Fundamental studies on the molecular breeding for flower color modification in Liliaceous ornamentals

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Chapter 1
General introduction

1. Background of the present study

In ornamental plants, flower color is one of the most important traits, and cultivars with various flower colors have so far been produced by traditional breeding methods such as intra- and interspecific hybridization and selection of spontaneous or artificially induced mutants. However, some flower colors are still lacking for many ornamental plants, since the gene pool is limited for any single species. For example, *Rosa* spp., *Dianthus caryophyllus* and *Dendranthema grandiflorum*, all of which are commercially very important ornamental plants, do not have blue-flowering strains and cultivars. Similarly, there are no yellow-flowering strains and cultivars in *Pelargonium* spp. and *Eustoma grandiflorum*, and flowers of *Gypsophila* spp. are mainly white (Ben-Meir et al., 2002). Recently, genetic transformation has commenced to be applied to plant breeding in order to overcome some limitations of the traditional breeding methods. Genetic transformation opens a new avenue to the introduction of agriculturally valuable traits such as novel flower colors into target plants from distantly-related species (Courtney-Gutterson, 1993; Robinson and Firoozabady, 1993). In addition, genetic transformation allows the introduction of only desired traits to pre-existing genotypes within a short period as compared to the traditional breeding methods (Robinson and Firoozabady 1993; Burchi et al. 1996). To date, transgenic plants with gene(s) for agriculturally valuable traits, such as novel flower colors and plant forms, increased flower longevity, controlled fruit maturation, male sterility, cold hardiness, herbicide tolerance, and resistance to diseases, insects or viruses, have been produced in a number of plant species, some of which have already been cultivated commercially (Dunwell, 2000; Deroles et al., 2002).

Flower coloration is due to the accumulation of plant pigments, i.e. flavonoids including anthocyanins, carotenoids, chlorophylls and betalains (Mol

et al., 1998; Davies, 2004). The first three classes of pigments are widespread, whereas betalains are found exclusively in one group of angiosperms, the Caryophyllales, but never in combination with anthocyanins (Stafford, 1994; Mol et al., 1998). Anthocyanins, a group of flavonoids, are major flower pigments in higher plants and have been studied extensively (Elomaa and Holton, 1994; Koes et al., 1994; Holton and Cornish, 1995; Mol et al., 1996). Anthocyanins absorb light at the longest wavelengths, and are the basis for orange, pink, red, magenta, purple, blue and blue-black flower colors (Schwinn and Davies, 2004). In addition to the accumulation of anthocyanins, various additional factors determine flower color, including co-pigmentation, vacuolar pH and cell shape (Mol et al., 1998).

2. Molecular breeding for flower color modification

To date, virtually all the genes encoding the enzymes of the flavonoid biosynthetic pathway (Figure 1) have been isolated based on extensive biochemical and genetic studies (Elomaa and Holton, 1994; Holton and Cornish, 1995; Mol et al., 1998). Extensive information provides a strong foundation for producing transgenic plants with novel flower colors, and numerous studies on flower color modification by engineering the flavonoid biosynthetic pathway have so far been reported (Ben-Meir et al., 2002; Schwinn and Davies, 2004). Two main strategies have successfully been employed for modifying flower color via genetic transformation. The first strategy is based on the up- or down-regulation of expression of the endogenous gene(s) involved in the flavonoid biosynthetic pathway (Ben-Meir et al., 2002; Schwinn and Davies, 2004). Flower color modification by this strategy was first reported for transgenic *Petunia hybrida*, in which expression of the endogenous CHS gene was reduced via the anti-sense technology leading to the production of white

flowers (van der Krol et al., 1988). Afterwards, regulation of the endogenous gene expression was also achieved via the co-suppression (van der Krol et al., 1990; Courtney-Gutterson et al., 1994; Gutterson, 1995) and RNAi (Fukusaki et al., 2004) technologies. The second strategy is based on the introduction of the exogenous gene(s), which encodes the enzyme lacking in the target plant, and thus allowing new branching of the flavonoid biosynthetic pathway (Ben-Meir et al., 2002). Usefulness of this strategy was first demonstrated for transgenic *Petunia hybrida*, which newly produced brick-red flowers via heterologous expression of the DFR gene derived from *Zea mays* (Meyer et al., 1987). In addition, Florigene Ltd., Australia, and Suntory Ltd., Japan, have recently succeeded in the production of transgenic violet-flowering cultivars of *Dianthus caryophyllus* by introducing the F3'5'H and DFR genes from heterologous plant species into a DFR-deficient, white-flowering strain (see review; Tanaka et al., 1998).

3. Application of molecular breeding for flower color modification to Liliaceous ornamentals

Almost all the Liliaceous ornamentals have limited flower colors as in the cases of most other ornamentals. For example, there are no blue-flowering strains and cultivars in most species including *Fritillaria* spp., *Gloriosa* spp., *Hemerocallis* spp., *Lilium* spp., *Ornithogalum* spp., *Sandersonia aurantiaca*, *Tricyrtis hirta* and *Tulipa* spp. Similarly, *Agapanthus* spp., *Hosta* spp. and *Muscari armeniacum* do not have yellow- and red-flowering strains and cultivars, and flowers of *Convallaria* spp. is mainly white. Since the flavonoid biosynthetic pathway in a wide range of species share a majority of common reactions (Holton and Cornish, 1995), strains and cultivars with novel flower colors could be produced in Liliaceous ornamentals by genetic engineering this

pathway as in the cases of *Petunia hybrida* and *Dianthus caryophyllus*.

To date, molecular breeding for flower color modification has been studied in dicot ornamentals, whereas there have been no reports on flower color modification via genetic transformation in monocot ornamentals including Liliaceous ones. One of the reasons for this may be poor accumulation of molecular biological knowledge of the flavonoid biosynthetic pathway in monocots. For example, the F3'5'H gene, one of the main structural genes in the flavonoid biosynthetic pathway, has not yet been isolated from monocots. Biochemical information on flower pigments including flavonoids is also very poor in monocot ornamentals compared with dicot ornamentals. Another reason for no application of molecular breeding for flower color modification in monocot ornamentals may be lack of reliable genetic transformation systems. As compared with dicot ornamentals, there have been only a few reports on the production of transgenic plants in monocot ornamentals (Deroles et al., 2002). Thus, it is indispensable to establish efficient genetic transformation systems as well as to accumulate biochemical and molecular biological knowledge of the flavonoid biosynthetic pathway for applying molecular breeding for flower color modification to Liliaceous ornamentals.

4. Aim of the present study and outline of the thesis

The present study aims at practical application of molecular breeding for flower color modification to Liliaceous ornamentals. In order to achieve the aim, it is prerequisite to establish efficient genetic transformation systems and to collect biochemical and molecular biological information on the flavonoid biosynthesis in target plants as described above. However, genetic transformation systems have so far been developed for limited Liliaceous ornamentals such as *Agapanthus praecox* ssp. *orientalis* (Suzuki et al., 2001),

Muscari armeniacum (Suzuki and Nakano, 2002a) and *Lilium* sp. (Hoshi et al., 2004) via *Agrobacterium*-mediated methods. Therefore, in Chapter 2, some experiments were carried out for establishing efficient genetic transformation systems in a wide range of Liliaceous ornamentals. Firstly, development of fast growing and highly regenerable callus cultures was examined, since such cultures may be most suitable as a target material for transformation in Liliaceous ornamentals (Suzuki and Nakano, 2002b). To date, regenerable callus cultures have been established in rather limited species of Liliaceous ornamentals, such as *Agapanthus* spp. (Supaibulwatana and Mii, 1997; Suzuki et al., 2002), *Lilium* spp. (Simmonds and Cumming, 1976; Stimart et al., 1980; Priyadarshi and Sen, 1992; Mii et al., 1994; Wickremesinhe et al., 1994; Godo et al., 1996, 1998; Arzate-Fernandez et al., 1997; Tribulato et al., 1997; Suzuki et al., 1998; Nakano et al., 2000), *Muscari armeniacum* (Suzuki and Nakano, 2001) and *Tricyrtis* spp. (Nakano et al., 2004). In Chapter 2, Experiment 1 and Experiment 2, callus induction from cultured explants and plant regeneration from induced calluses were newly examined in various species and cultivars of *Muscari* and *Lilium*, respectively. Secondly, in Chapter 2, Experiment 3, development of an efficient genetic transformation system in *Tricyrtis hirta* by using previously established regenerable callus cultures (Nakano et al., 2004) as a target material for *Agrobacterium*-mediated transformation. For perennial and vegetatively propagated crops including almost all the Liliaceous ornamentals, long-term and stable expression of transgene(s) is also indispensable for their successful improvement by genetic transformation. Thirdly, therefore, stability of transgene expression in transgenic plants of *Agapanthus praecox* ssp. *orientalis* and *Tricyrtis hirta* was examined after long-term cultivation in Chapter 2, Experiment 4 and Experiment 5, respectively.

In Chapter 3, some experiments were carried out to obtain the basic information on the flavonoid biosynthetic pathway. Firstly, anthocyanidins

and/or anthocyanins in floral organs of *Muscari* spp., *Agapanthus* spp. and *Tricyrtis* spp. were analyzed in Chapter 3, Experiment 1. Data on the anthocyanidin and/or anthocyanin composition may be valuable for molecular as well as traditional breeding for flower color modification. Secondly, isolation and characterization of genes involved in the flavonoid biosynthetic pathway were examined. Among enzymes in this pathway, F3'5'H and DFR are important key enzymes for the determination of flower color, and in particular, F3'5'H is essential for blue flower color (Figure 1). In addition, genes for F3'5'H and DFR have frequently been utilized as a transgene for flower color modification by genetic transformation in dicot ornamentals (Forkmann and Martens, 2001; Ben-Meir et al., 2002; Schwinn and Davies, 2004). Therefore, the F3'5'H and DFR genes were selected as a target for isolation in the present study. Isolation and characterization of the F3'5'H gene from the dicot ornamental *Vinca major* and the DFR gene from the Liliaceous ornamental *Agapanthus praecox* ssp. *orientalis* were described in Chapter 3, Experiment 2 and Experiment 3, respectively. Isolation and characterization of a cytochrome P450 gene from *Muscari armeniacum*, which might be involved in the flavonoid biosynthetic pathway, were also described in Chapter 3, Experiment 4.

In Chapter 4, flower color modification via genetic engineering was examined in Liliaceous ornamentals by using the transformation systems developed previously (Suzuki and Nakano, 2002a) or in Chapter 2 and the genes insolated in Chapter 3. Firstly, in Chapter 4, Experiment 1, *Tricyrtis hirta* was transformed with the F3'5'H gene from *Vinca major* and/or the DFR gene from *Agapanthus praecox* ssp. *orientalis* toward the production of blue-flowering cultivars. Secondly, in Chapter 4, Experiment 2, *Muscari armeniacum* was transformed with the F3'5'H gene from *Vinca major* to widen flower color variation. Finally, in Chapter 5, the general significance of the results obtained in the present study is summarized and discussed.

Chapter 2

Establishment of regenerable callus cultures and *Agrobacterium*-mediated transformation systems

Experiment 1

Somatic embryogenesis from callus cultures in various *Muscari* species and cultivars

Introduction

The genus *Muscari* consists of about 40 species, and some of these species are widely cultivated for pot and garden uses throughout the temperate regions of the world because of its beautiful, blue to white flowers. Establishment of highly embryogenic callus cultures (Suzuki and Nakano, 2001) and production of transgenic plants via *Agrobacterium*-mediated transformation of the embryogenic calluses (Suzuki and Nakano, 2002a) have already been reported for *M. armeniacum* 'Blue Pearl'. For wide application of molecular breeding in the genus *Muscari*, it is necessary to develop highly regenerable callus cultures in various *Muscari* species and cultivars, since such cultures have been shown to be most suitable as a target material for transformation in Liliaceous ornamentals (Suzuki and Nakano, 2002b). In the present experiment, therefore, callus induction from cultured explants and plant regeneration from induced calluses were examined in 19 genotypes in the genus *Muscari*.

