transgenic plants and wild-type plant was evaluated for five flowers per plant. Flower longevity was evaluated visually as the number of days taken from fully opening to wilting.

Floral architecture and seed yields

Five tobacco flowers per line were dissected just after opening, and the lengths of the filament, style, and ovary were measured. The stigma-anther distance, defined as the difference between the filament length and length of the style plus the ovary, was then calculated.

Flowers were artificially pollinated during anthesis prior to petal abscission by tapping freshly dehisced anthers of the same flower just above receptive stigma. The weight of the seeds from five flowers per transgenic line among the artificially pollinated and openly pollinated flowers was measured after the flowers had completely

dried.

STS treatment

Flowers of wild type were excised at the pedicel and immediately placed with their stem end in desired solution. I used a solution of silver thiosulfate (STS) (0.05, 0.1, 0.2 mM). A 20 mM STS was prepared by mixing 20 mL of 0.1 M silver nitrate solution and 80 mL of 0.1 M sodium thiosulfate solution. When the buds were flowering, the architectures of flowers were investigated as described above.

Pollen production

Following dehiscence, individual tobacco flower anthers were suspended in 100 μL of acetocarmine solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The pollen suspension was mounted on a glass slide and observed by light microscopy to determine the numbers of normal and abnormal pollen in 5 μL of each suspension. Five independent flowers from each plant line were used.

***In vitro* pollen germination**

In vitro germination of pollen was performed on a liquid germination medium (0.01% H_3BO_3, 0.01% KH_2PO_4, 0.1% $CaCl_2$, 15% sucrose). Small amounts of the medium were pipetted onto microscope slides and allowed to solidify, and the pollen was then allowed to germinate in a humid chamber at 25°C for 24 h. The percentages of germinated pollen grains from three independent flowers were determined by counting at least 500 pollen grains from each flower.

Histological and cytological staining

Anthers were fixed in FAA (63 % ethanol, 5 % glacial acetic acid, 2 % formaldehyde) and dehydrated in a graded alcohol series (1st; 40 % t(n)-butanol+30 % ethanol, 2nd; 55 % butanol+25 % ethanol, 3rd; 70 % butanol+20 % ethanol, 4th; 85 % butanol+15 % ethanol, 5th; 100 % butanol 2 times) at room temperature. The samples were then embedded in paraffin, sliced into 10-μm sections by rotary microtome HM325 (Microm, Germany). Sections were stained by using 0.05% toluidine blue. Imaging was done using a DC 300 (Leica, Germany) digital camera applied to a MZ FL III (Leica) microscope. Flower stage was determined by flower length and corolla appearance (Fig. 2), as described by Koltunow et al (1990).

Results

Production of transgenic tobacco plants containing the mutant ethylene receptor gene *Cm-ETR1/H69A* and their morphological and physiological characteristics

I generated transgenic tobacco plants containing the mutated melon ethylene receptor gene *Cm-ETR1/H69A* (Fig. 3A). Eleven independent transformants (T_0 plants; H69A transgenic plants) containing *Cm-ETR1/H69A* were selected on the basis of kanamycin resistance and PCR analysis (Fig. 3C). To investigate the relation to ethylene sensitivity of the transformants, the flower longevity was analyzed. The flower longevities on all transgenic lines were longer than those of wild-type flowers (Fig. 3B). In particular, five lines (H69A #2, 4, 7, 15, and 16) had much longer flower longevities varying from 4.6 to 6.1 days than those of wild-type plants. These results suggest that the sensitivity to ethylene of these five transgenic lines is significantly less than that of the wild type. In addition, the five transformants showed shorter plant height than the wild type. Although transformant H69A#4 displaying severe phenotype (Fig. 4C) accumulated a higher level of anthocyanin in transgenic petals than the wild type (Fig. 4B), flower morphology including petal shape and number of filaments show normal in all transgenic plants.

RT-PCR analysis using specific primers was performed to confirm the expression of *Cm-ETR1/H69A* in the above five H69A transgenic lines. The expected bands were amplified in all five lines but not in wild-type tobacco, indicating that the transgene was actively expressed (Fig. 4A).

Modification of floral architecture in the transgenic lines

The flowers of the five transformants (H69A #2, 4, 7, 15, and 16) displayed a modification of floral architecture with higher stigma than the anthers (Fig. 4C), whereas the anthers of wild-type flowers were positioned higher than the stigma (Fig. 4B). I measured lengths of filament and carpel in all transgenic plants, and the rates of length (filament/carpel) on transgenic plants were analyzed. The rate of three transformants (line 2, 4 and 7; value is 0.77 to 0.80) was smaller than that of wild-type (0.99), indicating that the modified phenotype was mainly derived from differences in the filament length. Therefore, the flowers of H69A transgenic plants have short filament architecture (SFA), and that of wild-type has normal filament architecture (NFA). The distance between the stigma and anthers in the wild-type plants was 1 mm

(Fig. 5). In three transformants (H69A #2, 4, and 7) with strongly modified floral architectures, the stigma-anther distances were 6 to 8 mm. However, the stigma-anther distance in the other two transgenic lines was about 2 mm.

These transgenic plants had longevity of flowers as shown in Fig. 3B. There was a significant negative correlation between flower longevity and stigma-anther distance (Fig. 6). Spearman rank correlation test was used at 1% level ($r=-0.8392$, $P=0.00471$).

Effect of STS on floral architecture of wild-type tobacco plant

I treated the flower buds of wild-type tobacco at a variety of flower size with STS to see the effects of ethylene action on floral architecture (Fig. 7). Two types of floral architectures were identified depending on the concentration of the chemical and the stage of flower buds treated. One was NFA as that of wild type flowers; the anther position was higher than stigma, and the other was SFA as that of H69A transgenic flowers; the anther position was lower than stigma (Fig. 4D). Treatment of flower buds with 0.05 mM STS, did not show any significant effect on the flower architecture as in wild-type uncut flower (Fig. 7A). The flower architectures were remarkably altered when the flower buds less than 2 cm (stage 5) were treated with STS concentration higher than 0.05 mM (Fig. 7B, C). These flowers showed SFA (Fig. 4D). These results showed that the degree of modified floral architecture depended on STS concentration and flower bud size at the start of STS treatment. When excised flower buds of wild type were treated with DW as control, floral abscission occurred within two days. STS prolonged flower longevity by about one week, indicating the effectiveness of this inhibitor for inhibiting ethylene action. These experiments of STS treatment showed that all flowers had a lot of normal pollen grains (data not shown), because a inhibition of pollen development take place before stage 2 in transgenic plants.

Seed yields of transgenic plants expressing *Cm-ETR1/H69A*

Under open pollination conditions, every wild type plant produced fruit containing seeds (average yield 143 mg/fruit; Fig. 8). Three transgenic lines (H69A #2, 4, and 7) produced fruit but without seed. In two transgenic lines H69A #15 and 16, one out of five fruits produced set seed, however the seed yield per fruit in these lines was much lower than the wild type. Artificial pollination with pollen from the same

transgenic plant, resulted in production of seeds in all the transgenic plants except H69A #4. The seed yields of lines H69A #15 and 16 were 120 and 118 mg/fruit, respectively, or 92.4 and 91.1% that of the wild type. The seed yields of artificially pollinated H69A #2 and 7 plants were far lower than that of the wild type. Pollination of line H69A #4 with wild-type pollen also did not result in seed production. These results demonstrate that the five transgenic lines display reduced fertility, with line H69A #4 appearing sterile.

When the T_1 seeds obtained by artificial pollination were sown in MS medium containing kanamycin, most of the seedlings of four lines (H69A #2, 7, 15, 16) showed kanamycin-resistance (95-100%). This result suggested that the four transgenic plants contained multi copies of the transgene.

Pollen production in H69A transgenic tobacco

The phenotypes of the pollen grains of the transgenic plants expressing *Cm-ETR1/H69A* were examined to investigate the cause of reduction of their seed yields. In contrast to the normal-looking pollen grains of the wild-type (Fig. 9A), H69A #4 produced pollen grains that appeared shriveled (Fig. 9B). The numbers of normal and abnormal (shriveled) pollen grains were counted from five anthers of each line (Fig. 10). Of the approximately 46,000 wild-type pollen grains, 98.2 % had a normal phenotype. Five transgenic lines (H69A #2, 4, 7, 15, and 16) showed reduced pollen production, with the total pollen grains amounting to 6.9-38.7% of that produced by the wild type. In lines H69A #2, 4, and 7, the abnormal pollen grains amounted to 79.5-97.7 % of the total. Line H69A #4 produced the lowest percentage of normal pollen grains (2.3 %). In summary, the transformants expressing *Cm-ETR1/H69A* showed not only reduced pollen production but also lower percentages of normal pollen grains.

To examine the viability of the pollen grains in three transgenic lines (H69A #4, 7 and 16), the pollen was incubated in liquid germination medium for 24 h. The germination rate of the wild-type pollen was 89.6±4.3 %. In contrast, pollen of the transgenic lines H69A #4, 7, and 16 showed germination rates of 0.04±0.09, 2.7±2.7, and 18.8±2.5 %, respectively. The abnormally shaped pollen grains did not germinate as compared to the normal pollen.

Histological observation of anthers

We observed tissue sections of anther to investigate how the transgenic plants had reduced pollen production. In anthers of wild-type plants, microspores were observed in the four pollen sacs at stage 2, when the tapetum had just begun to degenerate (Fig 11A, C). By stage 6, the tapetum had degenerated further and had almost disappeared (Fig. 11E). The H69A #4 anthers contained two or three collapsed pollen sacs lacking visible microspores (Fig. 11B). At stage 2, an undegenerated tapetum was present, and microspores and tetrads were observed (Fig. 11D). In addition, the microspores in the pollen sac appeared shriveled. At stage 6, the tapetum and connective cells in the wild-type plants had degenerated (Fig. 11E), but the degeneration of the H69A #4 tapetum was abnormal, that is, the tapetum was not degenerated in comparison with that of wild-type (Fig. 11F). No other abnormalities in the transgenic anther tissues were observed. A similar phenomenon was observed in H69A #7 and 16.

Discussion

Transgenic tobacco plants expressing the mutated melon ethylene receptor genes *Cm-ETR1/H69A* under the control of the constitutive CaMV 35S promoter showed increased flower longevity, as had previously been observed in transgenic *Nemesia* plants expressing *Cm-ETR1/H69A* (Cui 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 the *Cm-ETR1/H69A* gene 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 *Cm-ETR1/H69A* displayed pleiotropic phenotypes that included SFA, 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 *Cm-ETR1/H69A* gene and the four tobacco ethylene receptor genes. The mRNA expression levels and phenotypes were not correlated in the five transgenic lines. H69A #4 showed severe phonotype compared to other transformants while the level of mRNA expression was lower that those of other lines. It is likely that this inconsistency results from the interaction between *Cm-ETR1/H69A* gene and four endogenous ethylene receptor genes in tobacco plants. Investigation of expression profile of the five ethylene receptor genes and the phenotype of H69A transgenic plants will provede a clue to elucidate the inconsistency.

Our observations demonstrate that the reduced seed yields in the H69A transgenic plants were the result both of modifications in the floral architecture (Fig. 5) and reduced pollen production (Fig. 10). Therefore, it could not pollinate in five H69A transgenic plants (#2, 4, 7, 15, 16) under open pollination, because stigma-anther distances were long. Two transgenic lines (#2, 7) could not produce seed, because normal pollen grains were a little. H69A #4 was female sterility and produced no seed. The transgenic tobacco plants displayed SFA, in which the anthers are located below the stigma (Fig. 5). Chemical reduction of the ethylene sensitivity of non-transgenic tobacco plants by STS treatment also resulted in SFA (Fig. 7). This observation suggests that SFA of the transformants was due to their reduced ethylene sensitivity. In SFA flowers, the length of the filament was generally reduced rather than the length of the style. 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 ACC and promoted by cobalt ions, AVG, and STS. These reports conflict with our results. However, in inflorescences of immature carnation plants, ethylene promotes the elongation of floral organs such as styles (Camprubi and Nichols, 1979), and growth of *Gaillardia* filaments can be promoted by ethylene (Koning, 1983). These results appear similar to our results. It is likely that the ethylene sensitivity of filaments differs between plant species.

The reduction of normal pollen production could be caused by abnormal tapetum degeneration during anther development. Tapetal degeneration during anther development is a process of programmed cell death (PCD) (Wang et al., 1999). The inhibition of ethylene synthesis or perception blocks camptothecin-induced PCD in tomato suspension cells (De Jong et al., 2002). In pea carpels, the appearance of both DNA ladders and condensed nuclei can be prevented by ethylene action inhibitors, and

ethylene treatment accelerates DNA fragmentation (Orzáez and Granell, 1997). I found that tapetal degeneration was abnormal in anthers of the transformants (Fig. 6). It may be that reduced ethylene sensitivity in H69A transgenic plants causes abnormality of PCD in the tapetum.

Male sterility that results from tapetal cell abnormalities is categorized into two groups. In the first category, earlier degeneration or destruction of tapetum cells, as in examples of *TAZ1*-silenced plants (Kapoor et al., 2002) and plants in which the TA29 promoter is utilized (Cho et al., 2001; Mariani et al., 1990). In the second category, the tapetal cells show vacuolation during pollen development, as the *ms1* mutant (Wilson et al., 2001) and A9(tl)PR-transformed tobacco (Worrall et al., 1992). The H69A transgenic plants of the present study do not fit either of these categories. However, it is not known whether the abnormal tapetum degeneration was the cause of the reduced pollen production in these plants and this possibility requires further investigation.

In ethylene-insensitive tobacco plants that had been generated either by expression of the *etr1-1* gene or by treatment with the ethylene perception inhibitor 1-methylcyclopropene (MCP), the anthers showed delays in dehiscence, the degeneration of the stomium cells, and dehydration (Rieu et al., 2003). Although delays in anther dehiscence were often observed in the H69A transgenic plants, no delay in stomium cell degeneration was observed (data not shown). Delayed anther dehiscence is likely one of multiple phenotypes of plants with reduced ethylene sensitivity. In the future, it will be interesting to analyze the relationship between these phenotypes and the level of expression of the ethylene receptor protein.

In conclusion, I have demonstrated that the ethylene receptor gene is involved in stamen development, a major step in plant reproductive physiology. Transgenic tobacco plants constitutively expressing the mutated melon ethylene receptor gene *Cm-ETR1/H69A* and conferring severe phenotype showed both male and female sterilities, suggesting that the gene is useful for the generation of sterile transgenic plants.

Chapter III: Anther-specific expression of mutated melon ethylene receptor gene *Cm-ERS1/H70A* affected tapetum degeneration and pollen grain production in transgenic tobacco plants

Abstract

To develop a new system for inducible male sterility without any modification of the floral architecture in tobacco plants, a mutated ethylene receptor gene *Cm-ERS1/H70A* was fused either to the tobacco *Nin88* promoter known to function mainly in the tapetum and microspore or to the CaMV *35S* promoter known to be a constitutive promoter. The fusion genes *pNin88::Cm-ERS1/H70A* and *p35S::Cm-ERS1/H70A* were introduced in tobacco plants, which generated two independent transformants. Transformants with *35S::Cm-ERS1/H70A* produced less normal pollen and had modified floral architecture while those with *Nin88::Cm-ERS1/H70A* produced less normal pollen without modification of floral architecture. Histological observations of anthers at stage 2 showed abnormality of tapetum degeneration in NH70A #8 and H70A #2, strongly indicating that the expression of the mutated gene was involved in this abnormality. These results suggest that the tapetum-specific expression of a mutated ethylene receptor gene is a potential strategy for inducing male sterility in transgenic plants.

Introduction

Transgenic tobacco plants constitutively expressing *Cm-ETR1/H69A* showed a pleiotropic phenotype, a reduction in ethylene sensitivity, modification of floral architecture, a reduction in pollen production, and male and female sterility (Chapter II; Takada et al., 2005). In addition, the transgenic tobacco plants showed abnormal degeneration of the tapetum. A mutated ethylene receptor *Cm-ERS1/H70A* was also designated, which destroyed the ethylene-binding ability of the plants, and transgenic *Lotus japonicus* plants expressing the *Cm-ERS1/H70A* gene did not produce fruit (Nukui et al., 2004). It has been suggested that *Cm-ERS1/H70A* affected the fertility of plants in the same way as *Cm-ETR/H69A*.

It is expected that the tapetum-specific expression of *Cm-ERS1/H70A* will induce the abnormality of tapetum degeneration and result in pollen abortion. This chapter reports the generation and characterization of transgenic tobacco plants with the tapetum-specific expression of *Cm-ERS1/H70A* and shows that pollen production in the transgenic tobacco plants is reduced without the modification of floral architecture.

Materials and methods

Plant growth and analysis of transgenic plants

For all experiments *Nicotiana tabacum* cv. Sumsun plants and the transgenic plants (T_0 generation) were used. All experiments were repeated at least two times. Floral architecture, pollen number, seed yields, histological observation, and plant growth conditions were measured as described in chapter II. Flower stage was determined by flower length and corolla appearance (Fig. 2).

Constructions of *pNin88::Cm-ERS1/H70A*

Plasmid pBI*Cm-ERS1/H70A* (Fig. 12A) contained the *NPT II* gene under the control of the nopaline synthase (nos) promoter and *Cm-ERS1/H70A*, which introduced a missense mutation into the melon ethylene receptor gene *Cm-ERS1*, converting the His^{70} residue into Ala. This was fused to the cauliflower mosaic virus 35S promoter, and both were introduced in the T-DNA region (Nukui et al., 2004).

The genomic DNA fragment was used as a template and the promoter region of the *Nin88* gene (Goetz et al., 2001) was amplified using a polymerase chain reaction

(PCR) assay. A 3290bp promoter fragment was amplified using Nin88-PF primer (5'-CTC GAG CCA TTC ATG TTC AG-3') and Nin88-PR primer (5'-AAA CAG CTC CAT TTT CCC TA-3'). PCR amplification was performed at 95°C for 9 min, followed by 40 cycles for 1 min at 94°C, 1 min at 64°C, 2 min at 72°C, and a final 8 min at 72°C. The fragment was subcloned into the pGEM T-vector (Promega, Madison, Wisconsin, USA) (pGEM pNin88).

The fragment containing the complete promoter region of *Nin88* was excised from pGEM pNin88 using *Aat*II and *Pst*I. After this fragment and pBI*Cm-ERS1/H70A* (described above) that digested by *Hin*dIII and *Xba*I were converted to blunt-ended molecules by a DNA blunting kit (TaKaRa, Kyoto, Japan), the 3.3kb promoter region was cloned in the blunt-ended pBI*Cm-ERS1/H70A* (*pNin88::Cm-ERS1/H70A*; Fig. 12B). A *pNin88::GUS* construct was made from pBI121 as well as the procedures of pBI*Cm-ERS1/H70A* construction (Fig. 12C). All constructs were verified by sequence analysis.

Production and confirmation of transgenic plants

Nicotiana tabacum cv. Samsun was transformed using the *Agrobacterium tumefaciens* strain C58C1Rif^R with pBI *pNin88::Cm-ERS1/H70A*, and the transformants with kanamycin resistance were maintained in an MS medium, according to the procedure in Maliga et al. (1995). To confirm the presence of the transgene *pNin88::Cm-ERS1/H70A*, PCR was performed with total DNA from the putative transgenic tobacco plants using the primers Nin88PM (5'-GTT CAG TTT CTT TCT CCA CA-3') and Cm-ERS1+588R (5'-GTA GAC TTA GTC CAT TCC GT-3'). The confirmation of *pNin88::GUS* was performed by using primers Nin88PM and GUS+516R (5'-TGG TGT AGA GCA TTA CGC TG-3'). PCR amplification was performed at 95°C for 9 min, followed by 40 cycles for 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, and a final 8 min at 72°C.

Isolation of mRNA and reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from five anthers of the same flower at stage 2 with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized using a Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The resultant first-strand cDNA was used as the

template for amplification using PCR (40 cycles for 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, followed by 8 min at 72°C). To check the integrity of the cDNA, control PCRs were conducted using actin primers (actin-f: 5'-CAA AAG GAT GCA TAT ATT GGT-3', actin-r: 5'-ATC TAC ATC ACA CTT CAT GAT-3').

Histochemical analysis of GUS activity

Excised organs were immersed in a substrate solution containing 2 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-Glu), 50 mM sodium phosphate buffer (pH7.0), 10 mM EDTA, 0.1% triton X-100, 0.1% sarkosyl, and 10 mM ß-mercaptoethanol, and incubated overnight at 37°C. Ethanol was added to stop the reaction and to remove the pigments, and sections were observed. Histochemical staining was conducted according to Altamura et al. (1991) on anthers and pistils, either whole or cut in half.

Results

Production of transgenic tobacco plants expressing the mutant ethylene receptor gene *Cm-ERS1/H70A* in a tapetum-specific manner

To confirm whether the expression of the GUS reporter gene driven by pNin88 takes place specifically in the tapetum, transgenic tobacco plants were produced with *pNin88::GUS* (NGUS transgenic plants) (Fig. 12C). Histochemical observation of the NGUS transgenic plant revealed GUS activity in the anther at stage 1 (Fig. 13A) and in the pollen grain at stage 12 (Fig. 13B). GUS activity in other organs, including the leaf, root, and stem was not observed (data not shown), indicating that the *Nin88* promoter isolated in this study could drive the fused gene in a tapetum-specific manner, as shown by Goetz et al. (2001). NGUS transgenic plants showed similar morphology and fertility to the wild type plants (data not shown).