Materials and methods

Plant materials and callus induction

Nineteen *Muscari* genotypes (Table 1) were used. Plants of all genotypes were cultivated in the field. Preparation of explants and *in vitro* culture were essentially performed according to Suzuki and Nakano (2001). Young leaves of

19 genotypes and flower buds of 16 genotypes were harvested from field-grown plants just before anthesis and used for callus induction. They were surface-sterilized with 70% (v/v) ethanol for 30 s followed by a 1% (w/v) NaOCl solution containing 0.1% (v/v) Tween20 (Wako, Japan) for 15 min and rinsed three times with sterile, distilled water. Leaves were cut transversely into segments about 5 mm in length, and flower buds were cut lengthwise into halves. These explants were placed on MS medium (Murashige and Skoog, 1962) containing 54 μM NAA (Kanto Chemical, Japan), 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Wako, Japan). The pH of all culture media used was adjusted to 5.8. Ten explants were inoculated per plastic Petri dish (90×20 mm) containing 30 ml of medium, and each experiment was replicated three times. Cultures were incubated at 25°C under continuous illumination with white fluorescent light (35 μmol m^{-2} s^{-1}). Data on the percentage of explants forming calluses were recorded 8 weeks after culture initiation without subculturing.

Ten weeks after culture initiation, calluses were isolated from the explants and subcultured every 4 weeks under the same conditions as for callus induction. For each subculture, ten calluses, about 0.1 g FW each, were placed per plastic Petri dish (90×20 mm) containing 30 ml of fresh medium of the same composition as for callus induction. Callus growth was determined at the fourth subculture, and each experiment was replicated three times.

Somatic embryo induction from callus cultures

Somatic embryo induction was examined using calluses subcultured for 8 months. Calluses were transferred onto MS medium lacking PGRs but containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum. Ten callus clusters, about 0.1 g FW each, were placed per plastic Petri dish (90×20 mm) containing 30 ml of medium, and each experiment was replicated three times. Cultures were

maintained at 25°C under continuous illumination (35 μmol m^{-2} s^{-1}) with fluorescent light. Data on the percentage of callus clusters producing somatic embryos and the number of somatic embryos per embryo-producing callus clusters were recorded 4 weeks after transfer without subculturing.

Results and discussion

Callus formation from explants

Effects of explant type (leaf, peduncle, bulb scale, and root) and auxin (2,4-D, NAA, and PIC) on the establishment of regenerable callus cultures were previously examined in *M. armeniacum* 'Blue Pearl', and it was showed that leaf explants on MS medium containing 54 μM NAA produced embryogenic calluses most frequently (Suzuki and Nakano, 2001). Therefore, in the present experiment, the same medium was used for inducing embryogenic calluses in various *Muscari* species and cultivars, flower bud explants were newly examined in addition to leaf explants for their potential to produce regenerable calluses. In the present experiment, callus induction from leaf explants was examined in 19 genotypes, but flower bud explants were examined only in 16 genotypes because of the shortage of materials. Irrespective of genotype and explant source, calluses were produced mainly at the cut end of explants 4–5 weeks after culture initiation, and were generally creamy-white and soft in appearance. When leaves were used as an explant, callus formation was obtained in 16 out of 19 genotypes. The percentage of leaf explants forming calluses was highest in *M. neglectum* 'Dark Eyes' (100%), followed by five genotypes of *M. armeniacum* (80.0–90.0%) and *M. tubergenianum* (83.3%), whereas leaf explants of *M. azureum*, *M. botryoides* 'Album', and *M. paradoxum* never produced calluses (Table 1). When flower buds were used as

an explant, on the other hand, callus formation was obtained in all the 16 genotypes examined. More than 80% of flower bud explants produced calluses in five genotypes of *M. armeniacum*, *M. botryoides* 'Superstar', *M. neglectum* 'Dark Eyes', *M. paradoxum*, *M. tubergenianum*, and *Muscari* 'Sky Blue' (Table 1). For *M. azureum*, *M. botryoides* 'Album' and *M. paradoxum*, callus formation was obtained only from flower bud explants. Thus, flower bud explants might be more suitable than leaf explants for callus induction in a wide range of *Muscari* species and cultivars on a medium containing 54 μM NAA.

All of the leaf- and flower bud-derived calluses were subcultured to fresh medium of the same composition. After 3–8 weeks of subculture, white to creamy-yellow and friable calluses occasionally containing small globular structures were developed from the primary calluses (Figure 2A) irrespective of genotype and original explant type. Since the appearance of these newly developed calluses was similar to that of leaf-derived embryogenic calluses of *M. armeniacum* 'Blue Pearl' (Suzuki and Nakano, 2001), these calluses were selected and gathered together for each genotype–explant type combination during the first and second subculture. Growth of leaf- and flower bud-derived calluses was determined at the fourth subculture (Table 1). Twofold or more increases in callus mass in 4 weeks were obtained in leaf-derived calluses of five genotypes of *M. armeniacum*, *M. neglectum* 'Dark Eyes', and *M. tubergenianum*, and flower bud-derived calluses of five genotypes of *M. armeniacum*, *M. tubergenianum*, and *Muscari* 'White Beauty'. However, only no or slight growth was observed in leaf-derived calluses of two genotypes of *M. azureum*, two genotypes of *M. botryoides*, *M. latifolium*, *M. macrocarpum*, two genotypes of *M. moschatum*, *M. paradoxum*, and *Muscari* 'Sky Blue', and flower bud-derived calluses of two genotypes of *M. azureum*, *M. botryoides* 'Album', *M. latifolium*, and *M. macrocarpum*.

Somatic embryo induction from callus cultures

Somatic embryo induction was examined for leaf-derived calluses of nine genotypes and flower bud-derived calluses of 12 genotypes (Table 2), in which sufficient amounts of calluses were obtained. After 8 months of subculture on a medium containing 54 μM NAA, calluses were transferred to PGR-free medium, which was used for inducing somatic embryos from leaf-derived embryogenic calluses of *M. armeniacum* 'Blue Pearl' (Suzuki and Nakano, 2001). Within 3–4 weeks after transfer to PGR -free medium, calluses produced somatic embryos and/or adventitious shoots. Somatic embryo production was observed in leaf-derived calluses of four genotypes of *M. armeniacum* and *M. neglectum* 'Dark Eyes', and flower bud-derived calluses of four genotypes of *M. armeniacum* and *M. tubergenianum* indicating that these calluses were embryogenic. It should be noted that the frequency of somatic embryo production of more than 60% and the number of somatic embryos per embryo-producing callus cluster of ten or more were obtained in leaf-derived calluses of *M. armeniacum* 'Blue Pearl', and flower bud-derived calluses of four genotypes of *M. armeniacum*. For *M. armeniacum* genotypes, flower bud-derived calluses might have high potential to produce somatic embryos compared with leaf-derived calluses. Although white to creamy-yellow and friable calluses were selected as a putative embryogenic one, calluses with such appearances did not produce somatic embryos in some genotype–explant type combinations. Further studies are needed to clarify the histological difference between calluses with the same appearances but different embryogenic potential. Irrespective of genotype and initial explant type, somatic embryos obtained in the present experiment (Figure 2B) were globular to club-shaped, white under continuous illumination, and easily detached from the callus as previously reported for *M. armeniacum* 'Blue Pearl' (Suzuki and Nakano, 2001). In

addition, they were confirmed to have a capacity to develop into plantlets. On the other hand, leaf-derived calluses of *M. comosum* var. *plumosum*, and flower bud-derived calluses of *M. armeniacum*, *M. latifolium*, and *M. tubergenianum* produced adventitious shoots at low frequencies (3.3–20.0%). These shoots were green under continuous illumination and tightly attached to the callus.

Effect of explant type on regenerable callus induction was previously examined in *M. armeniacum* 'Blue Pearl', and leaves were shown to be superior to peduncles, bulb scales, and roots (Suzuki and Nakano, 2001). In the present experiment, however, flower bud explants were more suitable than leaf explants for establishing embryogenic callus cultures in *Muscari* species and cultivars. Famelaer et al. (1996) suggested that the presence of juvenile or meristematic tissue and/or the physiological condition of explant organs possibly accounted for the induction of regenerable calluses. Thus, in *Muscari* species and cultivars, some tissues in the flower bud might possess the physiological condition more suitable for callus production than the other organs. Young flower organs have also been used as an explant for inducing embryogenic calluses in other Liliaceous ornamentals such as *Agapanthus africanus* (Supaibulwatana and Mii 1997), *Lilium longiflorum* (Tribulato et al. 1997) and *Tricyrtis* ssp. (Nakano et al., 2004), and Supaibulwatana and Mii (1997) suggested that flower head tissues probably have the undifferentiated meristematic nature. Further studies are needed to identify tissues and organs forming embryogenic calluses in flower bud culture of *Muscari* species and cultivars.

In the present experiment, induction of embryogenic calluses in a wide range of *Muscari* species and cultivars was examined by culturing leaf and flower bud explants on a medium containing 54 μM NAA, which had initially been developed for leaf explants of *M. armeniacum* 'Blue Pearl' (Suzuki and

Nakano, 2001). The results indicated a considerable difference in the production of embryogenic calluses on this medium among different *Muscari* genotypes. Embryogenic calluses were obtained from both leaf and flower bud explants of four genotypes of *M. armeniacum*, leaf explants of *M. neglectum* 'Dark Eyes', and flower bud explants of *M. tubergenianum*, whereas embryogenic calluses were not produced in the other 13 genotypes. As suggested by Suzuki and Nakano (2001) and Nakano et al. (2004) for Liliaceous ornamentals, the type and concentration of auxin appropriate for inducing somatic embryogenesis may be different according to the species. Therefore, further studies should be directed to determine the optimum type and concentration of auxin for inducing embryogenic calluses in each *Muscari* genotypes, especially other than *M. armeniacum*.

In summary, embryogenic callus cultures were established in several *Muscari* species and cultivars. This is the first report on somatic embryo production in two *Muscari* species, *M. neglectum* and *M. tubergenianum*. The suitability of flower buds as an explant for inducing embryogenic calluses was also identified. The embryogenic callus cultures of *M. armeniacum*, *M. neglectum*, and *M. tubergenianum* established in the present experiment may be suitable as a target material for *Agrobacterium*-mediated transformation.

Experiment 2

Callus formation and plant regeneration in various *Lilium* species and cultivars

Introduction

The genus *Lilium*, which consists of about 100 species distributed throughout the cold and temperate regions of the Northern Hemisphere. This genus contains many beautiful ornamental species, and more than 3,000 cultivars have been bred mainly by intra- or interspecific hybridization, which are now widely cultivated throughout the world (Godo and Mii, 1996). Recently, a system for producing transgenic plants has successfully been established in the Oriental hybrid lily (*Lilium* sp.) 'Acapulco' by using regenerable calluses as a target material for *Agrobacterium*-mediated transformation (Hoshi et al., 2004). For wide application of molecular breeding in the genus *Lilium*, it is necessary to develop highly regenerable callus cultures in various *Lilium* species and cultivars. In the present experiment, callus induction from cultured explants and plant regeneration from induced calluses were examined in 33 *Lilium* genotypes belonging to various sections and hybrid groups.