To produce transgenic plants having a reduced pollen number without modifying the floral architecture using this specific promoter, the coding region of *Cm-ERS1/H70A* was fused to *pNin88*, and the construct *pNin88::Cm-ERS1/H70A* (Fig. 12B) was used to transform tobacco plants (NH70A transgenic plants). The NH70A transgenic plants were selected on the basis of kanamycin resistance and PCR analysis (Fig. 12D). As a result, 11 independent transgenic plants NH70A were examined. Two

lines (NH70A #8 and #9) among these tranformants had smaller fruits than those of the wild type, and the leaf yellowing and the plant height of NH70A #8 were the same as wild-type plant (Fig. 14). In addition, transgenic plants with *p35S::Cm-ERS1/H70A* (Fig. 12A) were produced as control plants with a modified floral architecture, i.e., SFA. Two lines of H70A transformants were selected with strong pleiotropic phenotypes that showed modification of the floral architecture, abnormal pollen grains, and delayed leaf yellowing.

RT-PCR analysis using specific primers was performed to confirm the existence and specific expression of the transgene *Cm-ERS1/H70A* in anthers. Total RNA was prepared from developing anthers at stage 2. The expected bands (700 bp) were amplified in two NH70A transformants but not in wild tobacco, indicating that the transgene was actively expressed in anthers (Fig. 15A). On H70A transformants with a constitutive expression of the *Cm-ERS1/H70A* transgene, the expected bands (620 bp) were also amplified (Fig. 15B), while specific bands were amplified in all plants using actin primer sets as an internal control (Fig. 15C). Flower longevity was analyzed to investigate the ethylene sensitivity of the transformants. The average longevity of wild-type flowers was 2.3 days, while the flower longevities of H70A #2 and #3 were also about 3.8 and 3.2 days, respectively (Fig. 16). In contrast, the longevities of NH70A #8 and #9 were about 4.3 and 3.1 days, respectively. No significant difference in flower longevities was observed between NH70A and H70A.

Two lines with a constitutive expression of *Cm-ERS1/H70A* (H70A #2, #3) showed SFA, similar to the transgenic plants with H69A (Chapter II; Takada et al., 2005), while wild-type flowers exhibited anthers above the stigma, NFA (Fig. 17A, B). The flower size of H70A #2 was smaller than that of both the wild type and NH70A #8, and no significant difference in flower size was observed between the wild type and NH70A #8 (Fig. 17A). H70A #2 and #3 exhibited significant modification of floral architecture; the stigma–anther distances were about 5 and 2.6 mm, respectively (Fig. 17B). In contrast, the modifications of floral architecture in NH70A transformants were less than in the H70A transformants. The stigma–anther distances of the NH70A #8 flower was about 1.6 mm, and that of NH70A #9 was about 0.2 mm. Because constitutive expression of *Cm-ERS1/H70A* gene affected whole flower, H70A transgenic plants showed SFA. However, NH70A transgenic plants showed NFA because of anther specific expression.

Seed yields of transgenic plants NH70A

Under open and artificial pollination, every wild-type plant produced fruit containing seeds, and the seed yields were 143 mg and 130 mg, respectively (Fig. 18). In contrast, H70A #2 produced no fruit with open pollination. The seed yield produced by artificial pollination with pollen from the same transgenic plants was 98.3 mg. The seed yield produced by cross-pollination with pollen from the wild type was 116 mg. The seed yields on H70A #3 were 18.8–65.3 mg, and the condition of cross-pollination produced the highest seed yield. In contrast, NH70A #9 produced about the same seed yields as H70A #2 under both artificial and cross-pollination conditions. The seed yield of the line on H70A #3 was 71 mg. NH70A #8 yielded less seed than the wild type under all conditions, with seed yields of 15.3–24.5 mg. These results demonstrate that NH70A #8 shows a more reduced male and female fertility than other lines.

When the T_1 seeds obtained by artificial pollination were sown in MS medium containing kanamycin, the seedlings of three line (NH70A #8, 9 and H70A #2) showed segregation ratios of Kan^r:Kan^s at 3:1. These ratios correlate with the expected Mendelian ratios for segregation of a single dominant gene. Transgenic progeny T_1 of H70A #3 did not be investigated.

Pollen production in transgenic tobacco

When the pollen grains were observed, the wild-type plant had almost normal pollen grains, and the number of total pollen grains was about 70,000 per anther; the number of normal pollen grains was 99.9% of the total pollen grains (Fig. 19). The two H70A transformants with a constitutive expression of *Cm-ERS1/H70A* had about 20,000 normal pollen grains per anther and 9,000–18,000 abnormal pollen grains per anther. In contrast, NH70A #8 had about 20,000 abnormal pollen grains per anther, and about twice that of normal pollen. Normal pollen on NH70A #9 was 46,000 per anther, and abnormal pollen was 21,000 per anther. The transgenic plants expressing *Cm-ERS1/H70A* had not only less pollen production but also lower proportions of normal pollen grains.

Cytological observation of anthers

Tissue sections of anthers were examined at stage 2 to investigate whether the

degeneration of the tapetum in the H70A and NH70A transformants was abnormal. In anthers of wild-type plants at stage 2, the tapetum had just begun to degenerate (Fig. 20A), and the degeneration of the tapetum in anthers of H70A and NH70A transformants was abnormal (Fig. 20B, C).

Discussion

It was demonstrated that the constitutive expression of the *Cm-ERS1/H70A* conferred a reduced ethylene sensitivity similar to that of *Cm-ETR1/H69A* (Cui et al., 2004; Takada et al., 2005; Chapter II). However, the degree of reduced sensitivity in H70A transformants was lower than that of H69A transformants because the flower longevity of H70A #2 was shorter than that of H69A #4 (6.1 days). These results were in accordance with the report that H69A transformants produced fewer normal pollen grains (Takada et al., 2005; Chapter II). Therefore, it appears that the two mutated melon ethylene receptor genes, *Cm-ERS1/H70A* and *Cm-ETR1/H69A*, reduce ethylene sensitivity and result in abnormal development of the male gamete. The transgenic *L. japonicus* plants expressing the *Cm-ERS1/H70A* gene also did not produce fruit (Nukui et al., 2004). It has previously been reported that transgenic tobacco plants expressing *Cm-ETR1/H69A* showed, e.g., pleiotropic phenotypes, male and female sterility, and modified floral architecture (Takada et al., 2005; Chapter II). The present study found that transgenic tobacco plants expressing a *Cm-ERS1/H70A* gene driven by a 35S promoter yielded pleiotropic phenotypes, modified floral architecture, male and female sterility, and smaller flower size.

A major concern related to transgenic plants is transgene flow via pollen dispersal into the environment. To address this problem, a reliable method for preventing pollen dispersal is needed. A number of articles have been published describing systems for inducing male-sterile plants. For example, the system that uses the TA29 promoter in tobacco is well-known; however, the phenotype of the transformants expressing the TA29::*ipt* gene shows various alterations in floral architecture (Sa et al., 2002). The reports on the use of the TA29 promoter for inducing male sterility showed only phenotypes in the upper part of flower (Kriete et al., 1996; Huang et al., 2003), and therefore, the pleiotropic effect on the whole plant was not clear. This chapter describes the pleiotropic effects and suggests that use of the

transgene *Nin88::Cm-ERS1/H70A* could reduce both pollen production and the modification of floral architecture. For the practical application of inducible male-sterility in transgenic plants, it is important to know whether the pleiotropic effect of the transgene on the whole plant is severe or not.

The Nin88 protein is present only in the tapetal cell layer during the early stage of anther development, and when the tapetum begins to degrade, the Nin88 protein is detected in both the tetrad and the microspores (Goetz et al., 2001). However, RNA blot analysis shows that the *Nin88* gene is almost expressed in the anther and is less expressed in the petals and ovary, possibly causing female sterility in NH70A #8.

Abnormal tapetum degeneration was observed in the anther of transgenic plants with the Nin88 promoter and 35S promoter-driven *Cm-ERS1/H70A*, similar to transformants expressing *Cm-ETR1/H69A* (Takada et al., 2005; Chapter II). These results suggest that the constitutive or specific expression of *Cm-ERS1/H70A* resulted in abnormal tapetum degeneration. Tapetal degeneration during developing anther is known to be a process of programmed cell death (PCD) (Wang et al., 1999). Therefore, it is suggested that PCD of the tapetum was abnormal by the expression of a modified ethylene receptor gene in the tapetum. It was not previously clear whether there is a correlation between reduced normal pollen production, abnormal tapetum degeneration, and the constitutive expression of ethylene receptor genes. Because the specific expression of *Cm-ERS1/H70A* induced these phenomena, it is suggested that the expression of the mutated gene involved abnormal tapetal degeneration.

This chapter presents a new system for inducing male sterility without the modification of floral architecture in tobacco plants by using a mutated ethylene receptor gene, *Cm-ERS1/H70A*. Although the male gametes became abnormal without any modification of floral architecture, and seed yield was reduced in NH70A transformants, the expression of the transgene reduced both male and female fertility, possibly because *Nin88* gene expression is only specific in male gametes. To develop a better system for the induction of male sterility, a promoter must be screened with higher specificity in the tapetum layer.

Chapter IV: Stability of male sterility induced by the expression of mutated melon ethylene receptor genes in *Nicotiana tabacum*

Abstract

A major concern about the safety of genetically modified crops is transgene flow through pollen dispersal. In Chapter II and III, I demonstrated that overexpression of the mutated melon ethylene receptor genes *Cm-ETR1/H69A* or *Cm-ERS1/H70A* induces pollen abortion and altered flower architecture, resulting in sterility or reduced fertility in transgenic tobacco plants. To investigate the stability of these traits, three transgenic tobacco lines, H69A and H70A in which confer sterility or reduced fertility were grown in a greenhouse with variable environmental conditions. During the growth of the plants, the temperature ranged from 31°C at the beginning of September to 17°C at the beginning of November. The provided light was natural sunlight. The first group of plants flowered in late September, and the second group flowered in late October. The wild-type plants showed the NFA, whereas three transgenic lines showed the SFA. The floral architecture was stable during the different flowering periods. Pollen production was significantly reduced in two transgenic lines and completely aborted in one transgenic line, and these traits were also stable during the different flowering periods. These results suggest that the sterility or reduced fertility induced by the expression of mutated melon ethylene receptor genes in transgenic tobacco plants is stable under varying environmental conditions.

Introduction

Although many transgenic plants showing male sterility have been developed to prevent the transgene flow through pollen dispersal, those traits were mostly evaluated in a growth chamber under constant temperature and light conditions. For the practical application of the induced male sterility in the containment of transgenes from GM plants, it is necessary to elucidate the stability of the sterility under more variable environment conditions.

In this chapter, to evaluate the stability of the sterility or reduced fertility in transgenic plants conferred by the mutated melon ethylene receptor genes, transgenic tobacco lines that had been selected in Chapter II and III, which exhibited either sterility or reduced fertility, were grown in a semi-containment greenhouse under variable environmental conditions.

Materials and methods

Plant materials

The transgenic tobacco lines H69A#4 and H69A#7 of *Nicotiana tabacum* cv. Sumsun, containing the mutated melon ethylene receptor gene *Cm-ETR1/H69A* (Takada et al., 2005; Chapter II) and the line H70A#2, containing the mutated melon ethylene receptor gene *Cm-ERS1/H70A* (Takada et al., 2006; Chapter III), were used. The transgenes were driven by CaMV35S promoter. T_0 plants of these lines were maintained *in vitro* and clonally propagated for use. When grown in a growth chamber under constant environmental conditions of 23°C and a 16-hr light / 8-hr dark photoperiod, H69A#4 showed complete sterility and H69A#7 and H70A#2 showed significantly reduced fertility. The plants were maintained *in vitro* until use. All experiments were repeated at least two times.

Plant growth conditions

Transgenic plants and untransformed wild-type plants were grown under axenic conditions on solid MS medium (Murashige and Skoog, 1962) at 23°C and in a 16-hr light / 8-hr dark photoperiod. The plants were then clonally propagated *in vitro* by lateral bud elongation. The rooted plants were acclimatized in a containment growth

room at 25°C under a 16-hr light / 8-hr dark photoperiod. Following acclimatization, groups of the plants were transferred to a greenhouse on 29 July 2004 and 2 September, 2004. Two plants of each line were grown for each experiment. The temperature in the greenhouse was monitored using a Thermo Collector (RTC-20, ESPEC Environmental Test Technology Center, Co. Ltd, Osaka, Japan).

Evaluation of flower longevity

The longevity of flowers of the transformants and wild-type plants was evaluated using five flowers from each plant. The longevity was determined as the number of days from full opening to wilting. The data were subjected to a Dunnet test using the program Excel Statistics 2002 (Social Survey Research Information Co., Ltd, Tokyo, Japan).

Floral architecture and fertilization by artificial pollination

Five tobacco flowers per line were dissected just after opening, and the lengths of the filament, style, and ovary were measured. The stigma-anther distance, defined as the difference between the filament length and length of the style plus the ovary, was then calculated.

Flowers were artificially pollinated during anthesis prior to petal abscission by tapping freshly dehisced anthers just above the receptive stigma of the same flower.

Pollen production

Following dehiscence, individual tobacco flower anthers were suspended in 100 μL of acetocarmine solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The pollen suspension was mounted on a glass slide and observed by light microscopy to determine the numbers of normal and abnormal pollen grains in 5 μL of each suspension. Five independent flowers from each plant line were used.

Results

Monitoring of temperature during flowering

To investigate the effects of temperature on floral development in the transgenic and wild-type plants, the first group of plants was transferred from the containment growth chamber to the greenhouse on 29 July, and the second on 2

September. The temperature in the greenhouse was monitored from 31 August to 15 November (Fig. 21). This period covered the phase of floral development of both groups of plants, as the first group flowered from mid- to late September, and the second flowered beginning in late November. The average temperature in the greenhouse decreased from about 30°C to 17°C during the monitoring period (Fig. 21). The light conditions varied depending on the outdoor weather conditions.

Evaluation of flower longevity

In both the transgenic plants and the wild-type plants, the longevity of flowers of each line increased with decreasing temperature at the time of flowering (Fig. 22). The longevity of wild-type flowers increased from 2 to 3 days, whereas that of H70A#2 flowers increased from 4 to 6 days, that of H69A#4 flowers increased from 5 to 7 days, and that of H69A#7 flowers increased from 3 to 6 days. Although the longevity of both transgenic and wild-type flowers varied depending on the time of flowering, the longevity of the flowers of the three transgenic lines was always greater that that of wild-type flowers. Although the longevity of both wild-type flowers and flowers of transgenic lines expressing the mutated melon ethylene receptor genes *Cm-ERS1/H70A* or *Cm-ETR1/H69A* was influenced by changes in temperature, the longevity of the transgenic flowers was always greater than that of wild-type flowers.

Modification of floral architecture in the transgenic lines

The distance between the stigma and anthers (stigma-anther distance) of the transgenic and wild-type plants was measured (Fig. 23). The flowers of the three transgenic lines (H69A#4, H69A#7, H70A#2) displayed a SFA, with the stigma positioned higher than the anthers and stigma-anther distances of 4 to 6 mm, whereas the anthers of wild-type flowers were positioned higher than the stigma, NFA, with stigma-anther distances of 2 to 3 mm. Within each line, the floral architecture did not differ significantly at different flowering times; the wild-type plants always showed NFA and the transgenic plants always showed SFA.

These observations indicate that the SFA conferred by the mutated melon ethylene receptor genes *Cm-ETR1/H69A* and *Cm-ERS1/H70A* is relatively stable, even under conditions of varying environmental parameters, including temperature and light.

Pollen production in transgenic tobacco

The phenotypes of pollen grains of the transgenic plants were examined to investigate the cause of the reduced fertility. In contrast to the normal-appearing pollen grains of the wild-type plants, all plants of the transgenic lines produced pollen grains that appeared shriveled (data not shown). The numbers of normal and abnormal (shriveled) pollen grains from five anthers of each line were counted (Fig. 24). All wild-type flowers that flowered during any period produced approximately 75,000 pollen grains per anther, and the majority of pollen grains had a normal appearance. Flowers of the transgenic lines H70A#2 and H69A#7 that bloomed during any period produced approximately 30,000 pollen grains per anther, with normal pollen grains comprising about half or one-third of the total, respectively. Flowers of the transgenic line H69A#4 that flowered during any period did not produce any pollen grains under the greenhouse conditions, indicating that this line is sterile.

These observations demonstrate that the pollen-aborted phenotype of transgenic plants containing a mutated melon ethylene receptor gene is a stable phenotype, even in an open-air greenhouse in which the environmental conditions varied throughout the growth of the plants.

Floral architecture and fruit set

Wild-type plants showed NFA, and the transgenic plants showed SFA. Following both open and artificial pollination, the wild-type plants produced fruit in the open-air greenhouse (Fig. 25). The transgenic lines H69A#7 and H70A#2 did not set fruit following open pollination, but did so after artificial pollination, indicating that the floral architecture affects the fruit set. The transgenic line H69A#4 did not set fruit following either open or artificial pollination with wild-type pollen, indicating that this line is both male- and female-sterile. These results indicated that the frequencies of fruit setting were not changed by the varying environmental conditions throughout the growth of the plants.

Discussion

Although the longevity of both wild-type flowers and flowers of transgenic lines expressing the mutated melon ethylene receptor genes *Cm-ERS1/H70A* or *Cm-*

ETR1/H69A was influenced by changes in temperature, the longevity of the transgenic flowers was always greater than that of wild-type flowers. Flower longevity is an indicator of ethylene responsiveness in transgenic plants containing mutated ethylene receptor genes (Nukui et al., 2004; Takada et al., 2005; Cui et al., 2004; Wilkinson et al., 1997; Bovy et al., 1999); my present observations indicate that the ethylene responsiveness of these transgenic plants was always lower than that of the wild type, even when the environmental conditions such as light and temperature varied.

Wild-type flowers showed NFA, whereas flowers of all of the transgenic lines showed SFA. Both types of flower architecture were consistent at all times that flowering occurred, indicating that SFA induced in the transgenic plants was not a result of the varying environmental conditions. When sunflower plants close to anthesis are exposed to continuous white light, the filaments do not grow sufficiently far to allow the anthers to extrude from the corolla (Lobello et al., 2000). However, I did not investigate whether continuous light affects the floral architecture of the transgenic lines.

The pollen production of wild-type plants was always higher than that of the transgenic lines. In addition, the quality of the wild-type pollen was always higher than that of the transgenic pollen: the transgenic lines produced greater amounts of abnormal pollen grains than the wild type. The trait was stable in both the wild-type and transgenic lines in plants of the same line that flowered at different times.

The H69A#4 line showed male and female sterility while other transgenic lines showed male sterility only (Fig. 25). The H69A#4 line had slightly severe phenotype in flower longevity and flower architecture. In addtion, this line showed severe pollen abortion compared to other transgenic lines. It is likley that the transgene in the H69A#4 line is more effective in inducing the phenotypes. It will be interesting to analyze the relationship between the effectiveness of transgene and phenotypes of transformant.

Hot water treatment (43°C, 5 to 7 min) is used for the production of F_1 seeds during rice breeding. Two environmental genic male sterilities (EGMS) have also been reported in rice (Ku et al., 2001). The male sterility of these two EGMS rice lines, thermosensitive genic male sterility (TGMS) and photoperiod-sensitive genic male sterility (PGMS) lines, are controlled by temperature and photoperiod, respectively. The conditions for male fertility and male sterility in the TGMS line are 26°C/20°C (day/night) and 32°C/26°C (day/night), respectively. Additionally, the PGMS line shows

male sterility in a daylength of 14 h light. In contrast, the transgenic tobacco lines containing mutated ethylene receptor genes showed stable sterility or reduced fertility in varying temperatures (30°C to 17°C) and photoperiods, respectively. It the future, it will be interesting to grow the transgenic lines in various environments and investigate the stability of the phenotypes.

Although other types of inducible-male-sterility systems (Mariani et al., 1992; Cho et al., 2001; Huang et al., 2003; Kriete et al., 1996; Sa et al., 2002) that utilize the TA29 promoter have been reported, the stability of induced male sterility in these transgenic lines was not clearly observed or described. However, for the practical application of these male-sterility systems, it will be important to determine whether the induced male sterility is stable in varying environments. The present study has described stable male sterility induced by the expression of mutated melon ethylene receptor genes. These results should be of great use in the improvement of crop species.

In conclusion, SFA and pollen abortion induced by the expression of a mutated melon ethylene receptor in transgenic tobacco plants were stable, even under varying environmental conditions. These traits confer male sterility in the transgenic lines. The results of this study clearly indicate that this inducible male-sterility system could be a useful technology for preventing transgene flow through pollen dispersal. For the practical application of this system, a vector system for inducing the expression of other useful genes along with the mutated ethylene receptor genes needs to be developed. The usefulness of this system in other plant species should also be tested.

Chapter V: Heterologous expression of the mutated melon ethylene receptor gene *Cm-ERS1/H70A* produces stable sterility in transgenic lettuce (*Lactuca sativa* L.)

Abstract

The mutated melon ethylene receptor gene *Cm-ERS1/H70A* was introduced into tobacco and induced stable sterility in transgenic lines. This gene contains a missense mutation that converts the His^{70} residue to Ala in the melon ethylene receptor gene *Cm-ERS1*. To test the applicability of this inducible sterility system to other plants, lettuce (*Lactuca sativa* L.) was transformed with the gene using *Agrobacterium*, and putative transformants containing *Cm-ERS1/H70A* were obtained. Thirteen randomly selected putative transformants were grown in a growth room under constant conditions, and seven of the lines showed sterility or significantly reduced fertility. DNA gel blot analysis confirmed the integration of the *Cm-ERS1/H70A* gene into the genomes of the putative transformants, and RT-PCR and protein gel blot analysis confirmed the expression of *Cm-ERS1/H70A* mRNA and protein in all of the transformants. Five transformants showing sterility or reduced fertility when grown in a growth room under constant conditions were randomly selected to be grown in an open-air greenhouse under various environmental conditions. All five showed stable sterility under the various conditions. These results suggest that *Cm-ERS1/H70A* can induce sterility in heterologous transgenic plants.

Introduction

I demonstrated that the mutated melon ethylene receptor genes Cm-ETR1/H69A and Cm-ERS1/H70A could confer stable sterility or reduced fertility to transgenic tobacco plants (Takada et al., 2005; Takada et al., 2006; Ishimaru et al., 2006; Chapter II-IV), suggesting that these genes might be useful for regulating transgene flow through pollen dispersal.

In this chapter, to test the usefulness of these genes for inducing stable sterility in other plants, transgenic lettuce plants overexpressing *Cm-ERS1/H70A* were generated. The transgenic lettuce plants were evaluated for fertility both in a growth chamber under constant temperature and light and in a semi-containment greenhouse under variable environmental conditions.

Materials and methods

Plant growth conditions and statistical analysis

Lettuce seeds (*Lactuca sativa* cv Kaiser) were surface-sterilized and germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose and 0.2% (w/v) Gelrite under a 16-h light/ 8-h dark photoperiod at 25°C. The lettuce plants were transformed by infection with *A. tumefaciens* C58C1Rif^r harboring the binary vectors pIG121-Hm or pBICm-ERS1/H70A (Fig. 21A) according to the method of Sun et al. (2006). The primary transgenic plants were incubated *in vitro* at 25°C under a 16-h light/ 8-h dark photoperiod with fluorescent light at an intensity of 60 μmol $m^{-2}s^{-1}$. For all experiments untransformed wild-type and the transgenic plants (T_0 generation) were used. All experiments were repeated at least two times. All experiments were repeated at least two times. The data were subjected to Dunnet test by using the software 'Excel statistics 2002' (Social Survey Research Information Co., Ltd, Tokyo, Japan).

Southern hybridization analysis

Using the CTAB extraction method of Rogers and Bendich (1985), genomic DNA was extracted from 0.5 g of fresh young lettuce leaves grown in an open-air greenhouse. For DNA gel blot analysis, 10 μg of genomic DNA were digested with *BamHI*, electrophoresed on a 1% agarose gel at 50 V for 4 h, and transferred to a Hybond-N+

(Amersham Biosciences) nylon membrane under alkaline conditions. The membrane was hybridized overnight at 60°C with a ^{32}P-labeled *Cm-ERS1/H70A* gene fragment amplified by PCR using the primers TCTTGGATCTTTCAAGACTT and ACGAAGTCTTTAAACTTGAT. The membrane was then washed, with the final, most stringent wash in 2X SSC containing 0.1% SDS at 60°C for 15 min. Hybridization signals were detected using a BAS-5000 image analyzer (Fuji Photo Film, Japan).

Isolation of mRNA and reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated from young leaves of greenhouse-grown lettuce using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, CA) according to the manufacturer's instructions. The resulting first-strand cDNA was used as a template for amplification by PCR with the primers p35S-20f (5'-CGG GGG ACT CTA GAG GAT CC-3') and ERS+588r (5'-GTA GAC TTA GTC CAT TCC GT -3'). The PCR conditions used were 95°C for 9 min; 40 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and 8 min at 72°C. To confirm the integrity of the cDNA, control PCR reactions were performed with it using actin primers (LS-actin-f, 5'-TTT GCT GGG GAT GAT GCG CC-3'; LS-actin-r, 5'-GTG GTA CGA CCA CTG GCA TA-3').

Western blot analysis

Protein gel blot analysis was performed as described previously (Takahashi et al., 2002). In brief, microsomal membranes were prepared from lettuce leaves (1 g fresh weight) of either the wild type or transformants grown in an open-air greenhouse. Melon fruit microsomes (cv. Vedrantais) were used as a positive control. Equal amounts of proteins (15 μg) were separated by SDS-PAGE on an 8% gel. The primary and secondary antibodies used were rabbit anti-melon CmERS1 (1:1,000) generated against amino acid residues 117-327 (Takahashi et al., 2002), and anti-rabbit IgG-HRP (1:10,000; Santa Cruz Biotechnology), respectively. Signals were detected with a Peroxidase Stain Kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions.

Characterization of transgenic plants for the fertility

The plants were then clonally propagated *in vitro* by lateral bud elongation. The rooted plants were acclimatized in a containment growth room at 25°C under a 16-h light/ 8-h dark photoperiod and grown until flowering. Seeds were harvested at maturity.

For evaluation of the lines in a greenhouse following acclimation, groups of plants were transferred to a greenhouse in July and September 2004 and grown until flowering. Seeds were harvested at maturity. The seeds from 20 to 80 flowers from each plant were harvested to evaluate seed production. The temperature in the greenhouse was monitored using a Thermo Collector (RTC-20, ESPEC Environmental Test Technology Center, Co. Ltd, Osaka, Japan).

Histological observation of flowers

Flower buds of lettuce grown in an open-air greenhouse were fixed in FAA (63% ethanol, 5% glacial acetic acid, 2% formaldehyde) and dehydrated in a graded alcohol series (40% *t*(n)-butanol + 30% ethanol, 55% butanol + 25% ethanol, 70% butanol + 20% ethanol, 85 % butanol + 15% ethanol, and finally 100% butanol twice) at room temperature. The samples were then embedded in paraffin and sliced into 10-μm sections using a rotary microtome HM325 (Microm, Germany). The sections were stained with 0.05% toluidine blue. Imaging was performed using an MZ FL III microscope (Leica, Germany) equipped with a DC 300 digital camera (Leica).

Results

To validate the adaptability and applicability of the inducible male-sterility system using mutated melon ethylene receptor genes, *Cm-ERS1/H70A* was introduced into lettuce (*Lactuca sativa* L.) plants using the method of Sun et al. (2006), and fifty-one independent kanamycin-resistant shoots were obtained. None of the kanamycin-resistant plants showed obvious differences in shape or growth compared to wild-type plants. The presence of transgenes was confirmed in all kanamycin-resistant plants using genomic PCR (data not shown).

Thirteen transgenic plants randomly selected from among the PCR-positive plants were grown to flowering in a growth chamber under constant conditions. Non-

transformed plants, used as a negative control, and transformed plants containing pIG121-Hm, as a positive control, were also grown to flowering. Seeds from these plants were harvested at maturity (Fig. 27). Both non-transformed and transformed plants produced seeds. Five (2, 3, 5, 13, and 40) of the thirteen transgenic lines containing *Cm-ERS1/H70A* set seeds. Six transgenic lines (1, 8, 31, 33, 38, and 51) were sterile, and two lines (11 and 50) showed significantly reduced fertility. These results demonstrate that *Cm-ERS1/H70A* effectively induced sterility in transgenic lettuce plants, as shown previously in transgenic tobacco (Takada et al., 2006; Chapter III). Transgenic tobacco plants expressing the *Cm-ERS1/H70A* gene driven by the 35S promoter showed pleiotropic phenotypes, including modified floral architecture, reduced seed yields, reduced numbers of normal pollen grains, decreased plant height, greater anthocyanin accumulation, and delayed leaf yellowing (Takada et al., 2006; Chapter III). In contrast, transgenic lettuce plants expressing *Cm-ERS1/H70A* showed only reduced pollen production, resulting in the sterility of the lines, which was the only obvious pleiotropic phenotype.

Five independent transformed lines (11, 31, 33, 50, and 51) showing sterility or strongly reduced fertility were subjected to DNA gel blot analysis to determine the integration of the *Cm-ERS1/H70A* gene, using a PCR-amplified *Cm-ERS1* fragment as a probe (Fig. 26A). The DNA from a non-transformed plant was used as a negative control. All of the transformed plants produced a 2.0-kb band (Fig. 26B), indicating the insertion of the *Cm-ERS1/H70A* gene into the genomes of these plants. No hybridization signal was detected from the non-transformed control (Fig. 26B).

To investigate the expression of the introduced *Cm-ERS1/H70A* gene in the above five transgenic lines, RT-PCR analysis was performed (Fig. 28A). Although transgenic line 31 showed lower levels of *Cm-ERS1/H70A* mRNA than the other four transgenic lines, the transgene was expressed in all five independent transformants examined, but not in the wild-type plants. These results show that the transgenes are expressed stably in the transformants.

Expression of the CmERS1/H70A protein in the transgenic lines (11, 31, 33, 50, and 51) was detected by protein gel blot analysis using an anti-CmERS1 antibody (Fig. 28B). Microsomal membranes were prepared from lettuce leaves collected from the wild type and transformants showing sterility. An expected signal of 67-kDa

molecular mass was observed in both the positive control (melon fruit microsomes, Fig. 28B, lane 1) and the transgenic lines (Fig. 28B, lanes 3-7). A weak band was detected in the wild type (Fig. 28B, lane 2). This signal may have been caused by cross-reaction of the antibody with native ethylene receptors of lettuce. Taken together, these results suggest that the sterility observed in both the positive control (melon fruit microsomes, Fig. 28B, lane 1) and the transgenic lines (Fig. 28B, lanes 3-7). A weak band was detected in the wild type (Fig. 28B, lane 2). This signal may have been caused by cross-reaction of the antibody with native ethylene receptors of lettuce. Taken together, these results suggest that the sterility observed in the transgenic plants is linked to overepression of the *Cm-ERS1//H70A* transgene.

Although the *Cm-ERS1/H70A* transgene was expressed in five greenhouse-grown transformants that showed sterility or reduced fertility, transgenic line 31 showed lower expression of the *Cm-ERS1/H70A* mRNA than the other four transgenic lines. However, the Cm-ERS1/H70A protein was detected in the five transformants tested. These results suggest that the expression level of the transgene in transgenic line 31 was affected by other factor. A more detailed analysis, including examining the expression of endogenous ethylene receptors, should provide insight into these factors.

Two transgenic lines (11 and 50) that showed significantly reduced fertility and three lines (31, 33, and 51) that showed fertility under constant conditions were grown to flowering in a greenhouse under various conditions (Table 1). Wild-type plants set an average of 5.26 seeds per flower. In contrast, transgenic lines 11 and 50 set averages of 0.01 and 0.88 seeds per flower, respectively. Lines 31, 33, and 51 set no seeds. These results indicate that the transgenic lines expressing *Cm-ERS1/H70A* show stable sterility or reduced fertility under various environmental conditions.

To examine the production of pollen in sterile or less-fertile transgenic lettuce lines, longitudinal sections of flower buds at the stage just before flowering were prepared and their pollen production observed. Wild-type plants produced large amounts of pollen in anthers (Fig. 29A), whereas pollen production was lower in transgenic line 50 (Fig. 29B), which showed significantly lower fertility. Transgenic line 11 also showed lower pollen production (data not shown). The sterile transgenic line 31 produced almost no pollen (Fig. 29C), and the other sterile lines, 33 and 51, also showed reduced pollen production (data not shown). These observations indicate that

the transgene *Cm-ERS1/H70A* affects pollen development and results in pollen abortion in transgenic lettuce lines, in accordance with observations in transgenic tobacco expressing the same gene (Ishimaru et al., 2006; Chapter IV). These findings indicate that the inducible male-sterility system using mutated melon ethylene receptor genes is useful not only in lettuce and tobacco plants but also in other plant species under various environmental conditions.

Discussion

To validate the adaptability and applicability of the inducible male-sterility system using mutated melon ethylene receptor genes, *Cm-ERS1/H70A* was introduced into lettuce (*Lactuca sativa* L.) plants. *Cm-ERS1/H70A* effectively induced stable sterility in transgenic lettuce, as shown previously in transgenic tobacco (Takada et al. 2006; Chapter III). Eight of thirteen transgenic lettuce lines showed sterility or significantly reduced fertility. Transgenic tobacco plants expressing the *Cm-ERS1/H70A* gene driven by the 35S promoter showed pleiotropic phenotypes, including modified floral architecture, reduced seed yields, reduced numbers of normal pollen grains, decreased plant height, greater anthocyanin accumulation, and delayed leaf yellowing. In contrast, transgenic lettuce plants expressing *Cm-ERS/H70A* showed only reduced pollen production, resulting in the sterility of the lines, which was the only obvious pleiotropic phenotype.

Although the *Cm-ERS1/H70A* transgene was expressed in five greenhouse-grown transformants that showed sterility or reduced fertility, transgenic line 31 showed lower expression of the *Cm-ERS1/H70A* mRNA than the other four transgenic lines. However, the Cm-ERS1/H70A protein was detected in the five transformants tested. These results suggest that the expression level of the transgene in transgenic line 31 was affected by other factors. A more detailed analysis, including examining the expression of endogenous ethylene receptors, should provide insight into these factors.

When grown in an open-air greenhouse, the transgenic lines continued to show sterility or substantially reduced fertility under various environmental conditions. Furthermore, histological observations indicated that the transgene *Cm-ERS1/H70A* affects pollen development and results in pollen abortion in the transgenic lettuce lines, in accordance with observations in transgenic tobacco expressing the same gene

(Ishimaru et al., 2006; Chapter IV). These findings indicate that the inducible male-sterility system using mutated melon ethylene receptor genes is useful not only in lettuce and tobacco plants but also in other plant species under various environmental conditions.

In conclusion, we have demonstrated that *Cm-ERS1/H70A* can stably induce sterility in heterologous transgenic plants under various environmental conditions. This system to induce male sterility in transgenic plants using a mutated ethylene receptor could contribute to overcoming concerns about GM plants by preventing pollen dispersal from transgenic plants.

Chapter VI: General discussion

Plants expressing the mutated melon ethylene receptor genes *Cm-ETR1/H69A* and *Cm-ERS1/H70A* show not only a reduction in ethylene sensitivity, but also a reduction in fertility in heterologous plants (Cui et al., 2004; Nukui et al., 2004), suggesting that these genes could be useful for producing sterile plants. Possible transgene flow into the environment via pollen dispersal is a concern for commercial applications of transgenic plants. Therefore, the induction of sterility in transgenic plants is a potential strategy to prevent such transgene flow.

To investigate the potential of the mutated melon ethylene receptor genes for the induction of sterility in transgenic plants, I introduced these genes into tobacco and evaluated their effects on the development of transgenic tobacco. Transgenic tobacco plants expressing the mutated melon ethylene receptor genes under the control of the constitutive CaMV 35S promoter showed increased flower longevity compared to that of the wild type. Additionally, the yellowing of leaves during senescence was delayed. These results suggest that both mutant genes conferred reduced ethylene sensitivity to transgenic tobacco, just as the expression of ethylene receptor genes in heterologous plants confers insensitivity or reduced sensitivity to ethylene in several other 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-ERS1/H70A* or *Cm-ETR1/H69A* displayed pleiotropic phenotypes, including short filament architecture, reduced seed yield, reduced production of normal pollen grains, decreased plant height, increased anthocyanin accumulation in transgenic petals, and infrequent delay of anther dehiscence. Specifically, I suspected that the reduced fertility in transgenic tobacco plants with *Cm-ERS1/H70A* and *Cm-ETR1/H69A* was caused by both the modification of floral architecture and the reduction in normal pollen production.

The reduction in normal pollen production was likely caused by the abnormal tapetum degeneration during anther development observed in the transformants. It is likely that reduced ethylene sensitivity in these plants causes an abnormality in programmed cell death (PCD) in the tapetum. My results demonstrated that the mutated ethylene receptor genes are involved in stamen development, a major step in plant reproductive physiology.

To create transgenic plants that produce little pollen without altering the floral

architecture, I constructed mutated melon ethylene receptor genes that would be expressed specifically in the tapetum. However, a promoter with high specificity to the tapetum was required in this new system for the induction of male sterility, because previous systems have resulted not only in male sterility, but also in modification of floral architecture (Mariani et al., 1990; Kriete et al., 1996; Sa et al., 2002; Huang et al., 2003).

I selected 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, the Nin88 protein is detected both in the tetrad and in the microspores. I predicted that the tapetum-specific expression of *Cm-ERS1/H70A* would induce abnormal tapetum degeneration and result in pollen abortion without modifying 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, abnormal tapetum degeneration was observed in the anthers of transgenic plants with the *Nin88* promoter and 35S promoter-driven *Cm-ERS1/H70A* (Takada et al., 2006; Chapter III), which was similar to the results in transformants expressing *Cm-ETR1/H69A* (Takada et al., 2005; Chapter II). There was no modification of floral architecture in the NH70A transformants producing abnormal male gametes, and 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.

For the practical application of this phenomenon to transgene containment in genetically modified plants, the stability of these traits under more variable environmental conditions must be determined. The over-expression of mutated melon ethylene receptor genes conferred sterility or reduced fertility in transgenic tobacco plants (H69A and H70A) because of the alteration of floral architecture and the abortion of pollen production due to abnormal tapetum degeneration in the anther (Takada et al., 2005, 2006; Chapters II and III). I evaluated the traits of these transgenic plants in growth chambers under constant conditions; thus, the stability of these traits under naturally occurring variable environmental conditions (e.g., light, temperature, wind, and rainfall) in regions where is the possible area for the transgenic plants still required

investigation.

Therefore, 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 the environmental conditions varied (Ishimaru et al., 2006; Chapter IV). Observations of the transgenic plants demonstrated that traits including flower longevity, floral architecture, pollen production, and seed yield were stable under varying environmental conditions, and suggest that a system using ethylene receptor genes to induce male sterile may be useful in preventing transgene flow via pollen dispersal. However, for a commercial application of developed technology, the stability of these traits in open field needs to be investigated in detail.

Although other types of inducible male sterility systems using the TA29 promoter have been reported, the stability of the induced male sterility in these transgenic lines has not been clearly observed or described (Mariani et al., 1992; Kriete et al., 1996; Cho et al., 2001; Sa et al., 2002; Huang et al., 2003). However, for the practical application of these male-sterility systems, it is important to determine whether the induced male sterility is stable under environmentally variable conditions. My results indicated that the expression of mutated melon ethylene receptor genes induced stable male sterility. These results should be of great use in the improvement of crop species.

To evaluate the adaptability of the inducible male sterility system using a mutated melon ethylene receptor gene, *Cm-ERS1/H70A* was introduced into lettuce (*Lactuca sativa* L.) plants. Transgenic lettuce plants were grown in growth chambers under constant temperature and light conditions and in an open-air greenhouse under variable conditions. The transgenic lettuce showed sterility or substantially reduced fertility both in the growth chamber and in the variable outdoor environment, similar to the transgenic tobacco plants. These results suggest that the inducible male sterility system using mutated melon ethylene receptor genes is adaptable to various plant species.

I investigated flower longevity, pollen grain and seed production, and floral architecture in the transgenic plants H69A, H70A, and NH70A and found high variation within transgenic lines. For example, the flower longevity of H69A transgenic plants ranged from 2.5 to 6 days. There are two possible explanations for this variability: RNA

silencing (van der Krol et al., 1990; Napoli et al., 1990) or a positional effect of the transgene in the plant genome (Dean et al., 1988; Peach and Velten, 1991). Phenotypic differences in transgenic plants are a major issue for the practical application of genetically modified plants. Thus, it is necessary to introduce an insulator sequence into the *pNin88::Cm-ERS1/H70A* vector so that the transgenic plants show stronger phenotypes. DNA elements containing a chromatin insulator or boundary function to insulate the transgene from the repressive effects of neighboring chromatin have been proposed to prevent gene silencing (Meyer, 1998; Allen et al., 2000). Matrix-associated regions (MARs) are candidates for such insulating activity. The presence of the chicken lysozyme MAR element, known as the A element, around transgenes in tobacco results in the position-independent expression of the transgenes (Mlynarova et al., 1994, 1995; Jansen et al., 2002).

Perspective

I developed a unique system for the induction of male sterility in transgenic plants using *Cm-ERS1/H70A* and *Cm-ETR1/H69A* (Fig. 30). 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 in floral architecture. When I 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 compared to that of the wild type. Therefore, the development of a better system for inducible male sterility requires the identification of a promoter with greater specificity for the tapetum layer. The use of a more specific promoter may circumvent the complication of female sterility in producing transgenic plants for cross breeding.

Although various systems for inducible male sterility in transgenic plants have been reported recently, the systems were only designed to prevent transgene flow via pollen dispersal (Mariani et al., 1990; Spena, 1992; O'Keefe et al., 1994; Dotson et al., 1996; Kriete et al., 1996; McConn and Browse, 1996; Derksen et al., 1999; Sanders

et al., 2000; Goetz et al., 2001). To enhance the practical application of the inducible male sterility system, genes conferring useful traits for crops of interest could be introduced into transgenic plants, in addition to the mutated ethylene receptor genes. For example, by introducing *Bacillus thuringiensis* and a mutated melon ethylene receptor gene simultaneously, it may be possible to produce transgenic plants with both insect resistance and male sterility. The production of such transgenic plants should mitigate concerns about transgene flow via pollen dispersal while providing advantages 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.