Materials and methods

Plant materials and callus induction

Lilium genotypes used in the present experiment are shown in Table 3. Seeds of 17 *Lilium* species were obtained from Yurigahara Park in Sapporo, Japan. They were surface-disinfected with 70% (v/v) ethanol for 30 s followed

by a 1% (w/v) NaOCl solution containing 0.1% (v/v) Tween20 for 10 min. After three rinses with sterile distilled water, seeds were wounded with a scalpel to promote germination and placed on a callus induction medium. Expanding leaves and flower buds 5–7 days before anthesis were harvested from potted plants and washed in running water for 1 min. They were surface-disinfected with a 1% (w/v) NaOCl solution containing 0.1% (v/v) Tween20 for 10 min followed by three rinses with sterile distilled water. Stamens were isolated from flower buds, and anthers were removed. Leaves and filaments were transversely cut into segments of ca. 1 cm in length and placed on a callus induction medium. Bulb-scales were excised from *in vitro*-grown plantlets, which were maintained by subculturing every 2 months on half-strength MS medium lacking PGRs but containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum at 25°C under continuous illumination (50 μmol m^{-2} s^{-1}) with fluorescent light. The pH of all culture media used was adjusted to 5.8. Bulb-scales were transversely cut into halves and placed on a callus induction medium. A callus induction medium consisted of MS medium, 4.1 μM PIC (Wako, Japan), 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Suzuki et al., 1998; Nakano et al., 2000). Plastic Petri dishes (90×20 mm) were filled with 30 ml of medium, and 10–20 explants were placed in each plate. Cultures were maintained at 25°C in the dark. Data on the percentage of explants producing calluses were recorded 8 weeks after culture initiation without subculturing. Each experiment was replicated three times with at least ten explants.

Calluses were isolated from the explants and transferred to plastic Petri dishes (90×20 mm), each containing 30 ml of fresh medium of the same composition as for callus induction. They were subcultured every 4 weeks under the same conditions as for callus induction. Callus type (appearance) and callus growth were determined at the third subculture.

Plant regeneration from callus cultures

For callus lines showing sustained growth one year after the initiation of subculture (62 callus lines from 28 genotypes), callus clusters were transferred to shoot induction media consisting of MS medium, 30 g l^{-1} sucrose, and 2 g l^{-1} gellan gum, with or without 22 μM BA (Merck, Germany). Plastic Petri dishes (90×20 mm) were filled with 30 ml of medium, and ten callus clusters, about 0.2 g FW each, were placed per dish. Cultures were maintained at 25°C under continuous illumination (35 μmol m^{-2} s^{-1}) with fluorescent light. Data on the percentage of callus clusters forming shoots were recorded 8 weeks after transfer to shoot induction media without subculturing. Each experiment was replicated three times with at least ten callus clusters.

Regenerated shoots were excised from the calluses and transferred to test tubes (25×130 mm) each containing 10 ml of half-strength MS medium lacking PGRs but containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum. Cultures were maintained at 25°C under continuous illumination (50 μmol m^{-2} s^{-1}). Plantlets in test tubes with a well-developed bulblet were then subjected to a cold treatment at 4°C in the dark for more than 3 months. After that, scale-leaves and roots were removed from the plantlets, and the remaining bulblets were washed in running water to remove gellan gum and transplanted to pots. They were incubated in the greenhouse without heating.

Results

Callus formation from explants

When seeds were used as an explant, they started to germinate 3–4 weeks after culture initiation. After 8 weeks, seed germination was observed with

frequencies of 20–100% in all the genotypes except for *L. canadense*, *L. michiganense* and *L. pumilum*, in which no seed germination was observed 6 months after culture initiation. Most seedlings showed stunted abnormal growth on PIC-containing medium, and calluses started to be produced mainly at the shoot base of the seedlings 4–6 weeks after culture initiation. For *L. monadelphum*, seeds germinated 4–5 months after culture initiation and callus formation was observed about 4 weeks later. Bulb-scale, leaf and filament explants started to form calluses mainly at their cut end 2–3 weeks after culture initiation.

The percentage of explants producing calluses as affected by genotype and explant type is shown in Table 3. Callus formation was obtained in all the *Lilium* genotypes except for *L. canadense*, *L. michiganense* and *L. pumilum*, in which no seed germination was observed. Generally, the highest frequency of callus formation was obtained with bulb-scale explants (83.0–100%) followed by filament (82.1–98.6%) and leaf (43.6–100%) explants. No apparent genotypic difference was observed in the callus formation frequency.

Calluses were isolated from the explants, gathered together for each genotype–explant type combination, and subcultured every 4 weeks onto fresh medium of the same composition. Callus type and callus growth were determined at the third subculture (Table 3). Calluses were generally yellow and nodular (YN) in appearance, but yellow and friable (YF) calluses were also produced in addition to YN ones in *L. pardalinum* 'Red Sunset', *L. formosanum*, *L. longiflorum* 'Georgia', *L. wallichianum*, three Asiatic hybrid cultivars, and two Longiflorum hybrid cultivars. Generally, filament explants tended to form YF calluses. Production of YF calluses only was not observed in any genotype–explant type combinations. Seed-derived calluses of *L. tsingtauense* were brown and nodular (BN), and showed no growth in subculture. Callus lines showing twofold or more increases in callus mass in 4 weeks were obtained in

15 out of the 33 genotypes examined. For *Leucolirion* species, and cultivars of Asiatic hybrid, LA hybrid, Longiflorum hybrid, Oriental hybrid, and Trumpet hybrid, most callus lines showed such vigorous growth. No apparent effect of initial explant type was observed on the callus growth. For genotype–explant type combinations, in which both YN and YF calluses were produced, no apparent difference in the growth rate was observed between these two types of calluses, and YN and YF calluses were separated and subcultured individually following the fourth subculture. YF callus cultures often produce YN calluses, which were removed from YF callus cultures during subculture.

Plant regeneration from callus cultures

Sixty-two callus lines from 28 genotypes, which were marked with ++ or +++ in Table 3, kept their growth rate one year after the initiation of subculture. These lines were then examined for the ability to form shoots on media with or without 22 μM BA (Table 4). Shoot regeneration was observed in all the callus lines examined. The regeneration frequency of over 80% was obtained in 20 genotypes: three *Archelirion* species, two *Sinomartagon* species, three *Leucolirion* species, three Asiatic hybrid cultivars, two LA hybrid cultivars, two Longiflorum hybrid cultivars, three Oriental hybrid cultivars, and two Trumpet hybrid cultivars. Generally, PGR-free medium was superior to BA-containing medium for regeneration. The regeneration frequency of over 80% was obtained in 46 and 22 callus lines on PGR-free and BA-containing media, respectively. Origin of calluses (initial explants for callus induction) and callus type (YN or YF) had no effects on shoot regeneration. The number of shoots per shoot-producing callus cluster was 2–30 depending on the genotypes, and more than 20 shoots per shoot-producing callus cluster were obtained in *L. martagon*, *L. formosanum*, *L. wallichianum*, and two cultivars of *L.* × *formolongi* (data not

shown). In most cases, shoots were regenerated via organogenesis, whereas those of *L. formosanum* on PGR-free medium appeared to be regenerated via somatic embryogenesis.

Almost all of the regenerated shoots developed into complete plantlets following transfer to PGR-free medium. These plantlets, subsequently, formed a small bulblet at the basal region. After cold treatment, regenerated plantlets were transferred to pots, in which over 90% of them developed new scaly leaves within 4 weeks irrespective of genotype, callus origin, callus type, and shoot induction medium.

Discussion

In an *Agrobacterium*-mediated transformation system developed for the Oriental hybrid lily 'Acapulco' (Hoshi et al., 2004), calluses used as a target for transformation were induced and maintained on a medium containing 8.2 μM PIC and shoots were efficiently induced from the calluses on a medium containing 0.4 μM PIC and 0.04 μM BA. However, preliminary experiments using several different *Lilium* genotypes indicated that no apparent differences in the callus formation frequency, callus type and callus growth were observed among callus induction media containing 4.1, 8.2 or 20.5 μM PIC, and 0.4 μM PIC in shoot induction medium had no apparent effect on shoot regeneration from callus cultures. Therefore, in the present experiment, callus induction was examined using a medium containing 4.1 μM PIC and shoot induction was examined using a medium without PGRs and a medium containing 22 μM BA.

To date, establishment of regenerable callus or suspension cultures in lilies has been restricted to relatively few genotypes, mainly *Leucolirion* species (Priyadarshi and Sen, 1992; Wickremesinhe et al., 1994; Arzate-Fernandez et al., 1997; Tribulato et al., 1997; Suzuki et al., 1998; Nakano et al., 2000), and

Longiflorum (Mii et al., 1994; Godo et al., 1996, 1998) and Oriental (Simmonds and Cumming, 1976; Stimart et al., 1980) hybrid cultivars. We examined callus formation from different explants and plant regeneration from induced calluses under the same culture conditions in 33 *Lilium* genotypes belonging to various sections and hybrid groups. As a result, regenerable callus cultures were successfully established in 28 genotypes, which included species in the sections *Martagon*, *Pseudolirium*, *Liriotypus*, *Archelirion*, *Sinomartagon* and *Leucolirion*, and cultivars in Asiatic, LA, Longiflorum, Oriental and Trumpet hybrid groups. When leaves, bulb scales and filaments were used as explants, no apparent differences in the callus formation frequency were observed among different *Lilium* genotypes. On the other hand, there were some genotypic differences in shoot production from callus cultures. Generally *Sinomartagon* and *Leucolirion* species, and Asiatic, LA, Longiflorum, Oriental and Trumpet hybrid cultivars showed relatively high shoot regeneration abilities.

To date, bulb-scales have mainly been used as an explant for callus induction in lilies (Simmonds and Cumming, 1976; Stimart et al., 1980; Priyadarshi and Sen, 1992; Suzuki et al., 1998; Nakano et al., 2000), and immature leaves (Wickremesinhe et al., 1994), seeds (Mii et al., 1994), shoot tips (Godo et al., 1996, 1998), filaments with anthers (Arzate-Fernandez et al., 1997), styles and flower pedicels (Tribulato et al., 1997) have also been used. In the present experiment, we compared the callus formation ability of seeds, bulb-scales, leaves and filaments, since relatively large numbers of explants could be prepared from them. Among four types of explants examined, bulb-scales and filaments showed high abilities to form calluses in a wide range of *Lilium* genotypes. Although Arzate-Fernandez et al. (1997) reported for *L. longiflorum* that callus formation occurred only from filaments with anthers, a high frequency of callus formation was obtained from filaments lacking anthers in the present experiment. The difference may be due to different PGRs added to

callus induction media. In the present experiment, relatively low frequencies of callus formation were obtained with seed explants, which were mainly due to poor seed germination. More than 70% of germinated seeds produced calluses irrespective of *Lilium* genotype. Failure in seed germination might be caused by inappropriate culture conditions, and further studies are necessary to improve germination efficiency.

Usefulness of PIC for callus induction has already been demonstrated for several *Lilium* genotypes (Mii et al., 1994; Godo et al., 1996, 1998; Suzuki et al., 1998; Nakano et al., 2000), and confirmed in the present experiment. In lilies, PIC tended to induce nodular calluses (Mii et al., 1994; Godo et al., 1996, 1998; Nakano et al., 2000), whereas friable calluses were produced on media containing other auxins such as 2,4-D and dicamba (Stimart et al., 1980; Tribulato et al., 1997). In the present experiment, yellow and nodular (YN) calluses were produced on PIC-containing medium irrespective of genotype and explant type. In some cases, especially when filaments were used as an explant, yellow and friable (YF) calluses were produced in addition to YN calluses. Tribulato et al. (1997) reported that friable calluses were formed from styles on PIC-containing medium. Thus, flower organs might have an ability to form friable calluses on PIC-containing medium. Although there have been no comparative studies of nodular and friable calluses in lilies, no apparent differences in the growth and shoot production between these two types of calluses were observed in the present experiment.

Recently, a system for the production of transgenic plants in an Oriental hybrid lily has been established by co-cultivation of regenerable calluses with *Agrobacterium* (Hoshi et al., 2004). Therefore, callus cultures with high regeneration ability established in the present experiment may be suitable as a target material for *Agrobacterium*-mediated transformation. Further study should be concentrated to optimize transformation conditions for each genotype

following detailed investigation of the phenotypic and genetic fidelity of plants regenerated in the present experiment.

Experiment 3

Agrobacterium-mediated production of transgenic plants in *Tricyrtis hirta*

Introduction

Tricyrtis hirta is an autumn-flowering, perennial plant native to Japan. The plants have attractive arching stems with exotic starry flowers. *T. hirta* is sometimes called 'toad lily' and has recently become popular as an ornamental plant for pot and garden uses. Recently highly embryogenic callus cultures were established in *T. hirta* (Nakano et al., 2004). In several Liliaceous ornamentals including *Agapanthus praecox* ssp. *orientalis* (Suzuki et al., 2001), *Muscari armeniacum* (Suzuki and Nakano, 2002a) and *Lilium* sp. (Hoshi et al., 2004), regenerable callus cultures have been used as a target material for *Agrobacterium*-mediated transformation. Thus, in the present experiment, development of an efficient system for *Agrobacterium*-mediated transformation was examined in *T. hirta* by utilizing the established embryogenic callus cultures.

Materials and methods

Plant material

Tepal-derived embryogenic calluses of *T. hirta* (Nakano et al., 2004) were subcultured every 4 weeks at 25°C in the dark onto half-strength MS medium containing 4.5 μM 2,4-D (Tokyo Chemical Industry, Japan), 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum. The pH of all plant culture media was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121°C under a pressure of 1.2 kg cm^{-2} for 15 min.

The calluses developed numerous somatic embryos following transfer to half-strength MS medium lacking PGRs but containing 30 mg l^{-1} sucrose and 2 g l^{-1} gellan gum at 25°C under continuous illumination (50 μmol m^{-2} s^{-1}) with fluorescent light. Four- to six-month-old callus cultures were used for transformation.

Agrobacterium *strain*

A. tumefaciens strain EHA101/pIG121Hm was used in the present experiment. The T-DNA region of the binary vector pIG121Hm contains the NPTII gene under the control of the NOS promoter, the GUS gene with an intron (intron-containing GUS gene) fused to the CaMV35S promoter, and the HPT gene under the control of the CaMV35S promoter (Ohta et al., 1990). This bacterial strain was inoculated into liquid YEP medium (An et al., 1988) containing 50 mg l^{-1} kanamycin (kanamycin monosulfate; Wako, Japan) and 50 mg l^{-1} hygromycin (hygromycin B; Wako, Japan), and incubated for more than 24 h at 28°C with reciprocal shaking (150 cycles min^{-1}).

Inoculation, co-cultivation and selection of putative transformants

Cultured *Agrobacterium* cells were collected by centrifugation (2,000 *g*, 10 min) and suspended to an OD_{600} of 0.2 in liquid inoculation media, which consisted of half-strength MS medium, 4.5 μM 2,4-D and 30 g l^{-1} sucrose with or without 50 mg l^{-1} AS (Sigma-Aldrich, USA). After blotting of embryogenic calluses on sterile filter papers to remove excess culture medium, they were immersed into the bacterial suspension for 3 min. Calluses were then transferred to the same medium as for inoculation but solidified with 2 g l^{-1} gellan gum. They were incubated at 25°C in the dark for 3, 5, 7 or 14 days.

Co-cultivated calluses were transferred to half-strength MS medium lacking PGRs but containing 40 mg l^{-1} hygromycin, 300 mg l^{-1} cefotaxime (Claforan; Hoechst, Germany), 30 mg l^{-1} sucrose and 2 g l^{-1} gellan gum for selecting transgenic cells and tissues. They were subcultured every 2 weeks onto fresh medium of the same composition at 25°C under continuous illumination (50 μmol m^{-2} s^{-1}) with fluorescent light. After 8 weeks, developed somatic embryos were transferred for promoting their germination to half-strength MS medium lacking PGRs but containing 20 mg l^{-1} hygromycin, 100 mg l^{-1} cefotaxime, 30 mg l^{-1} sucrose and 2 g l^{-1} gellan gum, and cultured under the same conditions. Four to five weeks later, regenerated plantlets were transferred to half-strength MS medium lacking both PGRs and antibiotics but containing 30 mg l^{-1} sucrose and 2 g l^{-1} gellan gum, and cultured under the same conditions. Regenerated plantlets with a well-established root system were washed carefully with tap water to remove gellan gum and transplanted to pots. Following acclimatized for 2–3 weeks, they were cultivated in a growth chamber.

GUS histochemical assay

Histochemical localization of GUS gene expression in the embryogenic calluses during co-cultivation, and leaf and root segments of the control and putative transformed plantlets was detected according to Jefferson (1987) with several modifications. Tissues were incubated for 48 h at 37°C in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; Wako, Japan). To remove chlorophyll, materials were then soaked in 70% (v/v) ethanol for several days.

Isolation of plant DNA and PCR analysis

Total genomic DNA was isolated from leaf tissues of *T. hirta* according to Rogers and Bendich (1985). For PCR analysis, the GUS gene primer set, 5'-AAT TGA TCA GCG TTG GTG G-3' (GUS-1) and 5'-GGT GTA GAG CAT TAC GCT GC-3' (GUS-2), which yields a 0.45-kbp fragment inside of the GUS gene (Anzai et al., 1996), was used. PCR amplification reactions contained 20 ng of template DNA, 0.4 μM of each primer, 100 μM of a dNTP mixture, 1×*Taq* DNA polymerase reaction buffer and 1 U *Taq* DNA polymerase (Takara, Japan) in a 20 μl final volume. For amplifying the GUS gene, 30 cycles were performed using a programmed temperature control system (2400-R, Perkin-Elmer, USA) under the following conditions: 30 s at 94°C, 40 s at 60°C and 40 s at 72°C. Amplified products were analyzed by electrophoresis in a 1.5% (w/v) agarose gel.

Results and discussion

Transient expression of the GUS gene

Effects of the co-cultivation period and the addition of AS to both inoculation and co-cultivation media on the number of blue spots, which may result from transient expression of the GUS gene, per 0.5 g FW of embryogenic calluses were initially examined (Table 5). Preliminary experiments indicated that no endogenous GUS activity was observed in the control, non-co-cultivated calluses, and leaf and root tissues of the control plantlets. In the absence of AS, no blue spots could be detected in the calluses irrespective of the co-cultivation period. In the presence of AS, blue spots started to be detected 5 days after co-cultivation, and its number increased thereafter. The highest number of blue spots, over 350 spots per 0.5 g FW of calluses, was obtained 7 days after co-cultivation (Figure 3A). Positive effects of AS treatment on the efficiencies

of transient expression of a reporter gene and of stable transformation have already been reported for *Agrobacterium*-mediated transformation of several Liliaceous ornamentals such as *Agapanthus praecox* ssp. *orientalis* (Suzuki et al., 2001) and *Muscari armeniacum* (Suzuki and Nakano, 2002a). In the present experiment, AS treatment during co-cultivation was essential for transient expression of the GUS gene. Based on these results, embryogenic calluses of *T. hirta* were co-cultivated for 7 days in the presence of AS in subsequent experiments. Seven-day co-cultivation was also performed in other Liliaceous ornamentals, *Agapanthus praecox* ssp. *orientalis* (Suzuki et al., 2001) and *Lilium* sp. (Hoshi et al., 2004).

Production of transgenic plants

Embryogenic calluses co-cultivated for 7 days were successively subcultured onto PGR-free medium containing 40 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime. Preliminary experiments indicated that this level of hygromycin was sufficient to inhibit the formation of somatic embryos from the control, non-co-cultivated embryogenic calluses of *T. hirta*. Co-cultivated embryogenic calluses turned brown within 4 weeks on a medium containing 40 mg l^{-1} hygromycin, but hygromycin-resistant (Hyg^r) somatic embryos, white to light yellow in color, started to develop thereafter (Figure 3B). These Hyg^r somatic embryos were selected and transferred to the same medium but containing lower concentrations of antibiotics (20 mg l^{-1} hygromycin and 100 mg l^{-1} cefotaxime), on which over 90% of them germinated after 4–5 weeks (Figure 3C). Small plantlets thus obtained were further transferred to a medium without both PGRs and antibiotics, on which *Agrobacterium*-free plantlets with a well-developed root system were established within 4 weeks (Figure 3D).

Hyg^r somatic embryo-derived plantlets were subjected to PCR analysis for

confirmation of their transgenic nature (Figure 4). PCR using the GUS gene primer set showed the expected band of 0.45 kbp in 64 out of 93 plantlets analyzed. No amplified fragments were detected in the control, non-transgenic plantlets. These results indicated that the GUS gene was present in about 70% of the plantlets regenerated from Hyg^r somatic embryos. Leaf and root segments of ten independent transgenic plantlets were subjected to GUS histochemical assay: eight plantlets showed GUS gene expression in both leaves and roots (Figures 3E, F), whereas no GUS gene expression could be detected in the rest two plantlets. The luck of GUS gene expression in these plantlets might be resulted from genomic position effects, deletion of the promoter or GUS cording region, or gene silencing as suggested by Suzuki and Nakano (2002a) for *Agrobacterium*-mediated transformation of *Muscari armeniacum*.

Almost all of the plantlets regenerated from Hyg^r somatic embryos were successfully acclimatized. Over 30 independent transgenic plants have so far been cultivated in a growth chamber (Figure 3G). All of them exhibited normal phenotype at least at early stage of growth. Examination of the stability of transgene expression in these transgenic plants as well as their detailed morphological characterization are necessary for the practical application of the established transformation system for molecular breeding of *T. hirta*.

Experiment 4

Morphological characterization and long-term stability of transgene expression in transgenic plants of *Agapanthus praecox* ssp. *orientalis*

Introduction

Agapanthus praecox ssp. *orientalis*, which is commonly called 'agapanthus' or 'lily of the Nile', has recently become popular as a potted plant, for landscaping and as a cut flower because of its beautiful, blue-violet to white flowers (Mor et al., 1984). An *Agrobacterium*-mediated transformation system has recently been developed in this species by using a cultivar 'Royal Purple Select' (Suzuki et al., 2001). This system was subsequently applied to other *A. praecox* ssp. *orientalis* genotypes, and transgenic plantlets containing the GUS reporter gene were successfully produced in several genotypes including the experimental strain 1 ('ES-1'), in which transgenic plantlets were most efficiently obtained. They had subsequently been cultivated for 5 years, and produced flowers 3–5 years after transplantation to pots. For perennial and vegetatively propagated crops like *Agapanthus* spp., long-term and stable expression of transgene(s) is indispensable for successful application of molecular breeding. In the present experiment, morphological characterization, and examination of the ploidy level, pollen fertility and transgene (GUS gene) expression in various organs of these transgenic plants were carried out at the flowering stage for evaluating the validity of genetic transformation in the breeding programs of *A. praecox* ssp. *orientalis*.