My system for inducible male sterility can be applied to transgenic crops that are vegetatively propagated and from which neither fruits nor seeds are harvested. The development of fruits requires pollination; 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 the harvested product. To apply ethylene receptor genes to fruit or seed crops, it is necessary to use the CMS/*Rf* system. In this system, it is difficult to produce a change in the sequences of each species because the transgenes are integrated into the chloroplast genome. Stable chloroplast transformation depends on the integration of foreign DNA into the chloroplast genome by homologous recombination; therefore, the foreign gene that is introduced must be flanked by sequences homologous to those of the chloroplast genome (Staub and Maliga, 1992). Generally, homologous sequences of more than 400 bp in length are used on each side of the construct to obtain chloroplast transformants at a reasonable frequency. However, the frequency of obtaining chloroplast transformants is very low, except in tobacco plants. Moreover, it is thought necessary to introduce the anti-sense ethylene receptor gene in *Rf* lines, and the pollen diffusion in the *Rf* lines results in a new problem. However, my system may be advantageous in tuber crops (e.g., potato, sweet potato, and taro), trees, and perennial and bulb-forming plants from which cut flowers are harvested (e.g., carnation, chrysanthemum, lily, and gladiolus).

Chrysanthemum production is popular as commercial cut flowers in the world, and the development of a genetically modified chrysanthemum with pest resistance is

active in Japan. However, there are wild-relatives to chrysanthemum compatible to the transgenic plans, and therefore the commercial use of transgenic chrysanthemum has not been done from the concern of the transgene diffusion via pollen dispersal. Shinoyama et al. (personal communication) has developed a transgenic chrysanthemum that could not produce pollen using the system I developed in this study. This suggests my technology is useful for overcoming the problem in transgenic chrysanthemum development. The stability of traits in transgenic chrysanthemum under variable environmental conditions is now in progress.

Finally, I hope the methods developed in this study are used for the future development of transgenic crops, which contribute to overcome food production and environmental problems. I hope that genetically modified crops will help humans worldwide.

Summary

Ethylene receptor genes play significant roles in plant development. However, their function in plant reproduction is unclear. To investigate the role of an ethylene receptor gene in plant reproduction, I introduced the mutated melon ethylene receptor genes *Cm-ETR1/H69A* and *Cm-ERS1/H70A* into tobacco plants (*Nicotiana tabacum* cv. 'Sumsun'). These genes have mis-sense mutations in *Cm-ETR1* and *Cm-ERS1*, which are homologous to *Arabidopsis ETR1* and *ERS1*, respectively, and are expected to disrupt the ethylene-binding function and reduce ethylene sensitivity in transgenic plants. Transgenic plants expressing *Cm-ETR1* or *Cm-ERS1* showed pleiotropic phenotypes, including modified floral architecture, male and female sterility, delay in leaf yellowing, and abnormal tapetum degeneration. Additionally, flower longevity was greater than that in the wild type, indicating that the transgenic plants had reduced sensitivity to ethylene. The results suggest that stamen development is related to ethylene sensitivity.

Because a major concern regarding genetically modified crops is transgene flow via pollen dispersal, the induction of sterility in these plants may be a strategy for preventing such transgene flow. Accordingly, to develop a new system in tobacco plants to induce male sterility without modifying floral architecture, the *Cm-ERS1/H70A* gene was fused to the tobacco *Nin88* promoter, which functions mainly in the tapetum and microspores. The fusion gene *pNin88::Cm-ERS1/H70A* was introduced into tobacco plants, generating two independent transformants that produced reduced amounts of normal pollen without modification of the floral architecture. Histological observations of anthers at stage 2 showed that abnormal tapetum degeneration occurred in the transformants, but not in the wild type, strongly indicating that the expression of the mutated gene was involved in this abnormal degeneration. These results suggest that the tapetum-specific expression of a mutated ethylene receptor gene is a potential strategy for inducing male sterility in transgenic plants.

I demonstrated that over-expression of the mutated melon ethylene receptor gene *Cm-ETR1/H69A* or *Cm-ERS1/H70A* induces pollen abortion and altered flower architecture, resulting in sterility or reduced fertility in transgenic tobacco plants. For practical applications, it is important to determine whether these transgenic tobacco plants have stable traits, i.e., male and female sterility, in the field. To investigate the

stability of these traits, the transgenic plants were grown in a greenhouse with environmental conditions that varied depending on the outdoor conditions. Temperatures ranged from 31°C at the beginning of September to 17°C at the beginning of November; natural sunlight was provided. The wild-type plants showed normal flower architecture, whereas the transgenic plants showed modified flower architecture. The floral architecture was stable during the different flowering periods. Pollen production was significantly reduced in two transgenic lines and completely aborted in one transgenic line, and these traits were also stable during the flowering periods. These results suggest that the sterility or reduced fertility induced by the expression of mutated melon ethylene receptor genes in transgenic tobacco plants is stable under varying environmental conditions.

To test the applicability of this inducible sterility system to other plants, lettuce (*Lactuca sativa* L.) was transformed with the gene using *Agrobacterium*, and putative transformants containing *Cm-ERS1/H70A* were obtained. Thirteen randomly selected putative transformants were grown in a growth chamber under constant conditions; seven of the lines showed sterility or significantly reduced fertility. DNA gel blot analysis confirmed the integration of the *Cm-ERS1/H70A* gene into the genomes of the putative transformants. RT-PCR and protein gel blot analyses confirmed the expression of *Cm-ERS1/H70A* mRNA and protein in all of the transformants. Five transformants that showed sterility or reduced fertility in the growth chamber were randomly selected to be grown in an open-air greenhouse with varying environmental conditions. All five showed stable sterility under the varying conditions. These results suggest that *Cm-ERS1/H70A* can induce sterility in heterologous transgenic plants.

In conclusion, I developed a new system for inducing male sterility using a mutated ethylene receptor gene that is expressed stably in varying environmental conditions. A new system for the simultaneous expression of useful genes and the mutated ethylene receptor gene would assist in promoting the incorporation of an inducible male sterility system into a wide variety of plants.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Hiroshi Ezura for his invaluable suggestions and providing me this precious study opportunity in his laboratory.

I especially would like to express my deepest appreciation to my supervisors, Professors Hiroshi Kamada and Shigeo Nishimura, Associate Professors Ryo Ohsawa and Tsuyoshi Mizoguchi, and Assistant professor, Ken-ichi Yuhashi for his elaborated guidance, considerable encouragement and invaluable discussion that make my research of great achievement and my study life unforgettable.

I am very grateful to K. Tachibana, Y. K. Lee, K. Ishimaru, T. Sano for their valuable cooperation in my experiments.

Special thanks must be accorded to all members of the laboratory for much useful discussion of the results reported herein.

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Figures and Table

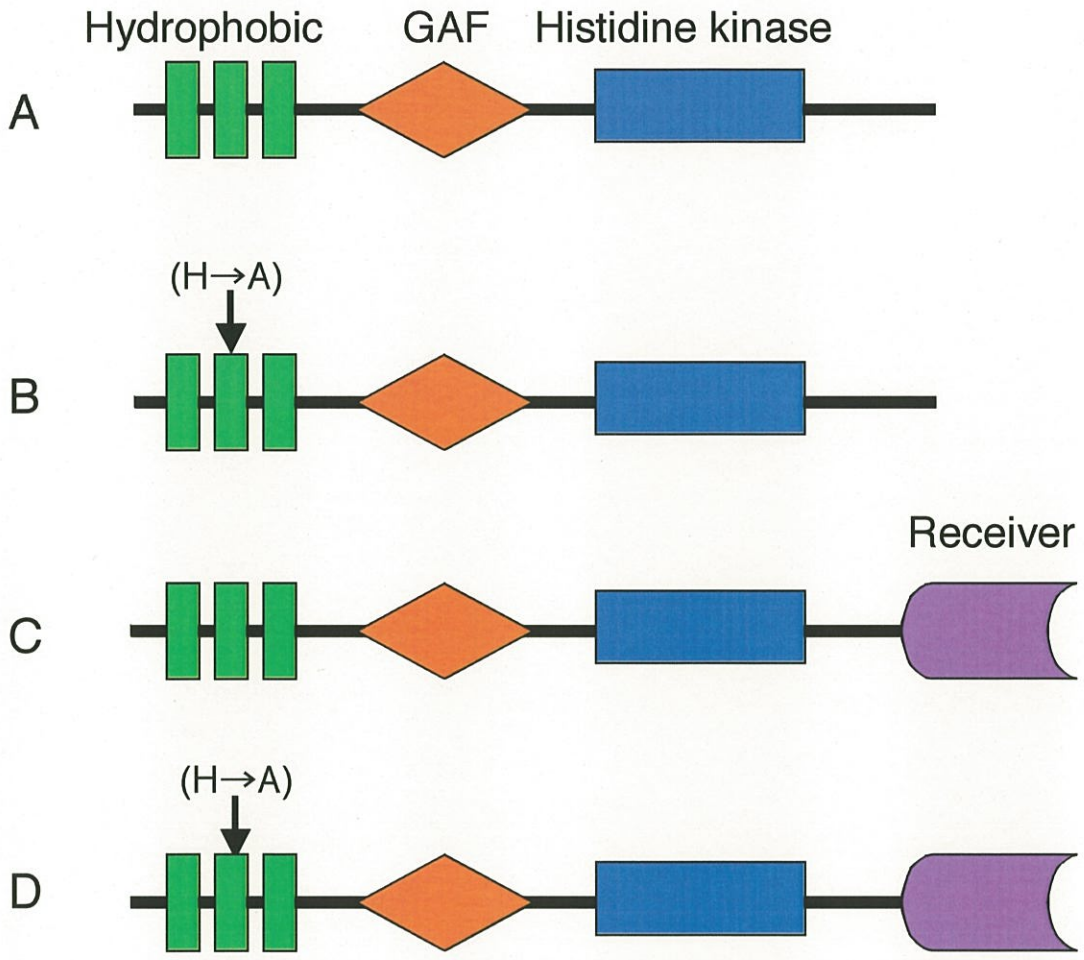

Fig. 1. Structural features of the melon ethylene receptors. A missense mutation (H→A) of Cm-ERS1/H70A and Cm-ETR1/H69A was introduced into the transmembrane domain, converting the His^{70} and His^{69} residue into Ala, respectively, affecting the binding of ethylene to these proteins. **A** Cm-ERS1. **B** Cm-ERS1/H70A. **C** Cm-ETR1. **D** Cm-ETR1/H69A.

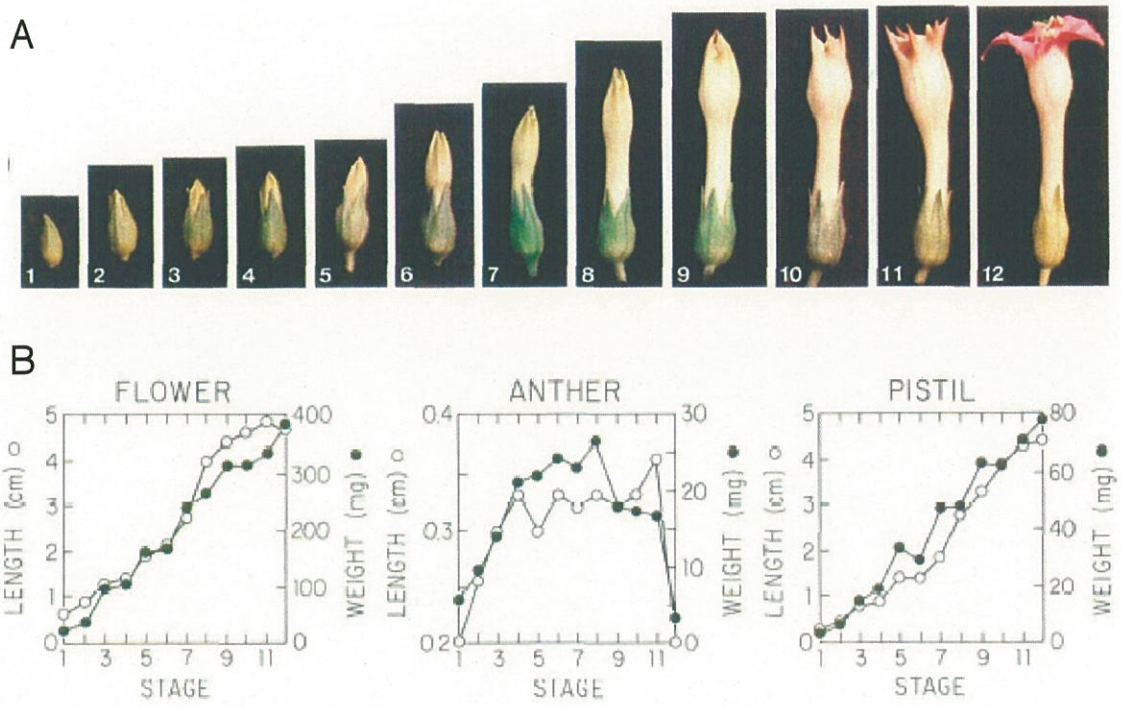

Fig. 2. Tobacco flower stage (Koltunow et. al., 1990). **A.** Flower development from 8-mm bud to opening. Flower buds were divided into 12 stages on the basis of size and morphological criteria. Stage 1 was designated as the period when tetrads are present within the anther and corresponds to an 8-mm flower bud. Stage 12 was designated as the time of flower opening and anther dehiscence. The transition from stage 1 to stage 12 occurred over a 1-week period during the summer. **B.** Changes in organ system weight and length during flower development. Five flower buds were picked at each development stage, and their lengths and fresh weights were measured individually. Similar measurements were made for the anthers and pistil of each flower bud. Data points represent the mean of these five measurements. Anther fresh weights represent the collective weight of all five anthers within the flower bud.

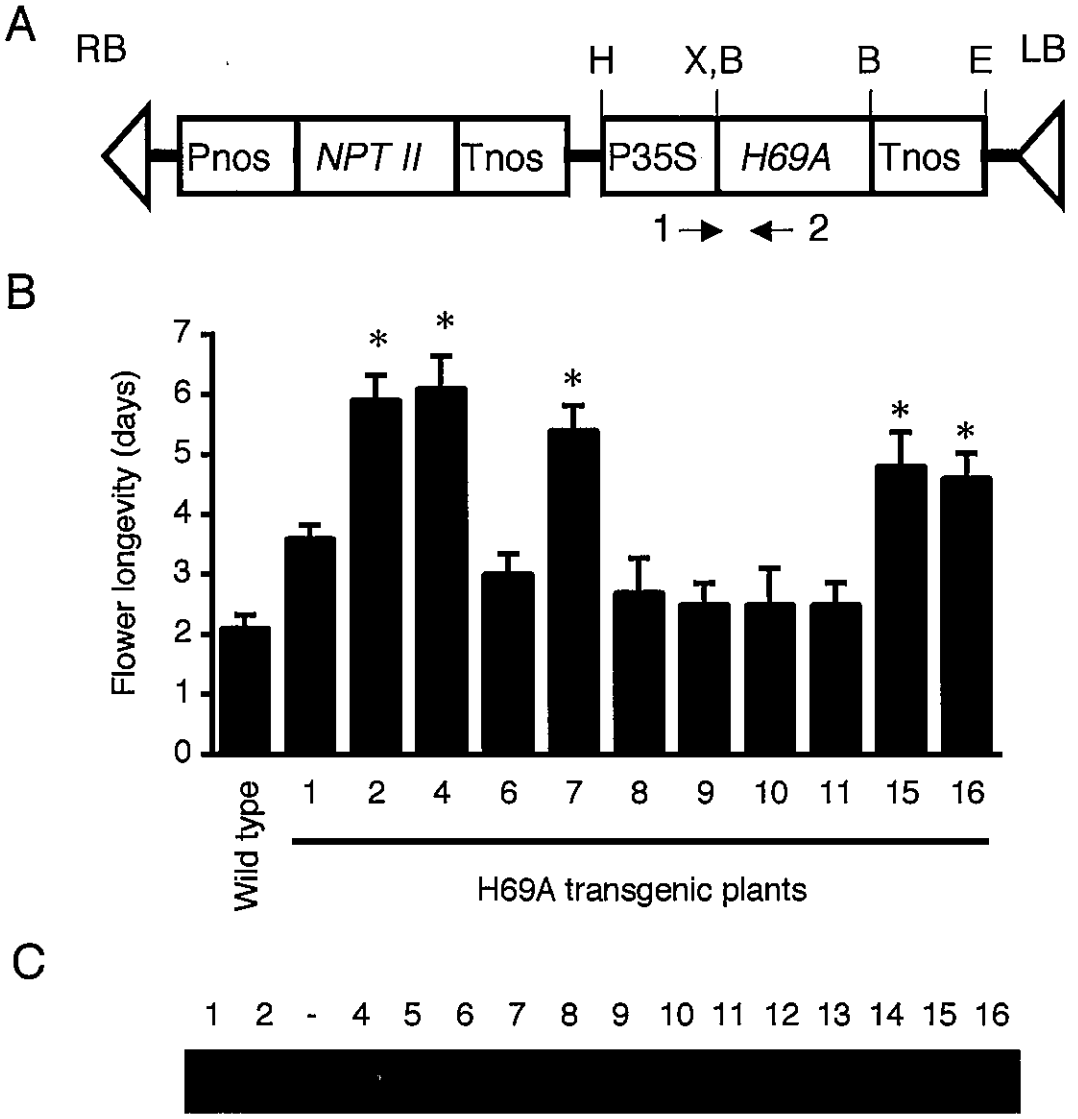

Fig. 3. **A** Construction of the transformation vector pBICm-ETR1/H69A. RB and LB, right and left borders of T-DNA; B, *Bam*HI site; H, *Hind*III site; X, *Xba*I site; Pnos, nopaline synthase gene promoter; *NPTII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; H69A, *Cm-ETR1/H69A* gene. Arrows 1 and 2 indicate the primers used in PCR and RT-PCR. **B** Flower longevity of transgenic tobacco plants with pBICm-ETR1/H69A. The flower longevity is expressed as the mean number of days from opening until the onset of corolla wilting. The flower longevities of five flowers of each line were determined. Error bars indicate standard error. Asterisk shows significant difference (Dunnet test for comparing wild-type to each of the transgenic lines. $P<0.05$). **C** Example of genomic PCR to confirm the presence of the *Cm-ETR1/H69A* transgene.

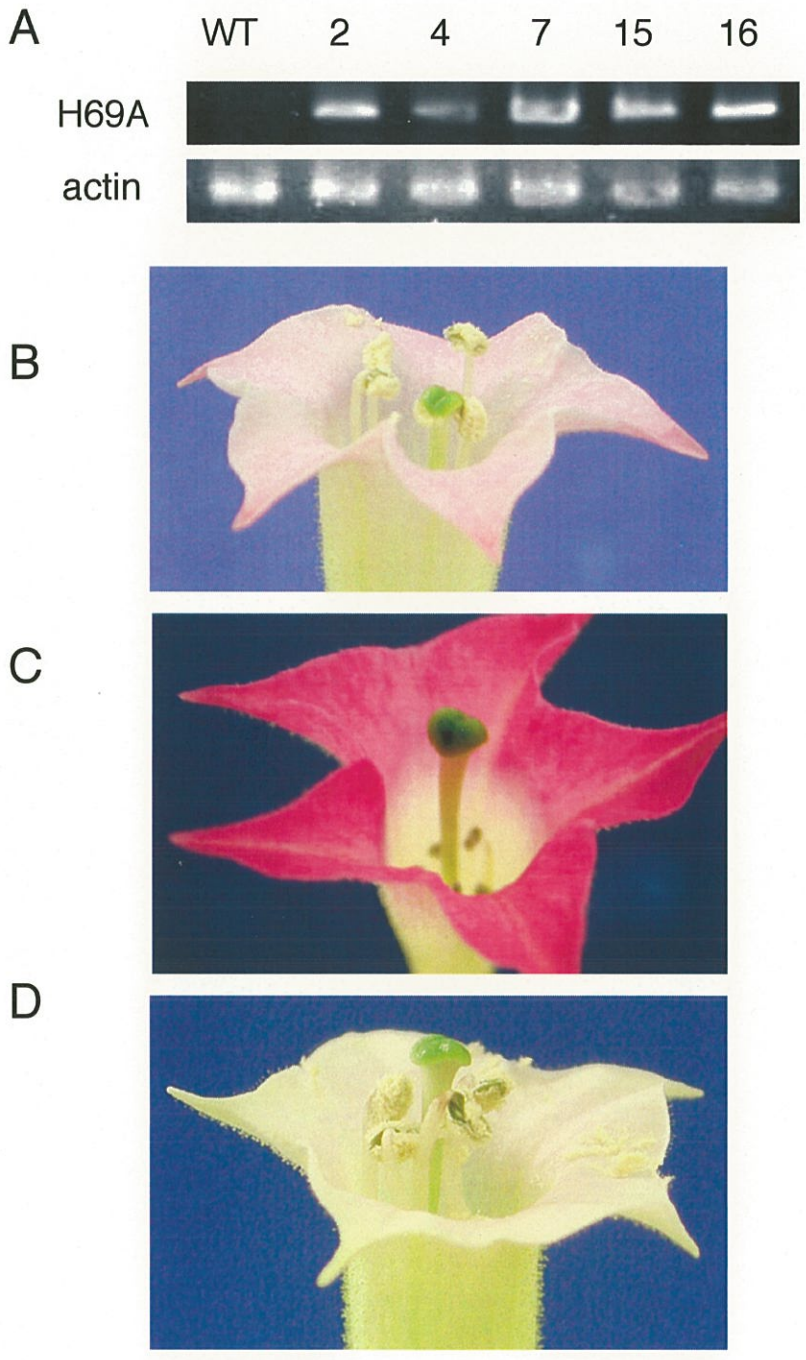

Fig. 4. **A** RT-PCR analysis of transgene expression using mRNA isolated from leaves. **B-D** Modification of floral architecture. **B** Wild-type flower. **C** H69A #4 flower. **D** Wild-type cut flower treated by 0.2 mM STS when the flower size was 2.3 cm (stage 6).