Materials and methods

Plant materials

One control, non-transgenic line (C1) and 5 independent transgenic lines (L7, L8, L9, L10 and E1) of *A. praecox* ssp. *orientalis* 'ES-1' were used in the present experiment. The transgenic lines L7, L8, L9 and L10 were obtained by co-cultivation of embryogenic calluses with *A. tumefaciens* strain LBA4404/pTOK233, whereas the transgenic line E1 was obtained by co-cultivation with *A. tumefaciens* strain EHA101/pIG121Hm according to Suzuki et al. (2001). Both *Agrobacterium* strains contained the NPTII gene under the control of the NOS promoter, the intron-containing GUS gene fused to the CaMV35S promoter, and the HPT gene under the control of the CaMV35S promoter in the T-DNA region of the binary vector (Ohta et al., 1990; Hiei et al., 1994). The non-transgenic line C1 was a division from the mother plants of *A. praecox* ssp. *orientalis* 'ES-1'. Plants of the transgenic and non-transgenic lines were transplanted to pots and cultivated for 5 years in a growth chamber. During cultivation the transgenic lines were divided into two to three plants. Thus, totally 12 transgenic plants derived from five independent lines and one non-transgenic plant (Table 6) were subjected to various analyses.

Morphological characterization and analyses of the ploidy level and pollen fertility

The number of leaves per inflorescence, length and width of leaves, length of inflorescence stalks, number of flowers per inflorescence, and length and width of flowers were measured using the earliest-flowering inflorescence of each plant. Ploidy level was determined by measuring relative DNA contents of nuclei isolated from leaf tissues using a flow cytometer PA (Partec, GmbH, Münster, Germany) as previously described (Saito et al., 2003a). To examine

pollen fertility, mature pollen grains were collected from flowers of the earliest-flowering inflorescence immediately after anthesis, stained with 1% acetocarmine, and observed under a light microscope (DMLB-100; Leica, Germany) (Nakano and Mii, 1993).

Histochemical and fluorometric GUS assays

Histochemical localization of GUS activity was detected in leaves, roots, tepals, pistils and stamens according to Chapter 2, Experiment 3. GUS activity was quantified in some plants by the fluorogenic 4-methl umbelliferyl glucuronide assay according to Jefferson et al. (1987). Approximately 100 mg FW each of leaf, root, tepal, pistil and stamen tissues were homogenized in 500 μl of an extraction buffer [10 mM EDTA (Kanto Chemical, Japan), 0.1% (v/v) Triton X-100 (Kanto Chemical, Japan), 0.1% (w/v) sarkosyl (Wako, Japan), 10 mM 2-mercaptoethanol (Wako, Japan) in 50 mM NaH_2PO_4, pH 7.0] using a mortar and pestle. The homogenates were centrifuged at 16,000 *g* for 10 min and the supernatants were stored on ice until use. An aliquot (150 μl) of the supernatant was added to 150 μl of an assay buffer [1 mM 4-methyl umbelliferyl-ß-D-glucuronide (Wako, Japan) in the extraction buffer]. After 0, 30 and 60 min of incubation at 37°C, an aliquot (100 μl) of the assay buffer with sample was added to 2,900 μl of 200 mM Na_2CO_3 in order to stop the enzymatic reaction. Three tissue samples were processed independently for each treatment. Fluorescence was measured with the Intelligent Spectrofluorometer 820-FP (Japan Spectroscopic, Japan). Protein concentration of the supernatants was measured using the Coomassie (Bradford) Protein Assay Kit (PIERCE, USA) according to the manufacturer's instructions.

Isolation of plant DNA and RNA

Total genomic DNA was isolated from leaves as described in Chapter 2, Experiment 3. Total RNA was isolated from leaves, roots, tepals, pistils and stamens of some plants by the guanidium thiocyanate-cesium chloride procedure (Sambrook et al., 1989).

Southern blot analysis

Thirty μg of genomic DNA was digested with *Hin*dIII and electrophoresed in a 0.6% (w/v) agarose gel. The DNA was then transferred to the Hybond-N^+ nylon membrane (Amersham Pharmacia, UK) under an alkaline condition. The GUS gene probe was generated by PCR (Suzuki and Nakano, 2002a) using pIG121Hm (Ohta et al., 1990) as a template and the primer set, 5'-CCA TTT GAA GCC GAT GTC ACG CCG TAT GTT ATT GC-3' (GUS1L) and 5'-GCT GCG GTT TTT CAC CGA AGT TCA TGC CAG TCC AG-3' (cGUS1L). The HPT gene probe was also generated by PCR using pIG121Hm as a template and the primer set, 5'-CGG CGA GTA CTT CTA CAC AGC-3' (hpt862F) and 5'-GAT GTA GGA GGG CGT GGA TA-3' (hpt862R). The amplified fragments were refined with the cartridge for DNA concentration and primers elimination, SUPREC™-02 (Takara, Japan), and labeled with an alkaline phosphatase-direct labeling using the AlkPhos Direct Labeling Reagents (Amersham Pharmacia, UK). Hybridization, washing and detection were performed according to the instruction manual of the AlkPhos Direct Labeling and Detection System with CDP-*Star* (Amersham Pharmacia, UK). Hybridization signals were detected using the Hyperfilm-ECL (Amersham Pharmacia, UK).

Real-time RT-PCR analysis

Total RNA was treated with RNase-free DNase I (Takara, Japan), and 0.3 μg of the DNase-treated RNA was reverse-transcribed in a total volume of 20 μl using Reverse Transcription Reagent (Takara, Japan) according to the manufacturer-supplied protocol. Serial dilution of RNA from leaves of the transgenic plant E1-1 was used to generate a standard curve. Real-time RT-PCR was carried out using the SYBR Premix Ex Taq (Takara, Japan) in the DNA Engine Opticon System (MJ Research, USA) for continuous fluorescence detection, the first-strand cDNA, and the primer sets: the GUS gene-specific primer set, 5'-CCC TTA CGC TGA AGA GAT GC-3' (GUS178F) and 5'-CTG TAA GTG CGC TTG CTG AG-3' (GUS178R); and *ApAct1* (actin gene of *A. praecox* ssp. *orientalis*; accession number AB196261 in the GenBank/EMBL/DDBJ databases)-specific primer set, 5'-AGC TGG TCT TGG ATG TTT CG-3' (AgAct216F) and 5'-CTG TCC CGA TCT ACG AAG GA-3' (AgAct216R). Each PCR reaction was performed in triplicate under the following conditions: 10 s at 95°C, 20 s at 58°C, and 20 s at 72°C, and plate read (detection of fluorescent product) for 40 cycles. To characterize the PCR products, the melting curve analysis (Ririe et al., 1997) was carried out by slowly raising temperature from 72°C to 95°C with fluorescence data at 0.5°C intervals. Relative amount of GUS gene transcripts was calculated using the comparative cycle threshold (Ct) method and the results were normalized to *ApAct1*.

Results

Southern blot analysis

Southern blot analyses using the HPT gene probe and the GUS gene probe

were carried out on the transgenic lines obtained with *A. tumefaciens* strain LBA4404/pTOK233 (L7, L8, L9 and L10) and that with strain EHA 101/pIG121Hm (E1), respectively (Figure 5). Transgenic plants of all the five lines showed a single band at different positions, indicating that these lines were independent and each had one copy of the transgene. The same banding pattern was obtained in different plants of the same transgenic line. No hybridization signal was detected in the non-transgenic line C1 for both probes.

Ploidy level

To determine the ploidy level of transgenic plants, flow cytometry analysis of relative nuclear DNA contents was carried out (Table 6). Plants of the four transgenic lines L7, L9, L10 and E1, as well as the control, non-transgenic line C1 were diploid, indicating that no chromosome doubling occurred in these four transgenic lines. On the other hand, plants of the remaining transgenic line L8 were tetraploid.

Morphology and pollen fertility

Transgenic and non-transgenic plants were transplanted to pots in 2000 and have been cultivated for 5 years. Morphological characterization of vegetative organs (number of leaves per inflorescence, and length and width of leaves) was carried out once for each plant (Table 6). Compared with the control, non-transgenic plant C1-1, the transgenic plants generally had decreased numbers of leaves per inflorescence. In addition, leaves of the transgenic plants were smaller and narrower than those of the control.

Transgenic as well as non-transgenic plants produced flowers 3–5 years after transplantation to pots: four plants (L8-3, L9-2, L10-1 and L10-2) flowered

in 2003; 11 plants (C1-1, L7-3, L8-2, L9-1, L9-2, L9-3, L10-1, L10-2, E1-1, E1-4 and E1-5) flowered in 2004; and six plants (C1-1, L7-2, L7-3, L8-3, E1-1 and E1-4) flowered in 2005 (Table 7). All the 12 transgenic plants showed morphological variations in flower organs compared to the control, non-transgenic plant C1-1. Large differences between non-transgenic and transgenic plants were observed especially in the length of inflorescence stalks and the number of flowers per inflorescence. All the transgenic plants had shorter inflorescence stalks, and the length of inflorescence stalks of the transgenic plants of L8, L9 and L10 lines were below half of C1-1. Although the number of flowers per inflorescence greatly varied with examined year, all the transgenic plants had fewer flowers per inflorescence than C1-1. Most of the transgenic plants had smaller flowers compared with C1-1. Generally no large differences in the morphology of flower organs were observed among the different transgenic plants in the same line. Typical flower phenotypes of each transgenic line are shown in Figure 6. Tepals of the transgenic plants of L7, L8 and E1 lines strongly reflexed backward. Transgenic plants of L10 and E1 lines occasionally developed flowers with twisted tepals.

In the control, non-transgenic plant C1-1, pollen grains were creamy-yellow in color, and the pollen fertility was over 70% as assessed with acetocarmine staining in both 2004 and 2005. On the other hand, most of the transgenic plants flowered in 2003 and 2004 produced brown pollen grains, and their pollen fertility was below 5%. Although all the transgenic plants flowered in 2005 produced creamy-yellow pollen grains similar to those of C1-1, their pollen fertility was also below 5% (Figure 7).

Histochemical and fluorometric GUS assays, and real-time RT-PCR analysis

Stability of GUS reporter gene expression in transgenic plants was

examined by three different methods, histochemical localization of GUS activity, fluorometric quantification of GUS activity and quantification of GUS gene transcripts by real-time RT-PCR analysis. Figure 8 shows leaf, root, tepal, pistil and stamen segments of the control, non-transgenic plant C1-1 and the transgenic plant E1-1 subjected to GUS histochemical assay. In C1-1, no GUS activity was detected in any organs assayed. On the other hand, all the organs of E1-1 turned clear-blue following the assay, indicating that this plant stably expressed the GUS gene in these organs. GUS histochemical assay was carried out on all the 12 transgenic plants, and the results are summarized in Table 8. All the transgenic plants showed GUS activity in all the organs examined. Although most of the samples turned clear-blue following the assay as those of E1-1 (Figure 8) (strong GUS activity; represented with + in Table 8), roots, tepals and stamens of the transgenic plants of L7 and L8 lines showed only a pale-blue staining (weak GUS activity; represented with ± in Table 8). There were generally few variations in the GUS activity pattern among the different plants in the same transgenic line. For the transgenic plants subjected to the assay in 2 or 3 different years, no apparent differences in the GUS activity pattern were observed between the years.

Fluorometric quantification of GUS activity in leaves, roots, tepals, pistils and stamens was carried out on the control, non-transgenic plant C1-1 and the transgenic plants L7-3, L8-3 and E1-1 (Figure 9). For C1-1, GUS activity was scarcely detected in all the organs examined. Although apparent GUS activities were detected in all the three transgenic plants, its level markedly differed among them. Generally L7-3 and L8-3 showed low activity levels in all the organs, whereas much higher levels of the activity were detected in all the organs of E1-1. For example GUS activity level in leaves of E1-1 was 115-fold higher than that of L7-3. No large differences in the activity level were observed among different organs for each transgenic plant.

To further examine GUS gene expression, GUS gene transcripts were quantified by real-time RT-PCR analysis in the same organs of the same plants as fluorometric GUS analysis described above (Figure 10). No detectable signals were obtained in any organs of the control, non-transgenic plant C1-1. Although GUS gene transcripts could be detected in all the organs of all the three transgenic plants, L7-3, L8-3 and E1-1, marked differences in the relative amount of GUS gene transcripts were observed among different transgenic plants as well as among different organs in the same transgenic plant. The level was generally low in all the organs of L8-3 compared with those of L7-3 and E1-1. Much larger amount of GUS gene transcripts were detected in leaves of L7-3 and leaves and stamens of E1-1. Common organ-specific accumulation pattern of the GUS gene transcripts was not clearly observed in the three transgenic plants.

Discussion

Transgenic plants of *A. praecox* ssp. *orientalis*, which contained the GUS reporter gene under the control of CaMV35S promoter, had been cultivated for 5 years after transplantation to pots. Although they had shown no apparent phenotypic alterations at early stages after transplantation (data not shown), all the transgenic plants examined exhibited various somaclonal variations with different degrees at the flowering stage. Somaclonal variations observed in the present experiment included polyploidization, decreased number of leaves per inflorescence, smaller leaves, shorter inflorescence stalks, decreased number of flowers per inflorescence, smaller flowers and reduced pollen fertility. Occurrence of these variations is a serious problem in the practical application of molecular breeding to *A. praecox* ssp. *orientalis*, although low or no pollen fertility of transgenic plants may possibly be valuable for preventing the spread

of transgene(s) via pollen to the environment.

Somaclonal variation has extensively been reported for transgenic plants (Imai et al., 1993; Gonsalves et al., 1994; Lynch et al., 1995; Singh et al., 1998; Cervera et al., 2000; Sala et al., 2000) as well as for non-transgenic, tissue culture-derived plants (Larkin and Scowcroft, 1981; Karp, 1995) in different species. The variations include stunted plant growth (Lynch et al., 1995; Singh et al., 1998), stem, leaf and flower abnormalities (Gonsalves et al., 1994), reduced fertility (Lynch et al., 1995; Singh et al., 1998) and polyploidization (Imai et al., 1993; Gonsalves et al., 1994; Singh et al., 1998; Cervera et al., 2000). Similar somaclonal variations were also observed in the present experiment for transgenic plants of *A. praecox* ssp. *orientalis*. Although typical phenotypic variations such as a deeper green leaf color, thicker leaves, and larger flowers have generally been reported in polyploid plants (Väinölä, 2000), such variations were not observed in the tetraploid transgenic *A. praecox* ssp. *orientalis* line L8.

Recently various molecular analyses have been carried out to examine genomic DNA changes in transgenic plants in relation to somaclonal variation in different plant species (Arencibia et al., 1999; Sala et al., 2000; Labra et al., 2001, 2004). Labra et al. (2004) investigated genomic changes in transgenic *Arabidopsis thaliana* plants produced by *in planta Agrobacterium*-mediated transformation, in which tissue culture processes were avoided, and concluded that somaclonal variation was essentially correlated with the stress imposed by tissue culture rather than with the integration of a foreign gene. Thus, somaclonal variations observed in the transgenic plants of *A. praecox* ssp. *orientalis* may also arise in tissue culture processes: induction and subculture of embryogenic calluses used as a target material for transformation and/or selection and regeneration of transgenic tissues following co-cultivation with *Agrobacterium*. The extent of somaclonal variation in transgenic plants may be

correlated with the extent and severity of tissue culture-derived stress (Sala et al., 2000; Labra et al., 2001), and therefore, it is necessary to minimize the tissue culture-derived stress, for example use of newly induced embryogenic calluses as a target material for transformation and reducing the period of time for selecting transformants, in order to produce transgenic plants of *A. praecox* ssp. *orientalis* without somaclonal variations.

Stability of transgene expression after long-term cultivation has previously been reported for transgenic plants of *Gladiolus*, in which expression of the GUS reporter gene under the control of several different promoters was detected in shoots and roots following three seasons of dormancy (Kamo, 2003). Similarly, stable GUS gene expression could be detected in leaves, roots, tepals, pistils and stamens of all the 12 transgenic plants of *A. praecox* ssp. *orientalis* after 5 years of cultivation. Thus transgene silencing may not be a common occurrence in transgenic plants of *A. praecox* ssp. *orientalis* containing the GUS gene under the control of the CaMV35S promoter.

In the present experiment, GUS gene expression in transgenic plants of *A. praecox* ssp. *orientalis* was examined by three different methods, histochemical localization and fluorometric quantification of GUS activity, and quantification of GUS gene transcripts by real-time RT-PCR analysis. The results obtained from the histochemical GUS assay were generally consistent with those from the fluorometric GUS assay. On the other hand, the GUS activity level measured by fluorometric assay did not necessary correlate to the relative amount of GUS gene transcripts determined by real-time RT-PCR analysis. For example, leaves of the transgenic plant L7-3 showed a much larger amount of GUS gene transcripts (Figure 10) but only a low GUS activity level (Figure 9). This may be resulted from a low rate of translation and/or inhibition of GUS enzyme activity. Low or no GUS enzyme activities despite the presence of large amounts of GUS gene transcripts have already been reported for transgenic plants of several

species (Schaart et al., 2002; Nagao and Obokata, 2003). Further analyses are necessary to clarify the disparity between the GUS gene transcription level and the GUS activity level in transgenic plants of *A. praecox* ssp. *orientalis*.

In conclusion, transgenic plants of *A. praecox* ssp. *orientalis* showed stable expression of the transgene (GUS reporter gene) even after 5 years of cultivation in pots, partly demonstrating the validity of genetic transformation for the improvement of *A. praecox* ssp. *orientalis*. However, for practical application of molecular breeding to *A. praecox* ssp. *orientalis*, some improvements in the transformation system are necessary to avoid the occurrence of somaclonal variation in transgenic plants.

Experiment 5

Long-term stability of transgene expression in transgenic plants of *Tricyrtis hirta*

Introduction

In Chapter 2, Experiment 3, an efficient *Agrobacterium*-mediated transformation system was established in *Tricyrtis hirta*, and a number of independent transgenic plants containing the GUS reporter gene were produced. Some of these transgenic plants had subsequently been cultivated in pots. In the present experiment, for evaluating the validity of molecular breeding in *T. hirta*, the stability of transgene (GUS gene) expression in the transgenic plants was examined after 1–2 years of cultivation by assaying GUS activity in various organs.

Materials and methods

Plant materials

One control, non-transgenic plant (C1) and four independent transgenic plants (T1, T2, T3 and T4) of *T. hirta* were used in the present experiment. These transgenic plants were produced in Chapter 2, Experiment 3, and had the intron-containing GUS gene under the control of CaMV35S promoter. The non-transgenic plant C1 was a regenerant from embryogenic calluses of *T. hirta*, which were used as a target material for transformation in Chapter 2, Experiment 3. Transgenic and non-transgenic plants in pots were cultivated for

2 years in a growth chamber.

Histochemical and fluorometric GUS assays

Histochemical GUS assay and fluorometric GUS assay were performed according to Chapter 2, Experiment 3 and Chapter 2, Experiment 4, respectively. Leaves, stems, roots, tepals, pistils and stamens of transgenic and non-transgenic plants were subjected to the assays.

Isolation of plant DNA and inverse PCR analysis

Total genomic DNA was isolated from leaves as described in Chapter 2, Experiment 3. Inverse PCR analysis was carried out according to Hoshi et al. (2004). The genomic DNA was digested with *Eco*RI, which cleaves at a site within the HPT gene, and ligated to generate circular molecules (Figure 11A). The first PCR was performed using the generated circular molecules as a template, the primer set, 5'-GGC CGT CTG GAC CGA TGG CTG TGT AGA AGT ACT CG-3' (P1) and 5'-TGC AGA ACA GCG GGC AGT TCG- GTT TCA GGC AGG TC-3' (P2), and the TaKaRa LA Kit (Takara, Japan). To amplify the fragment of the junctions of inserted DNA and plant genomic DNA (Figure 11A), 30 cycles were performed using a programmed temperature control system (2400-R, Perkin-Elmer, USA) under the following conditions: 10 s at 98°C and 10 min at 68°C. The second PCR was performed under the same conditions as the first PCR except for the template and primer set. In order to increase the specificity, the second PCR performed using the first PCR product as a template and the primer set, 5'-GAC GCC CCA GCA CTC GTC CGA GGG CAA AGG AAT AG-3' (P3) and 5'-ACA CCC TGT GCA CGG CGG GAG ATG CAA- TAG GTC AG-3' (P4) (Figure 11A). Amplified products

were analyzed by electrophoresis in a 1.2% (w/v) agarose gel.

Results

Cultivation of transgenic plants

One control, non-transgenic plant (C1) and nine independent transgenic plants were transplanted to pots in February 2003 and have been cultivated for 2 years in a growth changer. Among the nine transgenic plants, three (T1, T2 and T3) and four (T1, T2, T3 and T4) produced flowers in November 2003 and October 2004, respectively. C1 produced flowers in both years. The five plants that produced flowers (C1, T1, T2, T3 and T4) were subjected to histochemical and fluorometric GUS assays. Although both non-transgenic and transgenic plants were still small and produced only a few flowers after 2 years of cultivation, no apparent morphological variations were observed in these plants.

Inverse PCR analysis

To determine the transgene copy number in the transgenic plants, inverse PCR analysis, which amplified the fragments of the junctions of inserted DNA and plant genomic DNA, using the HPT gene-specific primers according to Hoshi et al (2004). As shown in Figure 11A, the distance between the LB and the *Eco*RI site of plant genomic DNA depends on the position where the transgene was inserted into the plant genome. Therefore, bands at different positions and of different numbers are expected to reflect the integration of the transgene(s) into different positions and to provide an estimate of the copy number in the genome of transformants. All the four transgenic lines showed a single band at different positions, indicating that these lines were independent

and each had one copy of the transgene (Figure 11B). The control, non-transgenic plant C1 yielded no amplified fragments.

Histochemical and fluorometric GUS assays

In order to examine the stability of transgene expression in transgenic *T. hirta* at the flowering stage, GUS activity in leaves, stems, roots, tepals, pistils and stamens was assayed histochemically and fluorometrically. Figure 12 shows each organ of the control, non-transgenic plant C1 and the transgenic plant T1 subjected to GUS histochemical assay. No GUS activity was detected in any organs of C1. On the other hand, all the organs of T1 turned clear-blue following the assay, indicating that this plant expressed the GUS gene stably in these organs. All the four transgenic plants were subjected to GUS histochemical assay, and the results are summarized in Table 9. All the transgenic plants showed GUS activity in all the organs examined. Although most of the samples turned clear-blue following the assay as those of T1 (Figure 12) (strong GUS activity; represented with + in Table 9), only a pale-blue staining was observed in stamens of the transgenic plants T1, T2 and T3 assayed in 2004, and pistils and stamens of the transgenic plant T4 assayed in 2005 (weak GUS activity; represented with ± in Table 9). For most organs, GUS activity was more strong in 2005 than in 2004.