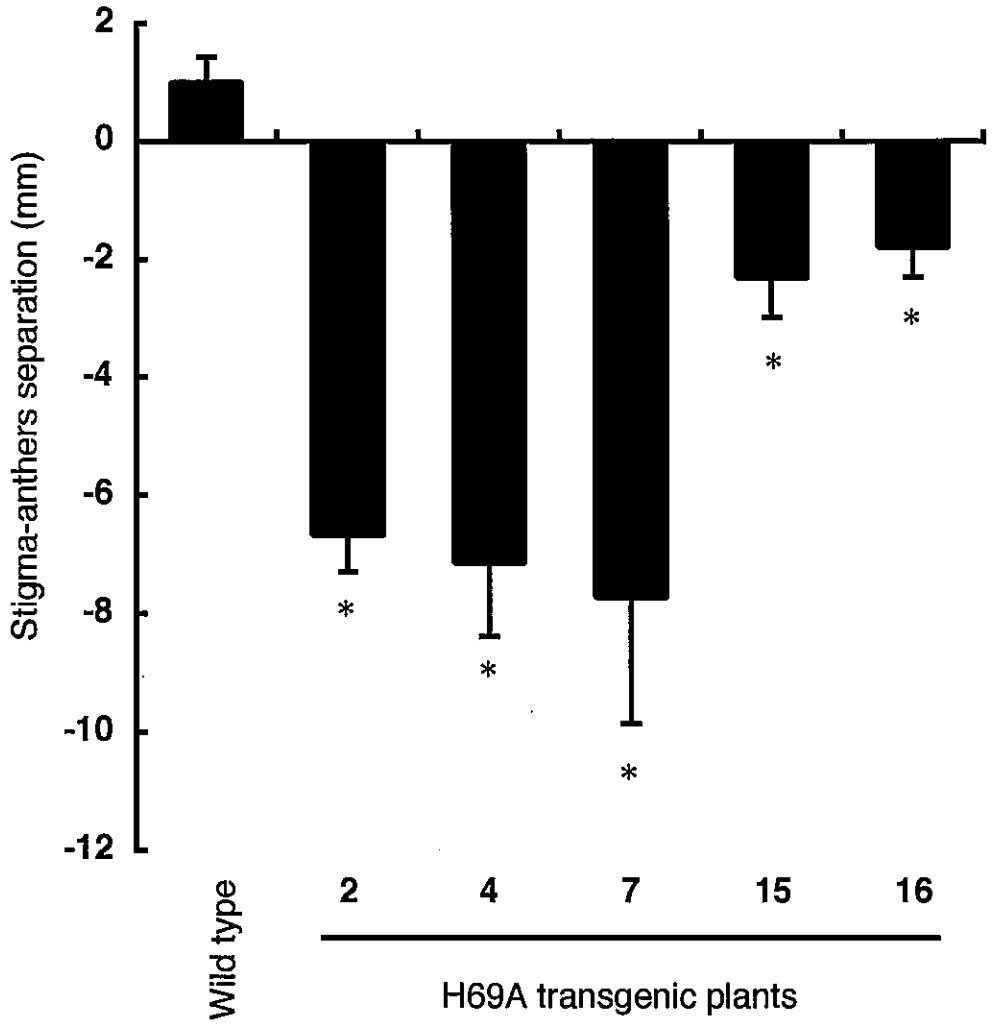

Fig. 5. Stigma-anther distance in transgenic tobacco plants expressing the *Cm-ETR1/H69A* gene. The stigma-anther distance was calculated by determining the difference between the stigma height and the height of all of the filaments. Asterisk shows significant difference (Dunnet test for comparing wild-type to each of the transgenic lines. $P<0.05$).

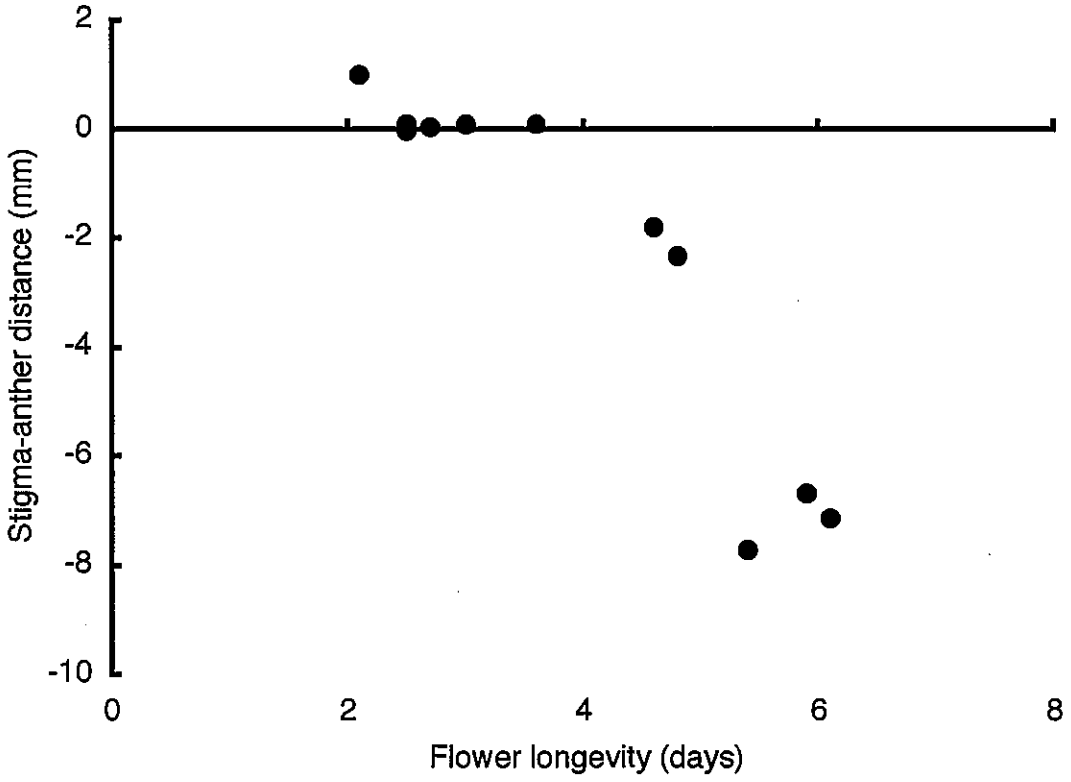

Fig. 6. Relationship between flower longevity and stigma-anther distance for all H69A transgenic lines. Spearman's rank correlation test was used at the 1% level (r=-0.8392, P=0.00471).

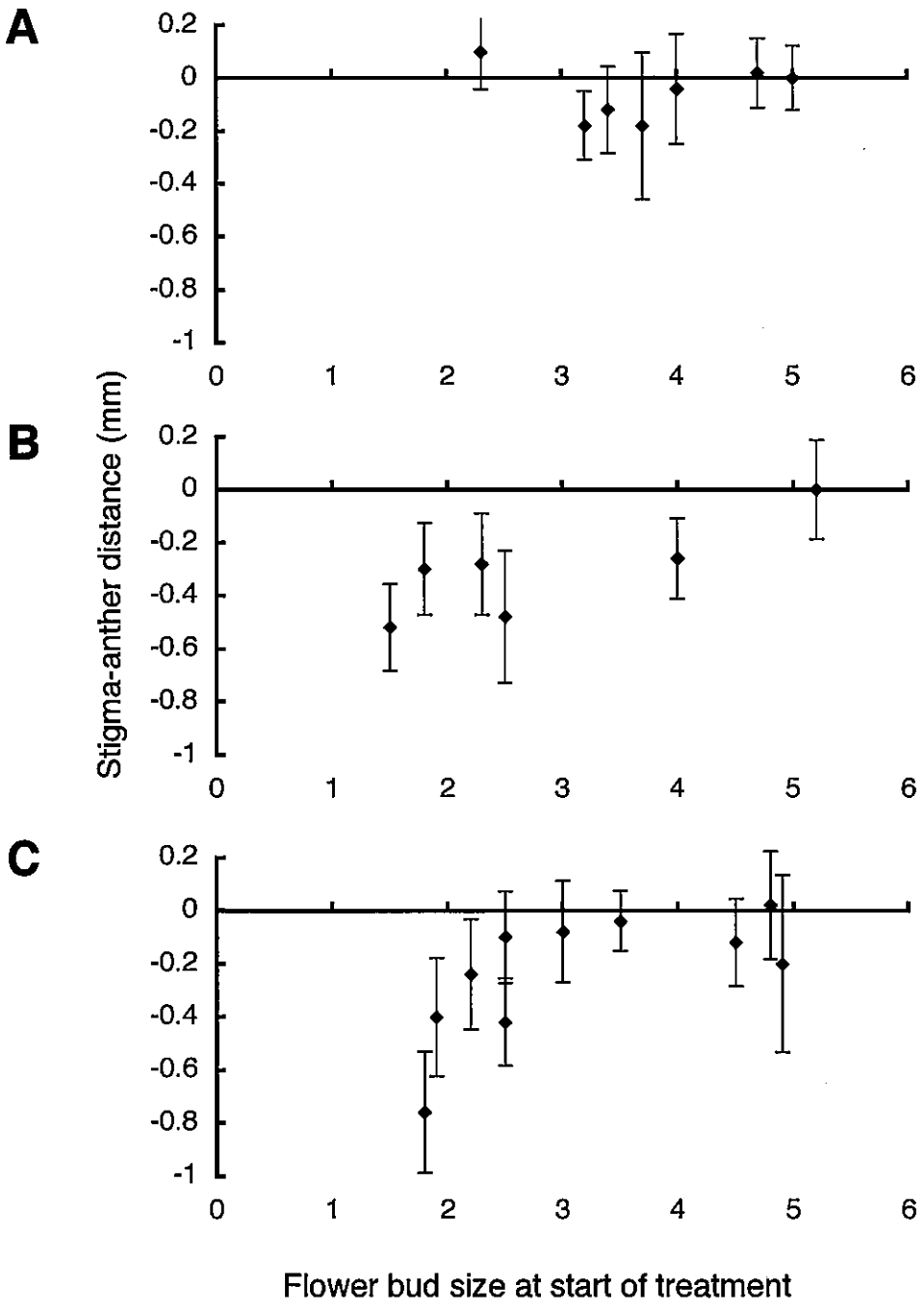

Fig. 7. Effect of STS on floral architecture in wild-type plants. Stigma-anthers distance on wild-type flowers with treatment of 0.05 mM STS (**A**), 0.1 mM STS (**B**) or 0.2 mM STS (**C**). Flower bud size was measured at start of STS treatment. Stigma-anthers separation was calculated by determining the difference between stigma height and the all anthers. Error bars indicate standard error.

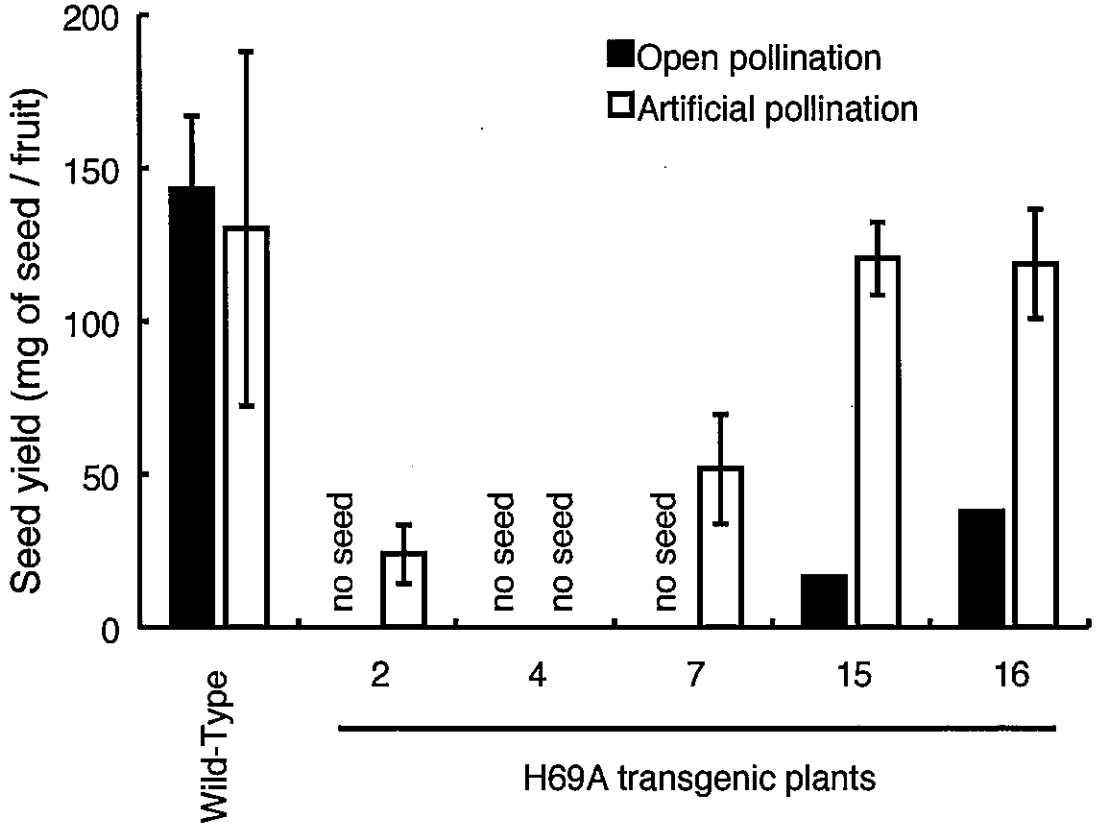

Fig. 8. Seed yields of transgenic lines. Five fruits of each line were examined during each treatment. Error bars indicate standard error.

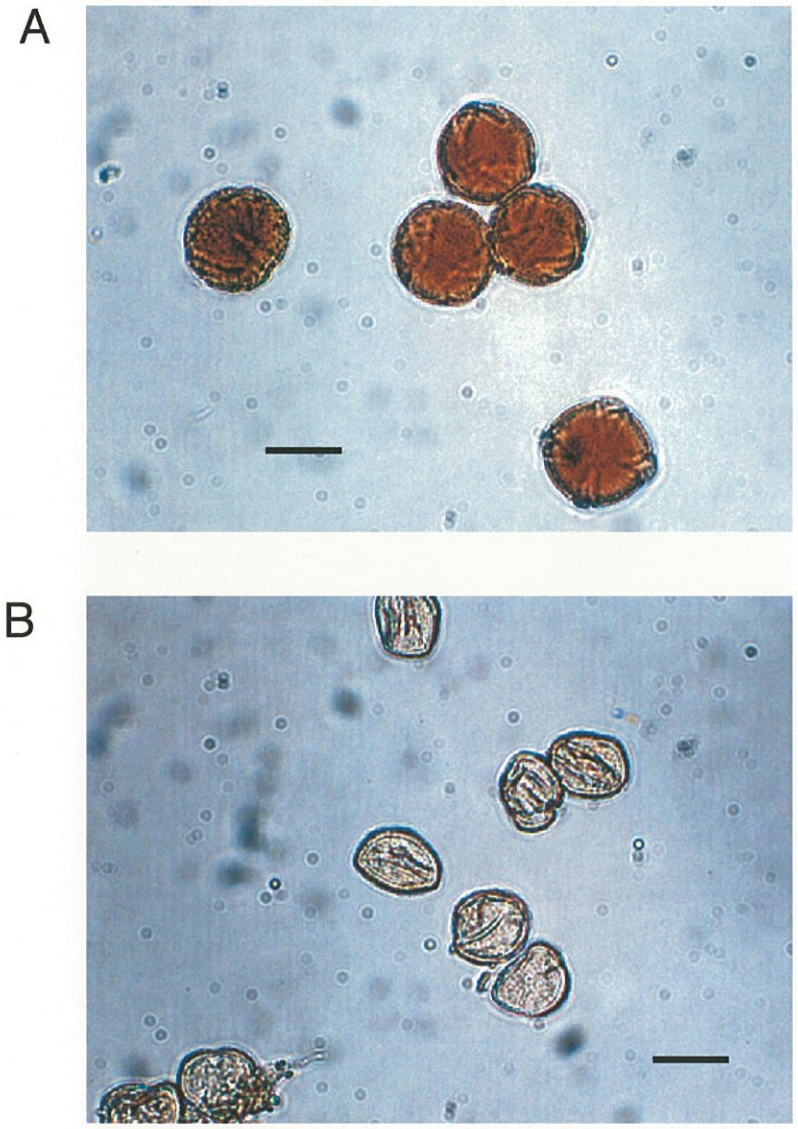

Fig. 9. Pollen grains of a wild-type plant (**A**) and transformant line H69A #4 (**B**) The pollen grains were stained with acetocarmine. Scale bars = 25 μm.

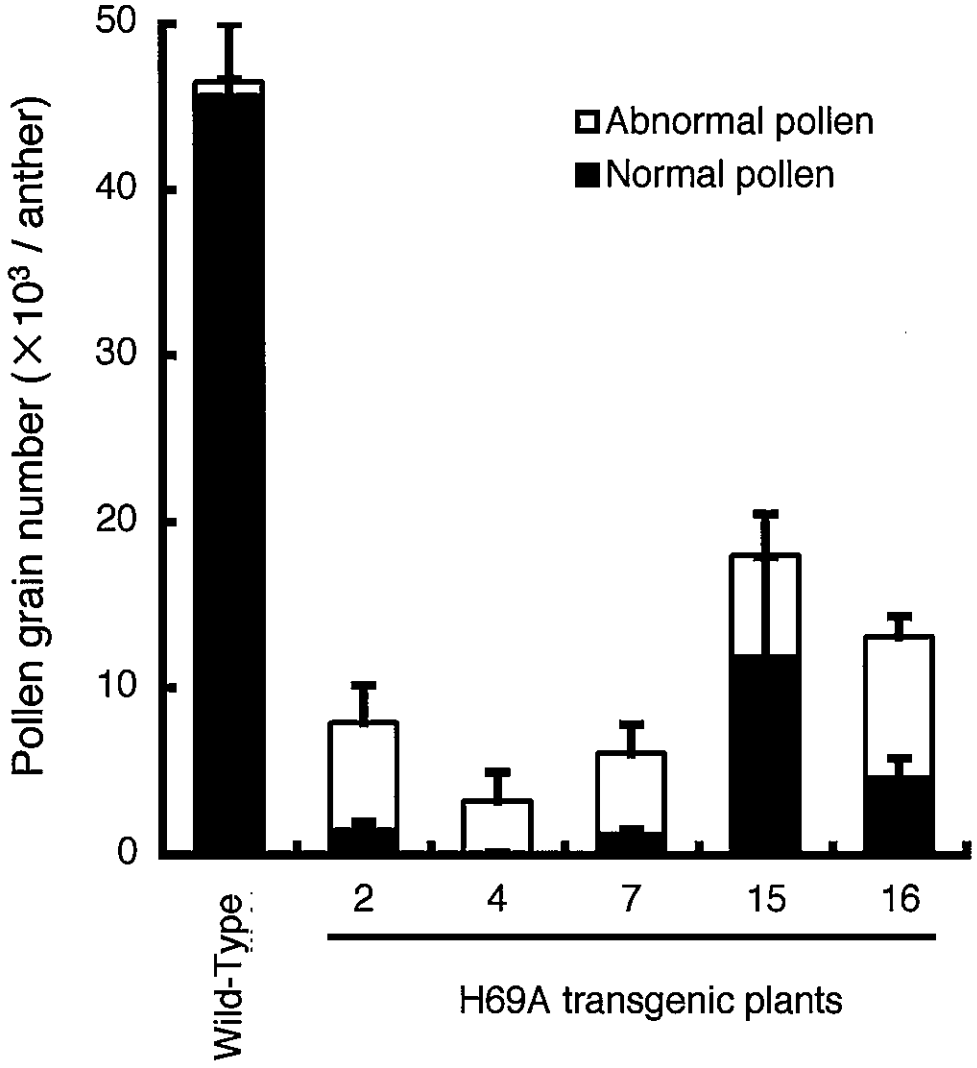

Fig. 10. Pollen production in the transgenic lines. Following anther dehiscence, one anther each from five independent flowers of each line was analyzed. Error bars indicate standard error.