Figure 13 shows the results of fluorometric GUS assay in leaves, stems, roots, tepals, pistils and stamens of the control, non-transgenic plant C1 and the transgenic plants T1, T2 and T3. For C1, GUS activity was scarcely detected in all the organs examined. On the other hand, apparent GUS activities were detected in all the three transgenic plants. However, marked differences in the GUS activity level were observed among different organs in each transgenic plant. Generally, stems, roots and tepals showed higher GUS activity levels

compared with the other organs in all the transgenic plants. No apparent differences in the GUS activity level were observed among different transgenic plants.

Discussion

Some of the transgenic plants of *T. hirta*, which was produced in Chapter 2, Experiment 3, and contained the GUS gene under the control of CaMV35S promoter, had subsequently been cultivated in pots. As described in Chapter 2, Experiment 4 for *Agapanthus praecox* ssp. *orientalis*, long-term stability of transgene expression is one of the indispensable requisites for applying molecular breeding to perennial and vegetatively propagated ornamentals like *T. hirta*. In the present experiment, all the four transgenic plants of *T. hirta* showed stable expression of the GUS gene in leaves, stems, roots, tepals, pistils and stamens following 2 years of cultivation. Although frequent occurrence of transgene silencing has been reported for various monocot species other than Liliaceous ornamentals (Iyer et al., 2000), transgene silencing may not be a common occurrence in transgenic *T. hirta* containing the GUS gene under the control of the CaMV35S promoter. Generally, transgene copy number has been demonstrated to influence greatly the transgene expression level and genetic stability (Yang et al. 2005). There is a tendency that single copies of a transgene are more stably expressed than multiple ones in various species (Bavage et al., 2002; Cervera et al., 2000). In the present experiment, all the four transgenic plants of *T. hirta* had one copy of the transgene, which may be one of the causes of stable expression of the transgene following long-term cultivation.

In Chapter 2, Experiment 4, stably expression of the GUS gene was also confirmed in transgenic plants of *Agapanthus praecox* spp. *orientalis* after 5 years of cultivation. Although marked differences in the GUS activity level were

observed among different transgenic lines of *A. praecox* spp. *orientalis* as detected by fluorometric GUS assay, there were no such differences in transgenic *T. hirta*. In addition, the GUS activity level in transgenic *T. hirta* plants was generally higher than that of transgenic plants of *A. praecox* spp. *orientalis*. Differences in GUS gene expression between these two species might be due to differences in the genetic and/or physiological backgrounds.

In conclusion, transgenic plants of *T. hirta* showed stable expression of the transgene (GUS reporter gene) after 2 years of cultivation in pots. This is essential for demonstrating the validity of genetic transformation for the improvement of *T. hirta*. Further experiments should be directed to detailed morphological characterization of the transgenic plants *T. hirta* when they grow into large plants (2–3 years later).

Chapter 3

Analyses of anthocyanidins and anthocyanins in flower organs, and isolation and characterization of genes for the flavonoid biosynthetic pathway

Experiment 1

Analyses of anthocyanidins and anthocyanins in flower organs of *Muscari* spp., *Agapanthus* spp. and *Tricyrtis* spp.

Introduction

The present study aims to apply molecular breeding for flower color modification to Liliaceous ornamentals. To achieve the goal, accumulation of biochemical information on flower pigments such as flavonoids is one of the indispensable requisites. However, there have been few reports on the analyses of flavonoids including anthocyanidins and anthocyanins in the Liliaceous ornamentals.

For Liliaceous ornamentals, genetic transformation systems have so far been established in *Agapanthus praecox* ssp. *orientalis* (Suzuki et al., 2001), *Muscari armeniacum* (Suzuki and Nakano, 2002a) and *Lilium* sp. (Hoshi et al., 2004). In addition, an *Agrobacterium*-mediated transformation system of *Tricyrtis hirta* was newly developed in the present study (Chapter 2, Experiment 3). Among these species, relatively high transformation efficiencies have been obtained in *A. praecox* ssp. *orientalis, M. armeniacum* and *T. hirta.* Therefore, in the present experiment, anthocyanidins and/or anthocyanins in flower organs of *A. praecox* ssp. *orientalis, M. armeniacum* and *T. hirta* and their related species were analyzed.

Materials and methods

Muscari *spp.*

Thirteen genotypes of *Muscari* spp., *M. armeniacum*, *M. armeniacum* 'Blue Pearl', *M. armeniacum* 'Blue Spike', *M. armeniacum* 'Fantasy Creation', *M. armeniacum* 'Valerie Finnis', *M. paradoxum*, *M. tubergenianum*, *M. latifolium*, *M. azureum*, *M. comosum* var. *plumosum*, *M. moschatum*, *M. moschatum* var. *flavum* and *M. botryoides* 'Album' were used (Figure 14). Plants of all genotypes except for *M. moschatum* var. *flavum* were cultivated in the field. Plants of *M. moschatum* var. *flavum* were cultivated in the flower garden of the Botanic Gardens of Toyama, Toyama, Japan. Inflorescences were harvested at anthesis, and fully opened flowers were collected. Tepals were isolated from the flowers and stored at -80°C until use. For *M. latifolium*, in which the color of flowers in the upper part of inflorescences is blue while that of flowers in the middle and lower parts is deep blue, tepals isolated from flowers of different colors were separately collected.

Agapanthus *spp.*

Four genotypes of *Agapanthus* spp., *A. praecox* ssp. *orientalis* 'Royal Purple Select', *Agapanthus* sp. 'Gayle's Lilac', 'Snow Storm' and 'White Dwarf' were used (Figure 15). Plants of all genotypes were cultivated in the field. Flower buds were harvested from field-grown plants just before anthesis. Tepals and stamens were isolated, collected and stored at -80°C until use.

Tricyrtis *spp.*

T. formosana and *T. hirta* were used (Figure 16). Plants of these genotypes were cultivated in the field. Flowers were harvested from field-grown plants at anthesis. Tepals were collected, dried overnight at 37°C and kept at room temperature until use. In some cases, tissues of dark reddish-purple spots were

isolated from tepals of *T. hirta*, and were dried and kept as described above.

Extraction and HPLC analyses

Analyses of anthocyanidins and anthocyanins were performed according to Asano et al. (2002). For extraction, ca. 0.3 g FW of tissues were incubated for 1 h in 0.3 ml of an acidic methanolic solution (0.1% HCl in methanol) at room temperature. A half of the crude extract obtained after centrifugation (3,000 cycles min^{-1} for 10 min) was used directly for anthocyanin analysis, and the rest was used for anthocyanidin analysis following full hydrolyzation with 2N-HCl for 30 min at 100°C for liberating anthocyanidins. The crude extracts and their hydrolysates were subjected to HPLC (L-7100 pump with L-7420 spectrophotometric detector; Hitachi, Japan) with linked analytical ODS column (Wakosil-II 5C18 AR, 4.6 mm i.d. × 250 mm; Wako, Japan), by eluting with 7% of solvent B (50% CH_3CN, 40% CH_3COOH and 0.5% CF_3COOH in H_2O; v/v) for the first 10 min, followed by elution with a linear gradient from 7 to 35% of solvent B in solvent A (0.5% CF_3COOH in H_2O; v/v) for 45 min, at a flow rate of 1.0 ml min^{-1} at 40°C. The elution pattern was monitored at 530 nm. For identification of each anthocyanidin, cyanidin, pelargonidin, delphinidin, petunidin and malvidin extracted from berry pericarps of *Vitis* × *labruscana* 'Kyoho', and peonidin from berries of *Fragaria* × *ananassa* 'Toyonoka', were used as standards.

Result and discussion

Analyses of anthocyanidins and anthocyanins in tepals of Muscari *spp.*

Data on the relative anthocyanidin composition in tepals of *Muscari* spp.

by HPLC analysis are summarized in Table 10. Some anthocyanidins, cyanidin, peonidin, delphinidin, petunidin, and/or malvidin were detected in 12 genotypes with colored flowers. In *M. moschatum* var. *flavum*, only two anthocyanidins, cyanidin and delphinidin were barely detected, and the amount of the former was slightly larger than the latter. No anthocyanidins were found in *M. botryoides* 'Album'. The main anthocyanidin in blue flowers (*M. armeniacum*, *M. armeniacum* 'Blue Pearl', *M. armeniacum* 'Blue Spike', *M. armeniacum* 'Fantasy Creation', *M. paradoxum*, *M. tubergenianum* and the upper part of inflorescences of *M. latifolium*) and pale blue flowers (*M. armeniacum* 'Valerie Finnis' and *M. azureum*) was delphinidin. On the other hand, two main anthocyanidins, cyanidin and delphinidin, were detected in reddish purple (*M. comosum* var. *plumosum*) and pale purple (*M. moschatum*) flowers. Generally, blue and violet flowers mainly contain 3',5'-hydroxylated anthocyanidin (delphinidin, petunidin and malvidin) derivatives, whereas red and pink flowers mainly contain derivatives of cyanidin or pelargonidin (Forkmann, 1991). A similar relationship between flower color and anthocyanidin composition was observed in *Muscari* spp.

Pelargonidin was never detected in flowers of all the *Muscari* genotypes analyzed. Similar observations have already been demonstrated for *Petunia hybrida* (Forkmann and Ruhnau, 1987) and *Cymbidium hybrida* (Johnson et al. 1999). In these species, flowers rarely produce pelargonidin, because DFR of this species does not catalyze the reduction of dihydrokaempferol to leucopelargonidin due to the substrate specificity (Forkmann and Ruhnau, 1987; Johnson et al. 1999). Therefore, DFR of *Muscari* spp. might be similar in the substrate specificity to *Petunia hybrida* and *Cymbidium hybrida*.

Tepal crude extracts from blue, deep blue, pale blue, reddish purple and pale purple flowers were subjected to anthocyanin analysis by HPLC, and totally 13 peaks were detected (Table 11). The peak No.9 was detected as a

main peak in all the flowers except for those of *M. azureum* and those in the middle and lower parts of inflorescences of *M. latifolium*. Therefore, this peak was presumed to be a delphinidin derivative. Furthermore, since the retention time of the peak No.9 was much later than that of delphinidin, this peak may be a delphinidin-(acyl)-glycoside. Further studies are necessary to identify each anthocyanin in flowers of *Muscari* spp.