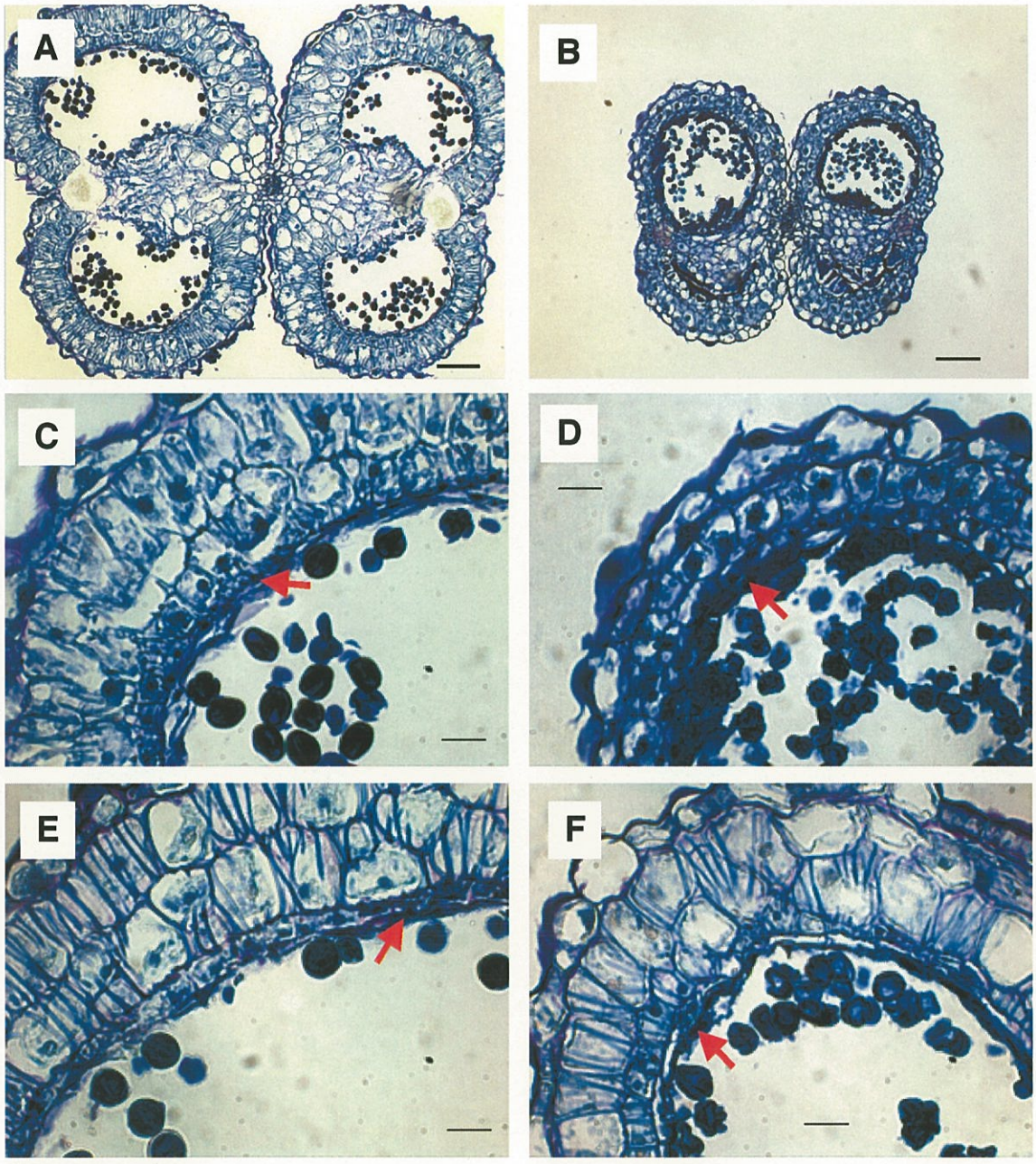

Fig. 11. Tapetum degeneration in tobacco anthers of wild-type (**A**, **C**, **E**) and transgenic line H69A#4 (**B**, **D**, **F**). Anthers were collected at stages 2 (**A-D**) and 6 (**E**, **F**). Lower magnification of anther Scale bars are 100 μm (**A**, **B**), 25 μm (**C-F**). Arrows indicate degenerated tapetum layer.

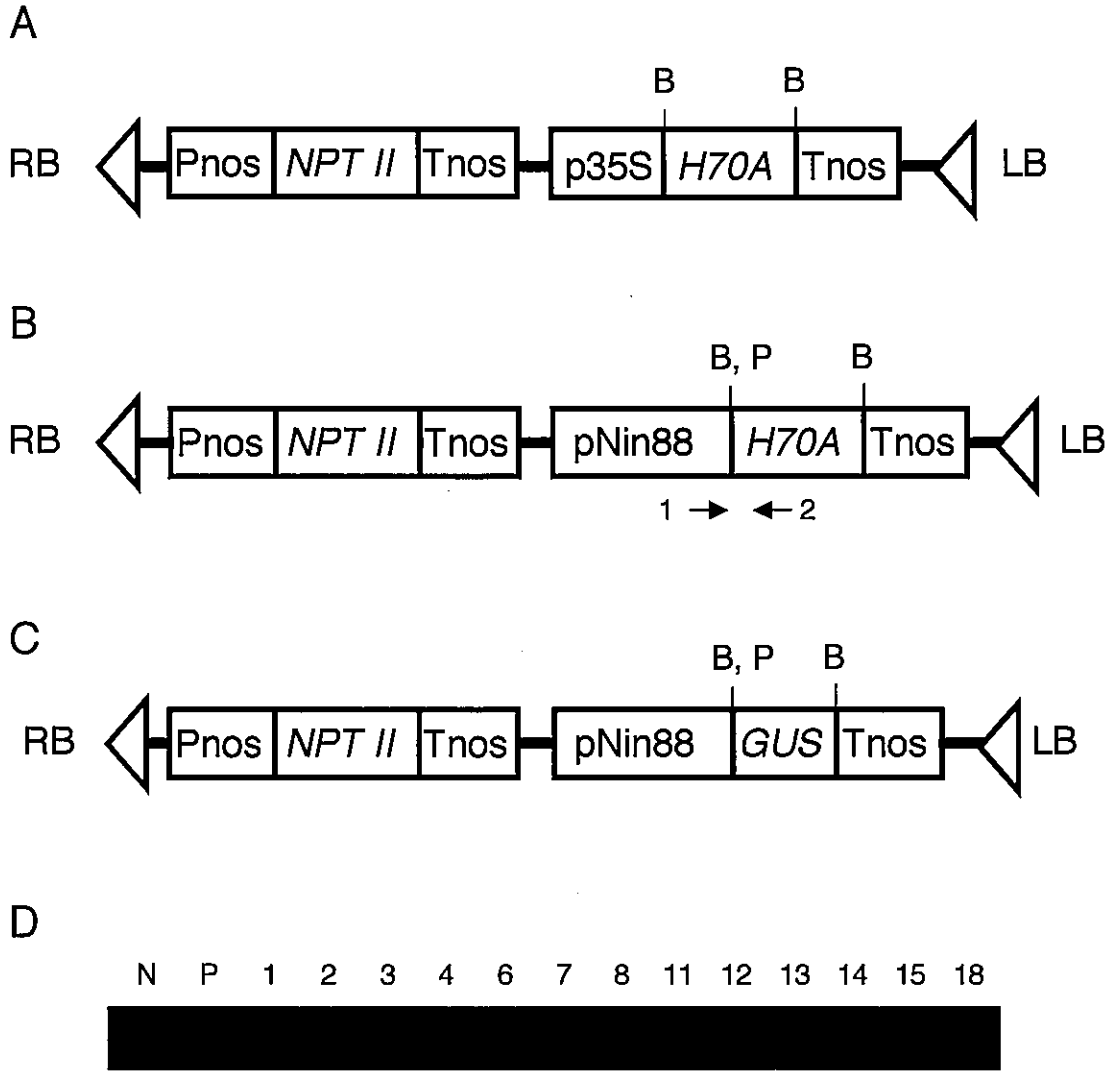

Fig. 12. Constructions of the transformation vector. **A** *p35S::Cm-ERS1/H70A*. **B** *pNin88::Cm-ERS1/H70A*. **C** *pNin88::GUS*. RB and LB, right and left borders of T-DNA; B, *Bam*H I site; P, *Pst* I site; Pnos, nopaline synthase gene promoter; *NPTII*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; P35S, CaMV 35S promoter; *H70A*, *Cm-ERS1/H70A* gene. Arrows 1 and 2 indicate the primers for PCR and RT–PCR. **D** Example of genomic PCR to confirm the presence of the *pNin88::Cm-ERS1/H70A* transgene. N, negative control; P, positive control.

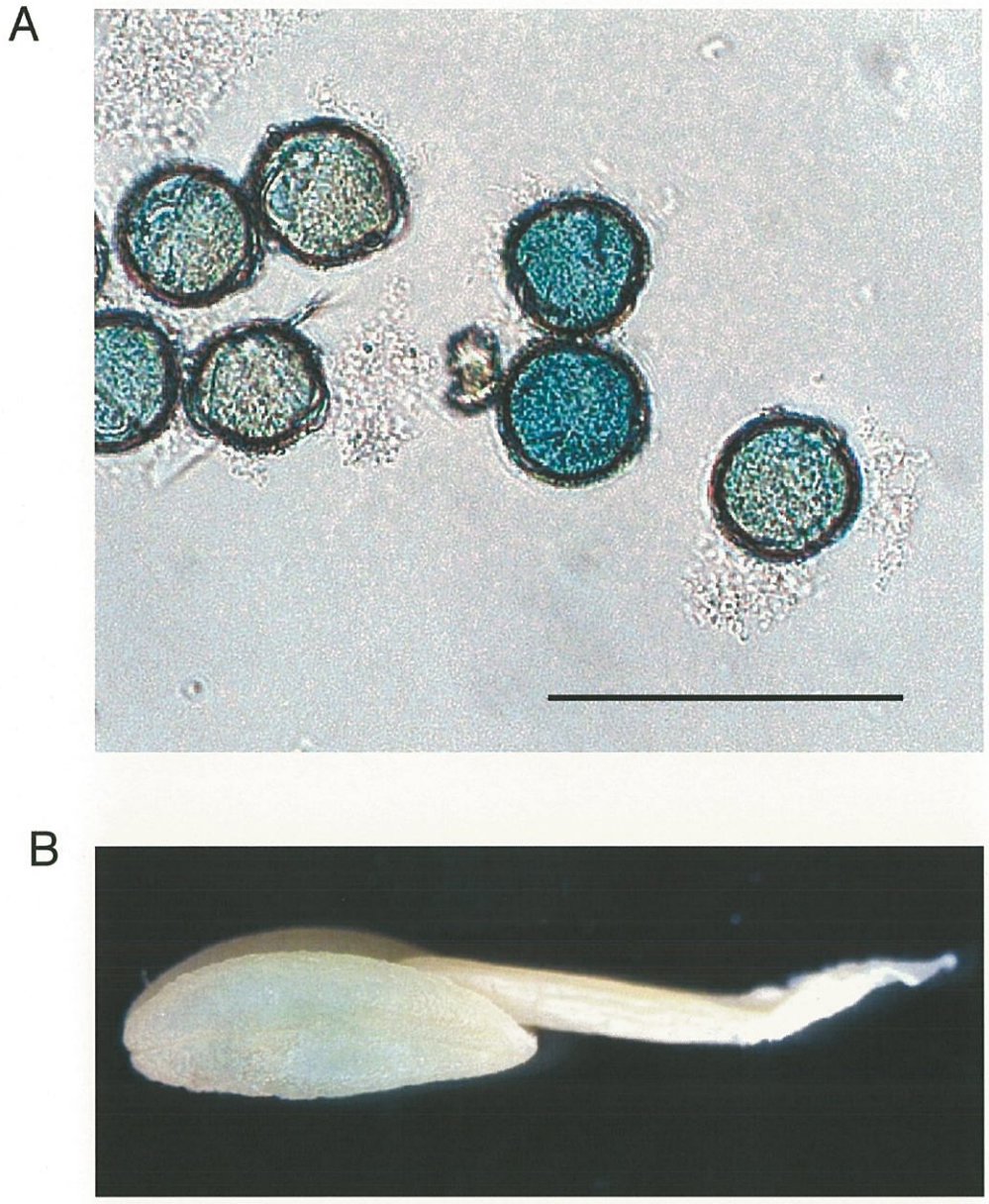

Fig. 13. **A** GUS staining of the stamen from the flower bud at stage 1 in NGUS tobacco plant. **B** GUS staining of pollen grains from the flower bud at stage 12 in NGUS tobacco plant. Bar = 100 μm.

Fig. 14. Phenotype of NH70A transformant. Left, NH70A #9; Right, wild type.

Fig. 15. RT–PCR analysis of transgenic tobacco lines; mRNA was isolated from the anther of the flower at stage 2. Expression of *pNin88::H70A* (**A**), *p35S::H70A* (**B**), and actin (**C**). WT, wild type; #8, NH70A #8; #9, NH70A #9; #2, H70A #2; #3, H70A #3

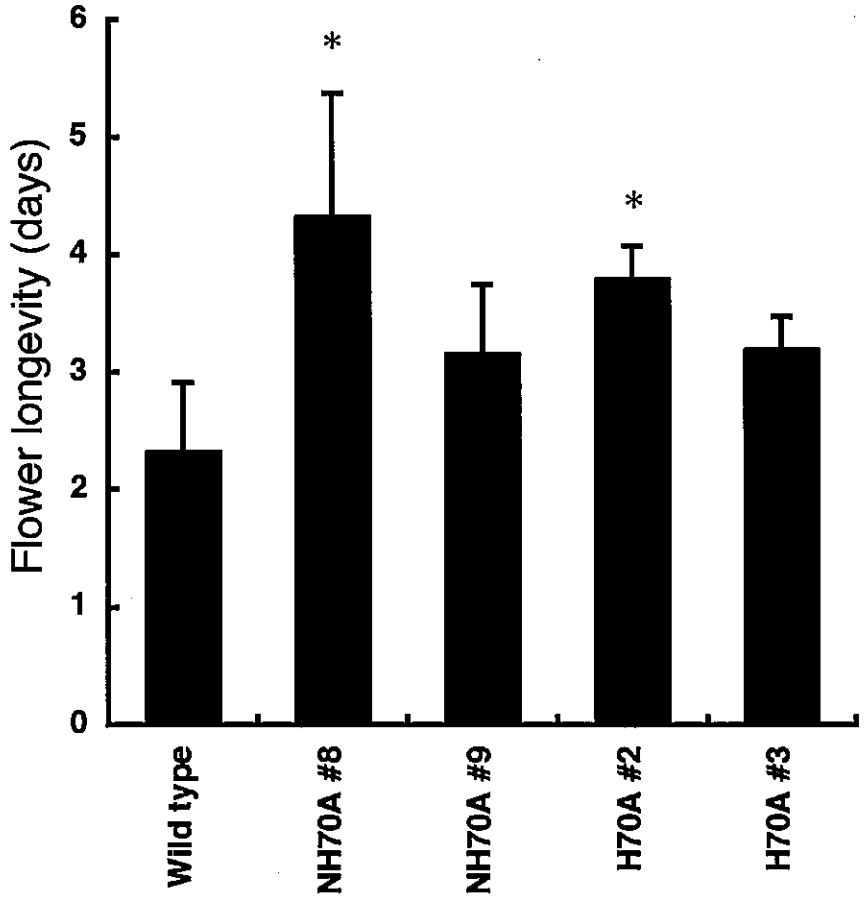

Fig. 16. Flower longevity of transgenic tobacco plants with expression of *pNin88::H70A* or *p35S::H70A*. The flower longevity is expressed as the mean number of days from opening until the onset of corolla wilting. The flower longevities of five flowers in each line were determined. Error bars indicate standard error. Asterisk shows significant difference (Dunnet test for comparing wild-type to each of the transgenic lines. $P<0.05$).

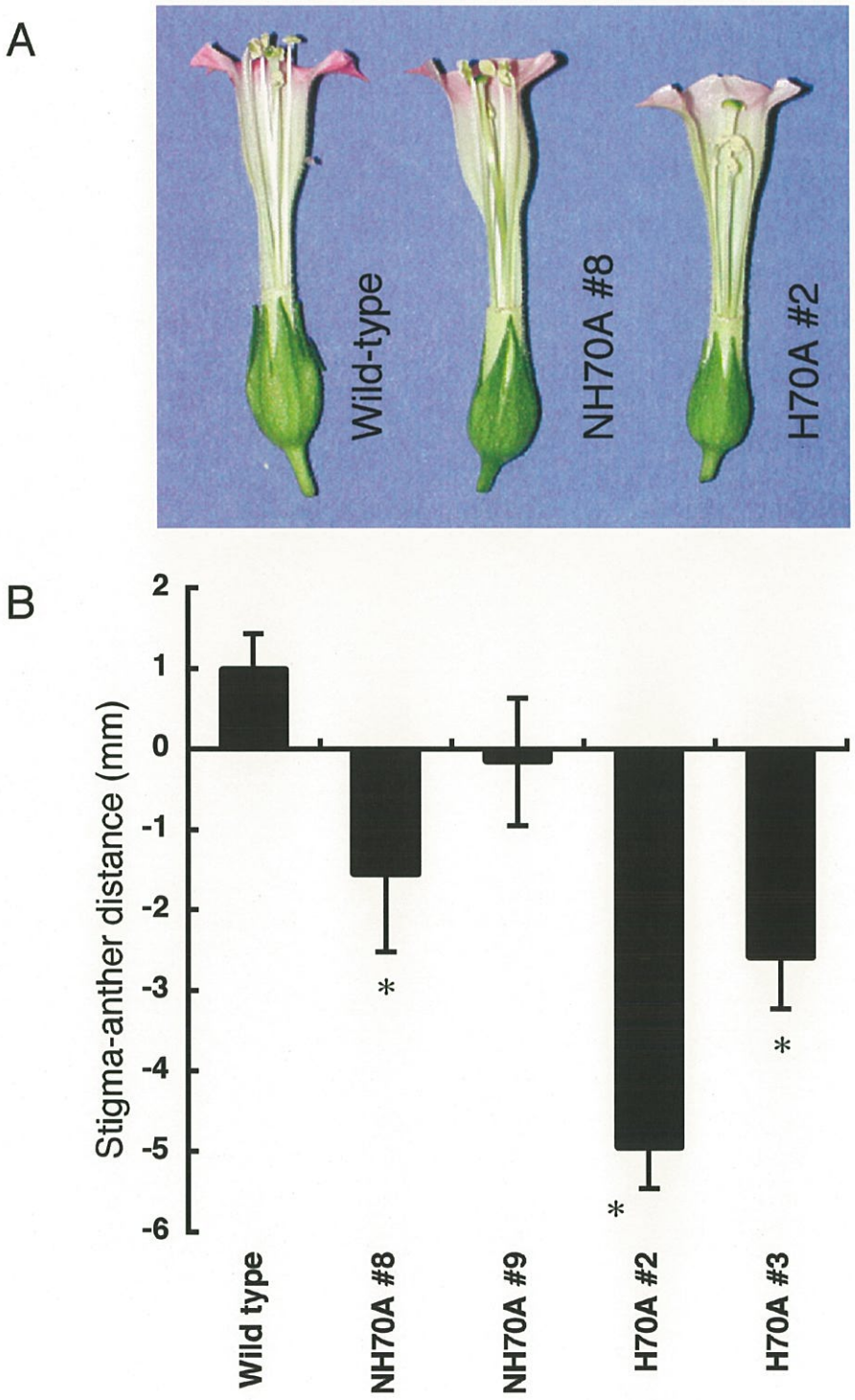

Fig. 17. The floral architectures in NH70A and H70A transformants. **A** Photograph of tobacco flowers. **B** Stigma–anther distance in transgenic tobacco plants. The stigma–anther distance was calculated by determining the difference between the stigma height and the height of all anthers. Asterisk shows significant difference (Dunnet test for comparing wild-type to each of the transgenic lines. $P<0.05$).

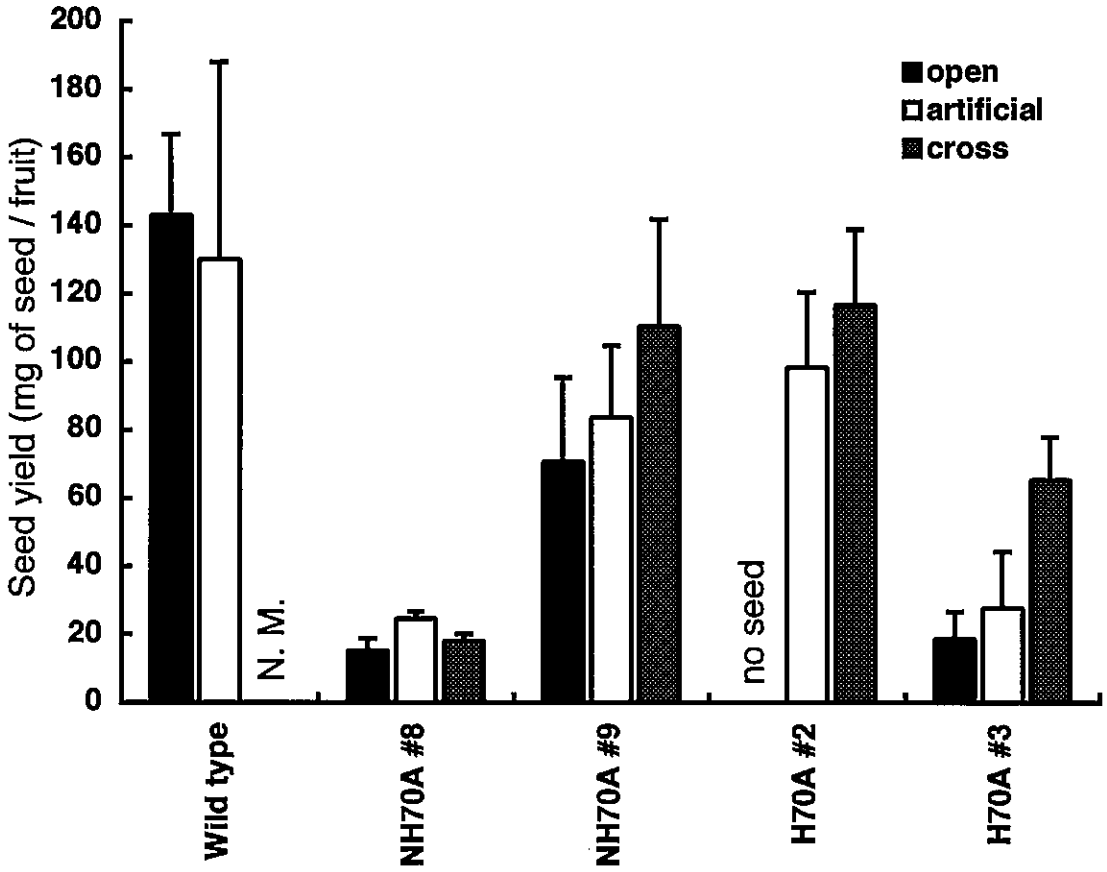

Fig. 18. Seed yields of transgenic tobacco lines. Five fruits from each line were examined during each treatment. Error bars indicate standard error. N.M., not measured.

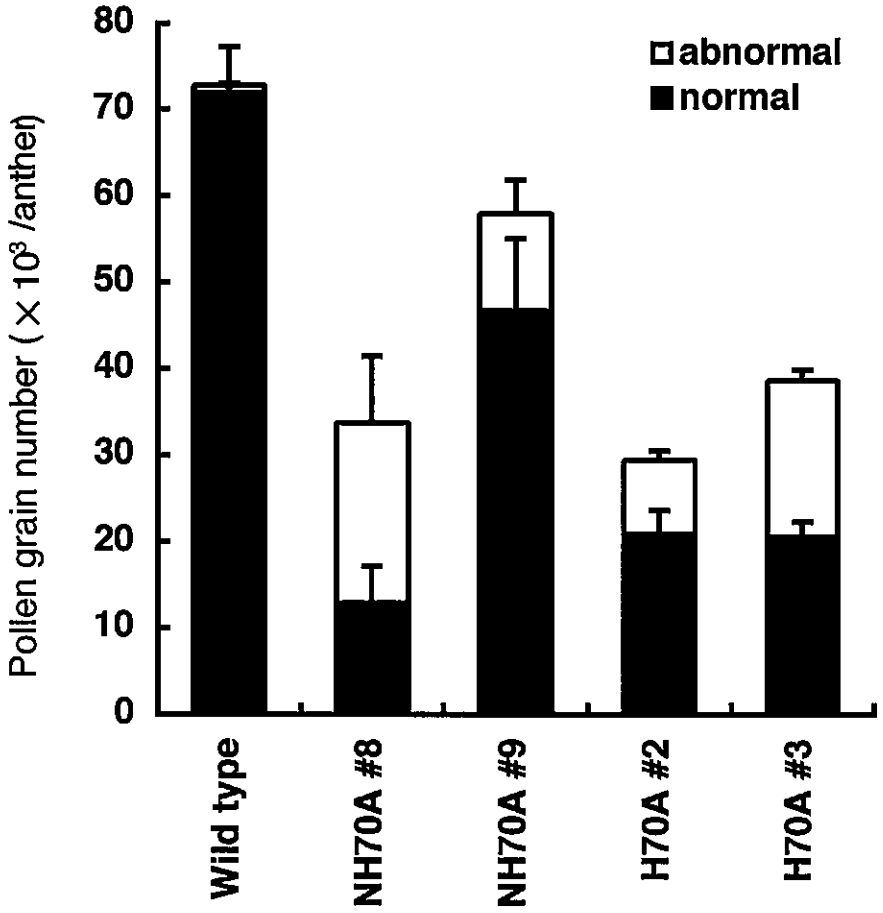

Fig. 19. Pollen production in the transgenic tobacco lines. Following anther dehiscence, one anther from five independent flowers of each line was analyzed. Error bars indicate standard error.

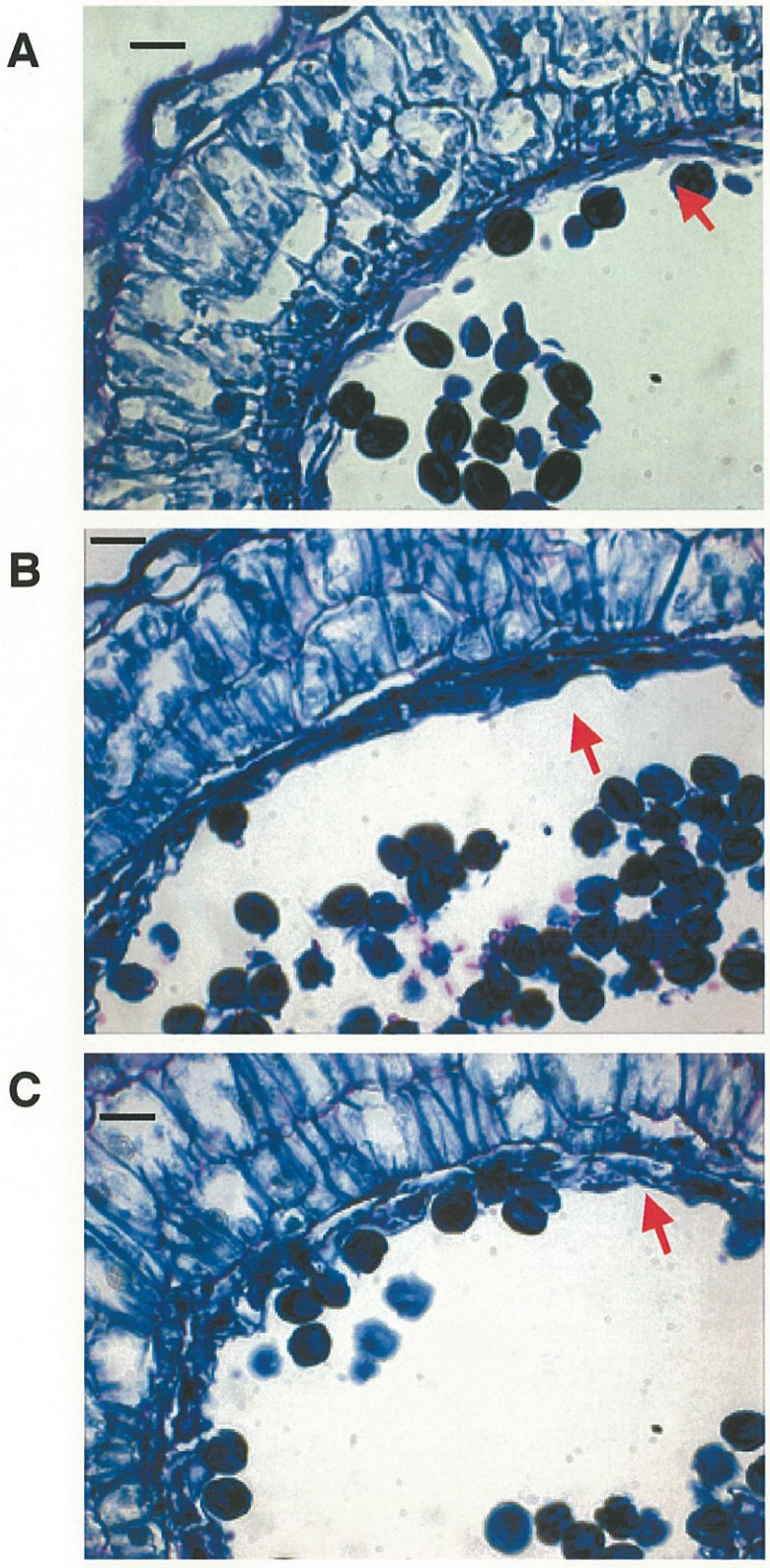

Fig. 20. Tapetum degeneration in the anthers at stage 2 in transgenic tobacco plants. **A** wild-type. **B** NH70A #8. **C** H70A #2. Scale bars = 25 μm. Arrows indicate the tapetum layer.

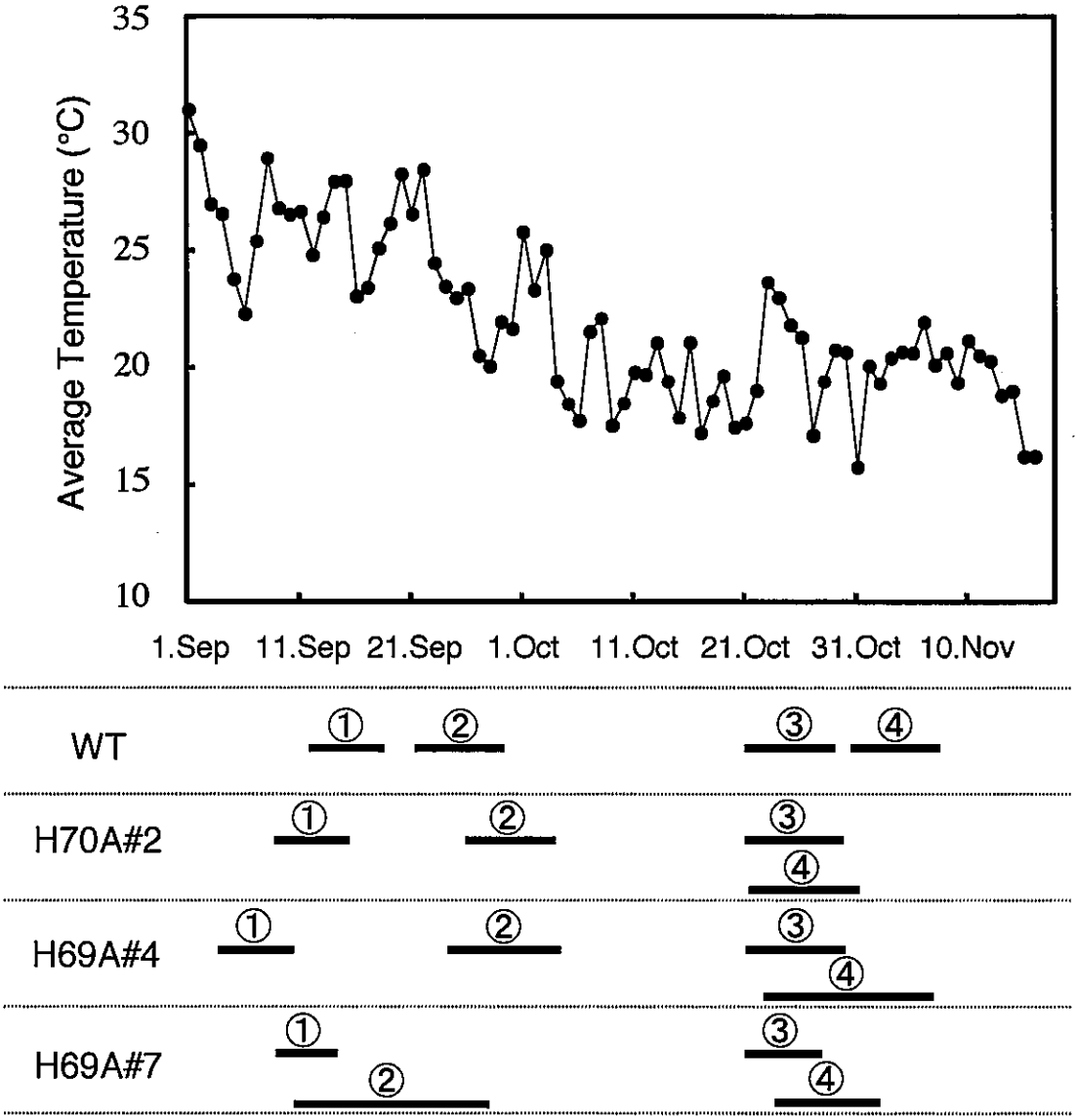

Fig. 21. Changes in temperature in a greenhouse during the flowering of transgenic tobacco plants. Each point indicates the average temperature on an individual day. Four plants of each transgenic line were grown, and the number over each line indicates each individual plant. The lines under the numbers indicate the flowering period of each plant.

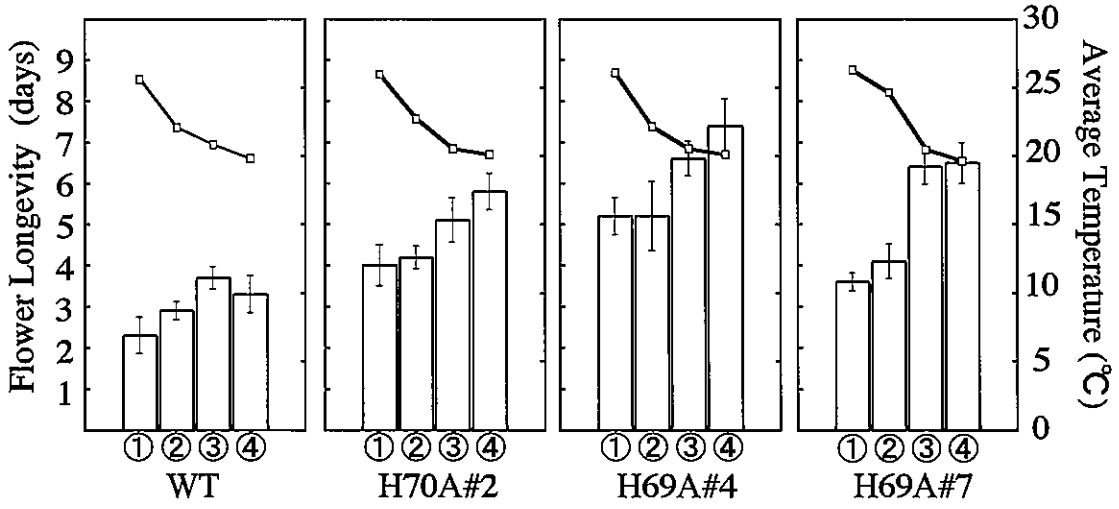

Fig. 22. Longevity of flowers of transgenic tobacco plants in relation to the temperature during flowering. The flower longevity is expressed as the mean number of days from opening until the onset of corolla wilting. The flower longevities of five flowers per plant were determined. Error bars indicate standard error.

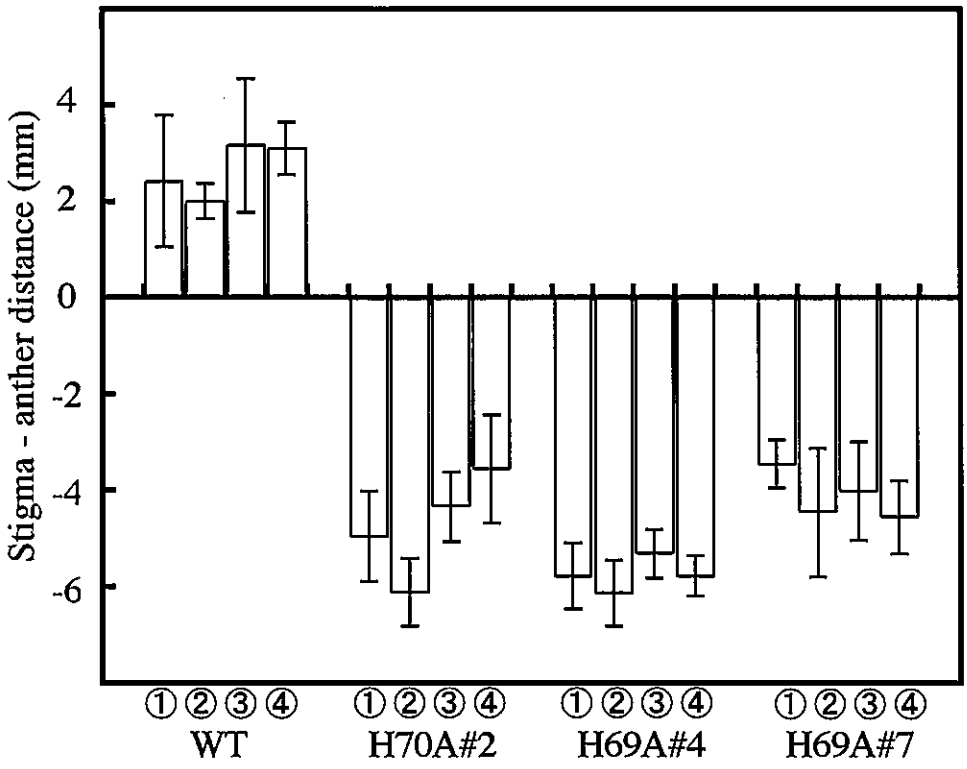

Fig. 23. Stigma-anther distance in transgenic tobacco plants expressing the *Cm-ERS1/H70A* or *Cm-ETR1/H69A* genes. The stigma-anther distance was calculated by determining the difference between the stigma height and the height of all of the anthers.

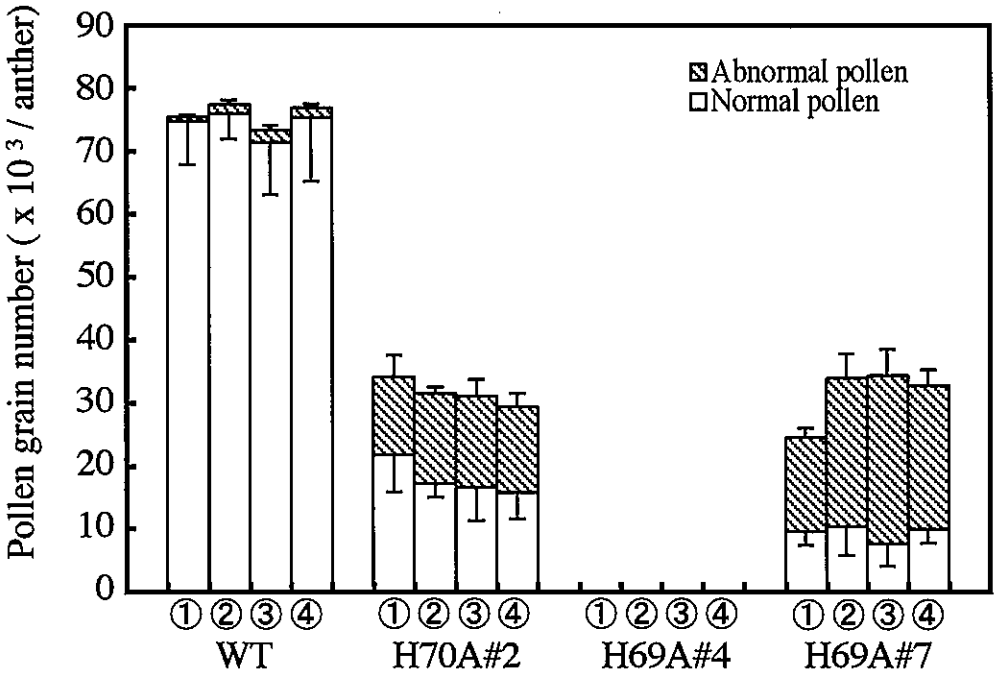

Fig. 24. Pollen production in the transgenic tobacco plants. Following anther dehiscence, one anther each from five independent flowers of each plant was analyzed. Error bars indicate standard error. Open and closed bars indicate normal and abnormal pollen, respectively.

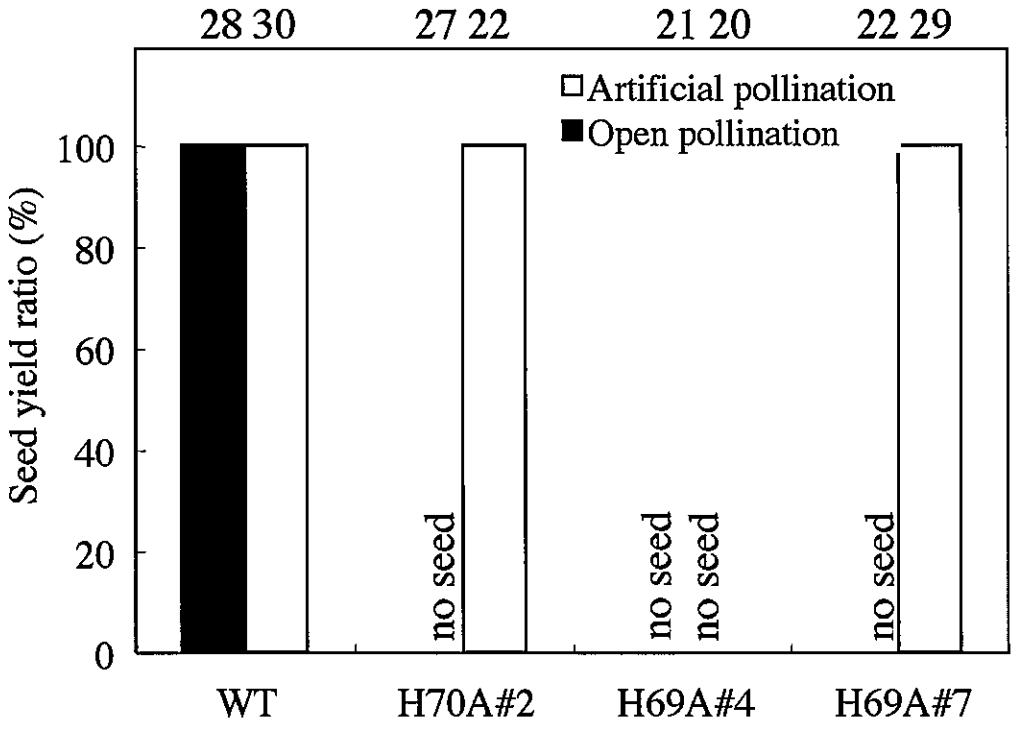

Fig. 25. Frequencies of fruit setting following open pollination or artificial pollination. Open and closed bars indicate artificial pollination and open pollination, respectively. The number over each bar indicates the number of flowers observed.

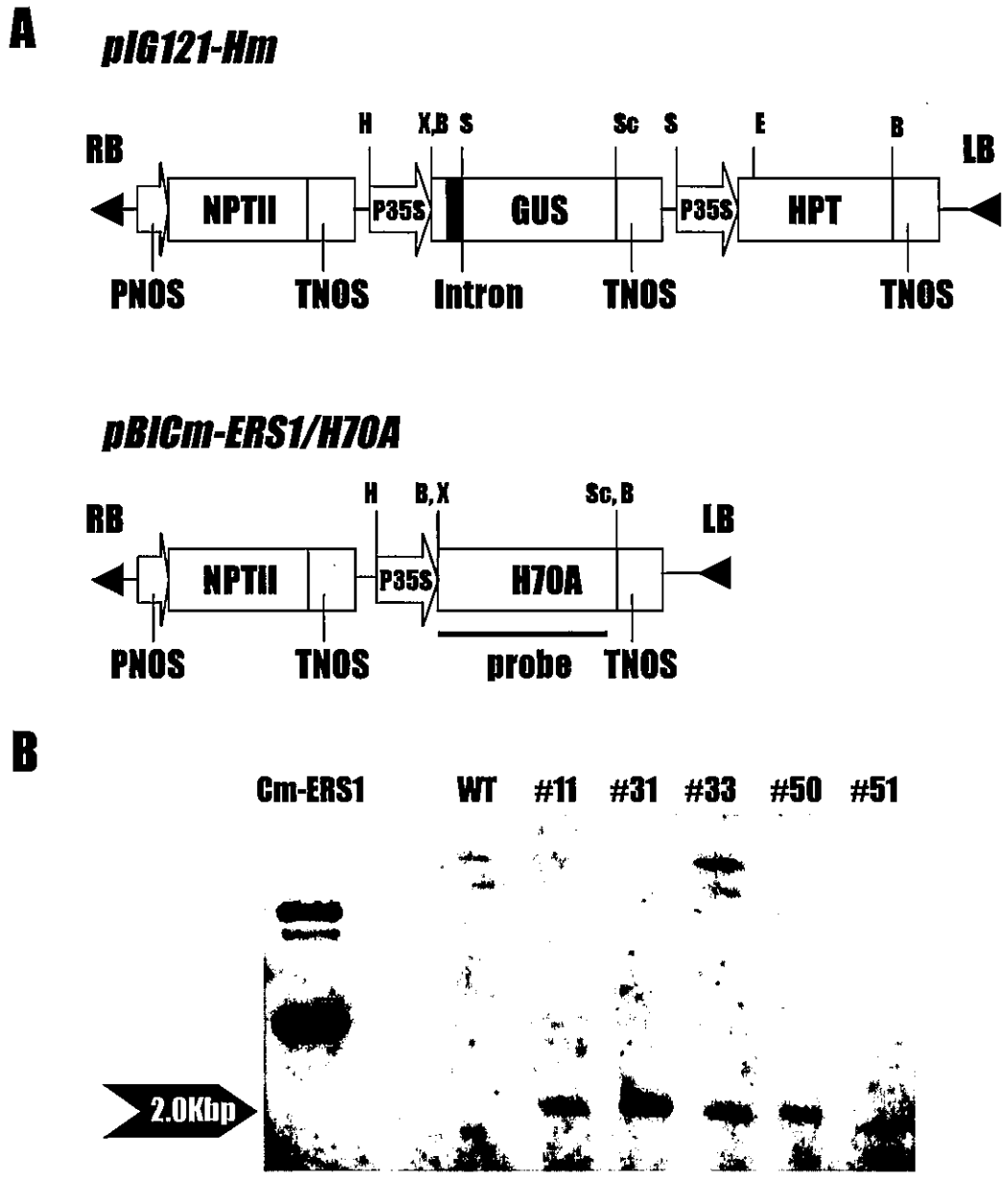

Fig. 26. **A** Map of the binary vectors, pIG121-Hm and pBICm-ERS1/H70A. The underlined portion of the *Cm-ERS1/H70A* gene, a 1-kb fragment amplified by PCR, was used as a probe. RB and LB, right and left borders of T-DNA; Pnos, nopaline synthase gene promoter; *NPT-II*, neomycin phosphotransferase gene; Tnos, nopaline synthase gene terminator; 35S, CaMV 35S promoter; *H70A*, *Cm-ERS1/H70A*; *GUS*, beta-gluculonidase gene; *HPT*, hygromycin phosphtransferase gene. **B** DNA gel blot analysis. Genomic DNAs from the five independent transgenic lines and wild-type plant were subjected to DNA gel blot analysis. Ten micrograms of genomic DNA were digested with *Hin*dIII, separated by electrophoresis, and hybridized with a 1.0-kb ^{32}P-labeled coding region amplified by PCR.

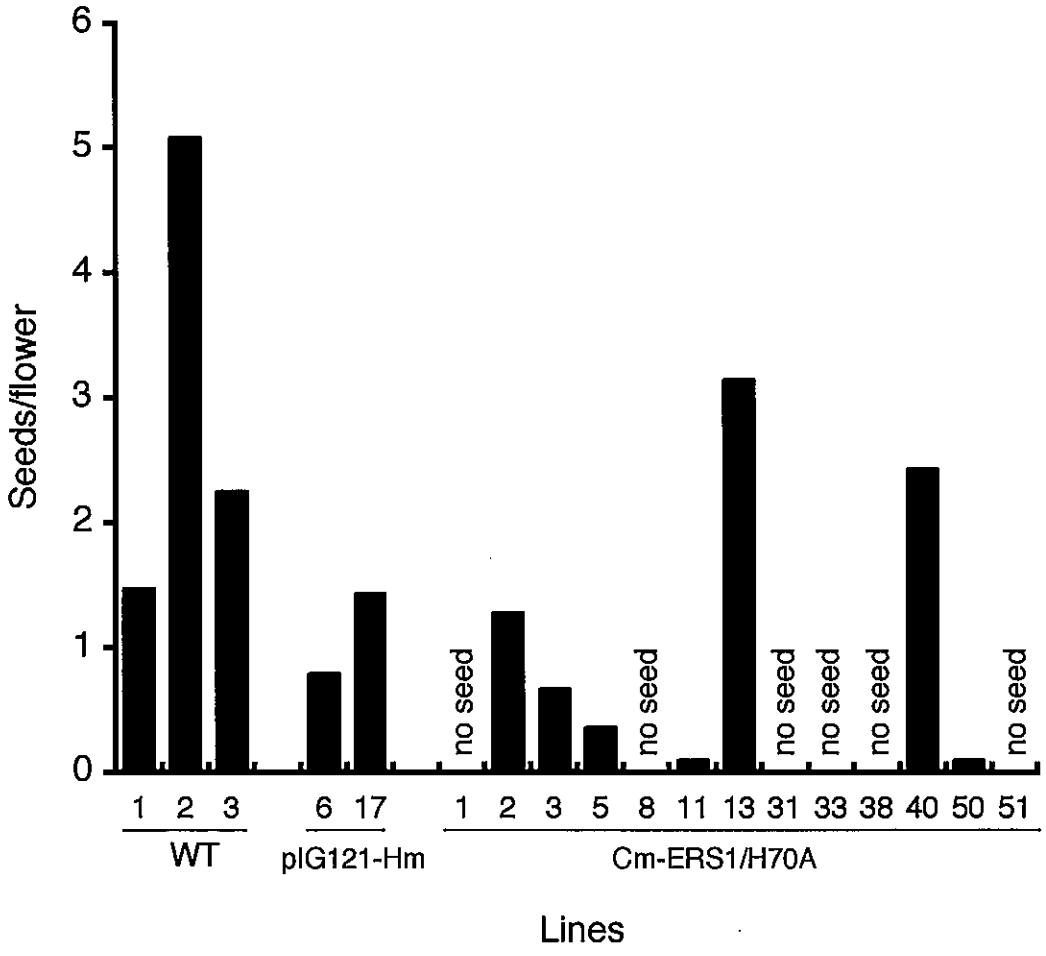

Fig. 27. Primary examination of the fertility of the transgenic plants. The wild type and the transgenic lines containing the pIG121-Hm and *Cm-ERS1/H70A* genes were grown in a growth chamber under constant light and temperature conditions until flowering. Seeds were harvested after the seeds reached maturity.

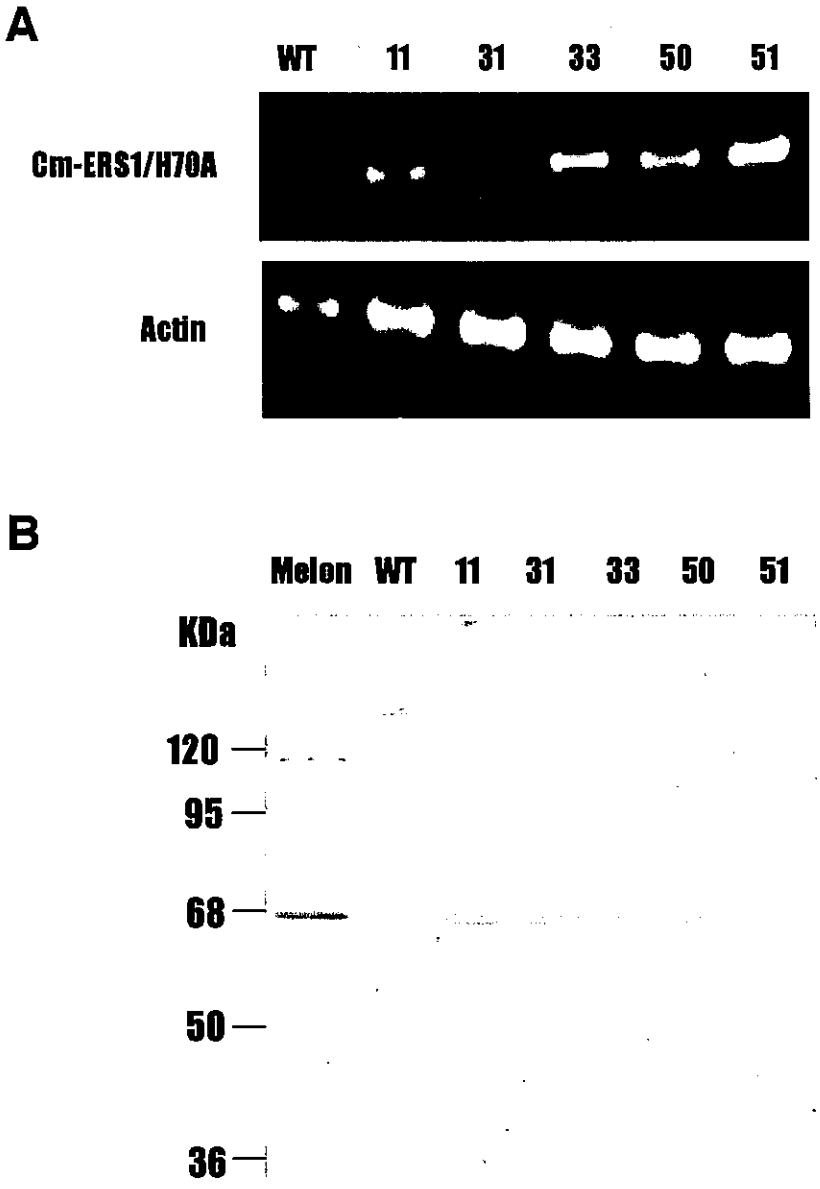

Fig. 28. Molecular characterization of transgenic plants containing *Cm-ERS1/H70A*. **A** RT-PCR analysis. RNA was extracted from young leaves of the wild type and transgenic lines, and 1 μg of total RNA was used for reverse transcription. The amplification of 600- and 500-bp cDNA products was expected for *H69A* and the actin, respectively. **B** Protein gel blot analysis. Microsomal membranes were prepared from lettuce leaves of either the wild type and transgenic lines. The proteins (15 μg) were separated by SDS-PAGE on an 8% gel. The primary and secondary antibodies used were rabbit anti-melon CmERS1 (1:1,000) and anti-rabbit IgG-HRP (1:10,000), respectively. Melon, melon fruit microsomes (cv. Vedrantais).

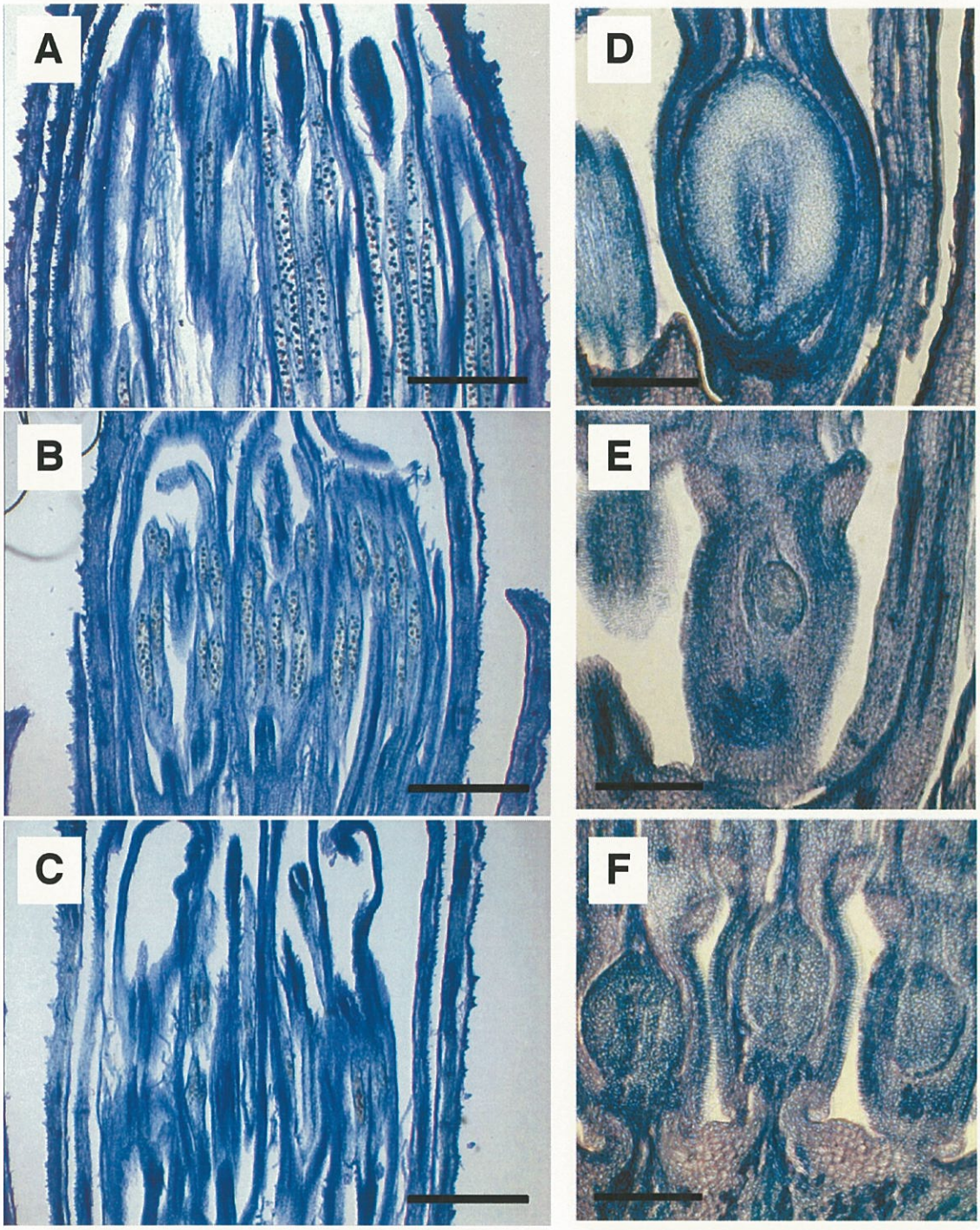

Fig. 29. Histological observations of pollen and ovule development in flower-bud before pollination in the wild type and sterile transgenic lettuce lines grown in an open-air greenhouse. **A** and **D**, Wild type; **B** and **E**, line 50; **C** and **F**, line 33. Bar = 0.2 mm.

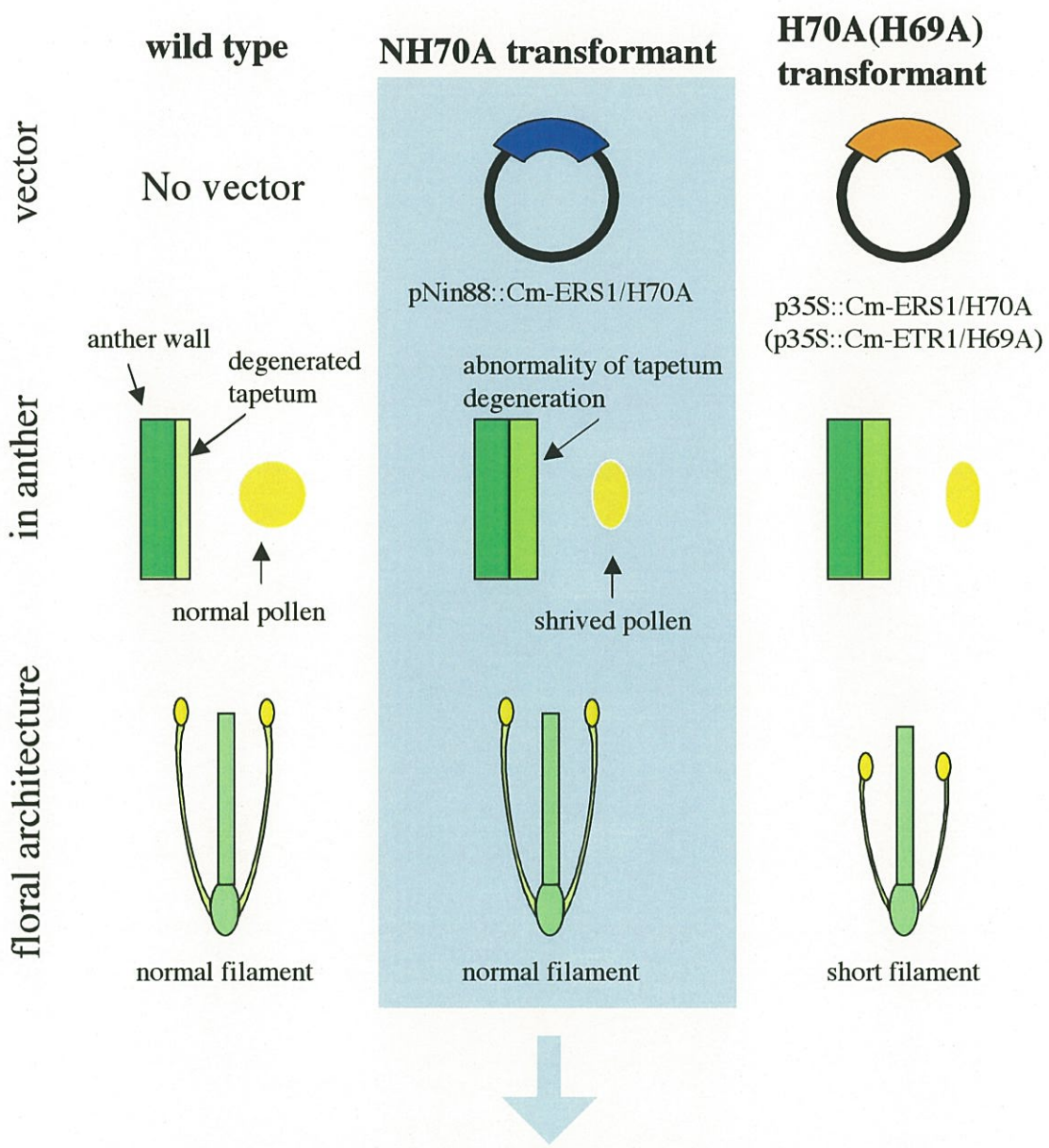

Fig. 30. A schematic representation of the inducible male sterility system using mutated melon ethylene receptor genes. Pollen production and flower architecture are affected when the genes are driven by a constitutive promoter. While only pollen production is affected when the genes are driven by a tapetum-specific promoter.

Table 1. Seed production in the wild type and five transgenic lettuce lines expressing the mutated melon ethylene receptor *Cm-ERS1/H70A* grown in an open-air greenhouse under various environmental conditions.

Line	No. of clones evaluated	Seeds/flower
WT	7	5.26
11	6	0.01*
31	5	0.00*
33	5	0.00*
50	8	0.88
51	9	0.00*

Asterisk shows significant difference. The significant differences for comparing wild-type to each of the transgenic lines were determined using Dunnet test at the 0.05 confidence level. The seeds of twenty to eighty flowers per plant were harvested.