Analysis of anthocyanidins in tepals and stamens of Agapanthus *spp.*

Anthocyanidin analysis was performed by HPLC using tepals and stamens of four genotypes of *Agapanthus* spp. (Figure 15). Some anthocyanidins, cyanidin, delphinidin, and/or petunidin were detected in tepals and stamens of three genotypes. In tepals of *A. praecox* ssp. *orientalis* 'Royal Purple Select', which produces blue flowers, and *Agapanthus* sp. 'Gayle's Lilac', which produces pale blue flowers, the main anthocyanidin was delphinidin followed by cyanidin and petunidin. Tepals of *Agapanthus* sp. 'White Dwarf', which are white with a small reddish part at the tip, mainly contained cyanidin. No anthocyanidins were detected in tepals of *Agapanthus* sp. 'Snow Storm', which produces pure white flowers. The main anthocyanidin in stamens with a blue anther of 'Royal Purple Select' was delphinidin followed by cyanidin and petunidin. Stamens with a dark brown anther of 'Gayle's Lilac' contained both cyanidin and delphinidin, and those with a reddish purple anther of 'White Dwarf' mainly contained cyanidin. No anthocyanidins were detected in stamens with a yellow anther of 'Snow Storm'. Thus a general relationship between flower color and anthocyanidin composition (Forkmann, 1991), described above for *Muscari* spp., was also observed in *Agapanthus* spp. As in the case of *Muscari* spp., pelargonidin was never detected in both tepals and stamens of all the *Agapanthus* genotypes examined. Therefore, DFR of *Agapanthus* spp. might

also have similar substrate specificity to that in *Petunia hybrida* and *Cymbidium hybrida.*

Analysis of anthocyanins in tepals of Tricyrtis *spp.*

Recently, anthocyanin analysis was carried out in flowers of *T. formosana* 'Fujimusume', which has mauve tepals with dark red spots, and two novel anthocyanins, 8-*C*-glucosylcyanidin 3-*O*-malonylglucoside (Saito et al., 2003b) and 8-*C*-sinapylglucosylcyanidin 3-*O*-malonylglucoside (Tatsuzawa et al., 2004) were isolated. *C*-glycosylated anthocyanins have not yet been isolated from plants other than *T. formosana.* According to Tatsuzawa et al. (2004), six peaks of anthocyanins including one unidentified anthocyanin were detected by HPLC in tepals of *T. formosana* 'Fujimusume'. Among these peaks, three peaks, all of which are common cyanidin derivatives, were detected as main pigments in tissues of dark red spots of tepals, whereas other two peaks, both of which are *C*-glycosylated anthocyanins described above, were main anthocyanins in mauve tepal tissues other then the dark red spot tissues. According to these results, Tatsuzawa et al. (2004) concluded that mauve tepal color might be resulted from the accumulation of *C*-glycosylated anthocyanins.

Differently from *T. formosana*, *T. hirta*, which is a related species of *T. formosana* and was used as a target plant for molecular breeding in the present study, has light reddish-purple tepals with dark reddish-purple spots. The relationship between flower color and pigment composition has not yet been clarified in *T. hirta*, and it has not yet been known whether tepals of *T. hirta* contain *C*-glycosylated anthocyanins or not. Therefore, in the present experiment, anthocyanin analysis was performed in tepals of *T. hirta* as well as *T. formosana.* In consequence, six peaks of anthocyanins were detected by HPLC in tepals of *T. hirta*, all of which had the same retention time as those

detected in tepals of *T. formosana* (Figure 17). Two *C*-glycosylated anthocyanins were also detected in tepals of *T. hirta*. In addition, two common cyanidin derivatives, cyanidin 3-rutinoside (peak No. 3) and cyanidin 3-*O*-malonylglucoside (peak No. 4), were detected as main anthocyanins in spot tissues of tepals, as in the case of *T. formosana*. Since *T. hirta* and *T. formosana* showed similar anthocyanin compositions but different flower colors, further experiments are needed to clarify the mechanism of flower coloration in these species.

In the present experiment, analyses of anthocyanidins and/or anthocyanins in flower organs of *Muscari* spp., *Agapanthus* spp. and *Tricyrtis* spp. were carried out. Data on the anthocyanidin and/or anthocyanin composition may be valuable for systematic breeding for flower color modification of these species by molecular as well as traditional methods.

Experiment 2

Isolation and characterization of the F3'5'H gene from *Vinca major*

Introduction

F3'5'H, a member of the cytochrome P450 family, is a key enzyme in the synthesis of the blue flower-specific anthocyanidins, 3',5'-hydroxylated anthocyanidins (delphinidin, petunidin and malvidin) (Figure 1). The F3'5'H genes have so far been cloned only from dicot species, such as *Petunia hybrida* (Holton et al., 1993a; Toguri et al., 1993; Shimada et al., 1999), *Catharanthus roseus* (Kaltenbach et al., 1999) and *Torenia hybrida* (Suzuki et al., 2000). In the present experiment, a genomic clone for the F3'5'H gene was isolated from *Vinca major* by a PCR-based strategy. *Vinca major*, a dicot species belonging to the family Apocynaceae, shows vigorous growth and produces attractive blue flowers. Activity of the F3'5'H gene isolated from *Vinca major* was examined by transformation of a heterologous plant species, *Petunia hybrida*, which has advantages of much existing information on the flavonoid biosynthetic pathway, high transformation efficiency, and a short period to flowering (Horsch et al., 1985; Mol et al., 1998).

Materials and methods

Plant materials

Potted plants of *Vinca major* were grown in the greenhouse. Plantlets of *Petunia hybrida* 'Polo Red Target' were cultured on half-strength MS medium lacking PGRs but containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum, and used for

Agrobacterium-mediated transformation.

Isolation of plant DNA and RNA

Total genomic DNA was isolated from leaves of *Vinca major* and *Petunia hybrida* according to Chapter 2, Experiment 3. Total RNA was isolated from petals and young leaves of *Vinca major* according to Chapter 2, Experiment 4.

Isolation of a F3'5'H cDNA clone from petals and a genomic F3'5'H clone from leaves

To amplify a DNA fragment specific to the F3'5'H gene of *Vinca major*, 3'-RACE was carried out using the Takara RNA LA PCR Kit (Takara, Japan), total RNA as a template isolated from petals of the stage 3 flowers (see Results and discussion), and a degenerate primer, 5'-GC(T/C/A/G) TA(T/C) AA(T/C) GC(T/C/A/G) CA(A/G) GA(T/C) ATG GT-3', which was designed from the conservative region of several published F3'5'H genes. The PCR products were subcloned into the pCR2.1 vector (Invitrogen, USA) using the TA cloning kit (Invitrogen, USA) and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using an automatic sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, USA). The nucleotide sequence was analyzed with the computer program DNASIS (Hitachi Software Engineering, Japan), and a cDNA clone with the highest homology to the published sequences was used to decide on primers and restriction enzymes for inverse PCR.

Inverse PCR (Ochman et al., 1988; Triglia et al., 1988) using total genomic DNA from leaves of *Vinca major* as a template was successively carried out three times. A complete genomic clone of the F3'5'H gene containing presumed promoter and terminator regions was isolated by PCR using the Takara LA PCR

Kit (Takara, Japan) and the primer set, 5'-ATT CAA GCT TGA CCT GCG GTT GGC ATA TAG CGT AG-3' (VFH4L) and 5'-TTG CTT ATT CTC CAC GAT TTC TCC GAC CAT CTC TG-3' (cVFH4L). The genomic clone finally obtained was subcloned into the pCR2.1 vector (Invitrogen, USA) using the TA cloning kit (Invitrogen, USA). DNA sequencing and nucleotide sequence analysis were performed as described above.

Southern hybridization

Southern hybridization was performed according to Chapter 2, Experiment 4. Genomic DNA (50 μg) of *Vinca major* was digested with either *Hin*dIII, *Xba*I, *Sca*I or *Sac*I. The genomic region of the *Vinca major* F3'5'H gene has no restriction sites recognized by these enzymes. The 0.82-kbp DNA fragment used as a probe was generated by PCR (Suzuki and Nakano, 2002a) using pNAVFH (described below) as a template and the primer set, 5'-TAT GGC TAT AGT TGA CTT CC-3' (VFH8) and 5'-ACA TAA TCA AGA AGG TCT GG-3' (cVFH8) (Figure 18A). The amplified fragment was purified with a cartridge for DNA concentration and primer elimination, SUPREC-02 (Takara, Japan), before using as a probe.

RT-PCR analysis for expression of the F3'5'H gene

RT-PCR was carried out using the Takara RNA LA PCR Kit (Takara, Japan), total RNA extracted from petals or leaves as a template, and the primer set, VFH8 and cVFH8 (Figure 18A). RT-PCR was performed according to the manufacturer's instructions. After one min heating at 94°C, 25 cycles were performed using a programmed temperature control system (2400-R; Perkin-Elmer, USA) under the following conditions: 20 s at 94°C, 45 s at 50°C,

and 30 s at 72°C. Amplified products were analyzed by electrophoresis in a 1.2% (w/v) agarose gel.

Transformation of Petunia hybrida

A DNA fragment was excised with *Hin*dIII and *Xba*I from the subcloning vector containing the complete genomic clone for the *Vinca major* F3'5'H gene. The fragment was inserted between the *Hin*dIII and *Xba*I sites of the plant expression vector pIG121Hm (Ohta et al., 1990) in exchange for the intron-containing GUS gene, yielding pNAVFH (Figure 18A). The DNA fragment containing the CaMV35S promoter was excised from pBI221 (Jefferson et al., 1987) with *Bam*HI and the 3' and 5' terminal regions blunted using the DNA Blunting Kit (Takara, Japan), followed by treatment with *Hin*dIII. The resulting DNA fragment was inserted between the *Hin*dIII and blunted *Spe*I sites of pNAVFH, in exchange for the promoter region of the *Vinca major* F3'5'H gene, yielding pNA-VFH-35S (Figure 18B). The T-DNA region of each binary vector contains the following genes: pNAVFH, the NPTII gene under the control of the NOS promoter, the F3'5'H gene of *Vinca major* (*VmFH1*) under the control of the original promoter of *VmFH1* (VFH-*VmFH1*), and the HPT gene under the control of the CaMV35S promoter; pNA-VFH-35S, the NPTII gene under the control of the NOS promoter, *VmFH1* under the control of the CaMV35S promoter (35S-*VmFH1*), and the HPT gene under the control of the CaMV35S promoter. pNAVFH and pNA-VFH-35S were each transferred to *Agrobacterium tumefaciens* strain EHA101, resulting in strains EHA101/pNAVFH and EHA101/pNA-VFH-35S, respectively.

Petunia hybrida 'Polo Red Target' was transformed with *A. tumefaciens* strains EHA101/pNAVFH or EHA101/pNA-VFH-35S according to Horsch et al. (1985). Kanamycin-resistant plantlets were acclimatized, transplanted to pots

and grown in a growth chamber at 25°C under a 16/8-h photoperiod with fluorescent light (50 μmol m^{-2} s^{-1}). Integration of the transgene was confirmed by PCR (Suzuki and Nakano, 2002a) using genomic DNA isolated from leaves of putative transgenic plants according to Chapter 2, Experiment 3 as a template, and the primer set, 5'-AAA TAT GGT CCG GTC ATG TAT CTC AAG GTT GGT AC-3' (VFH2L) and 5'-ACA TTC GAC CAG TCT TCG AGT GCT TTA CCA CCA AG-3' (cVFH1L) (Figure. 18A).

HPLC analysis of anthocyanidins

Anthocyanin extraction from petals, hydrolyzation of the crude extracts, and anthocyanidin analysis by HPLC were performed according to Chapter 3, Experiment 1. HPLC analysis was carried out using at least three flowers for each transgenic plant.

Results and discussion

Isolation of a genomic clone for the F3'5'H gene

A genomic clone for the F3'5'H gene of *Vinca major* was isolated by a PCR-based strategy and designated *VmFH1* (accession number AB078781 in the GenBank/EMBL/DDBJ databases). *VmFH1* contained an ORF of 1,518 bp, consisting of two exons (885 and 633 bp) encoding a polypeptide of 506 amino acid residues (Figure 19). The deduced amino acid sequence of *VmFH1* was compared with that of the previously reported F3'5'H genes (Figure 19). F3'5'H was a member of the cytochrome P450 proteins, which have several conserved regions in their amino acid sequences (Chapple, 1998). The most highly conserved region is the heme-binding domain centered around a cysteine (C)

residue that binds heme in the active site. The heme-binding domain of cytochrome P450 is generally represented by the highly conserved sequence FxxGxxxCxG, and this fingerprint motif was found in the deduced amino acid sequence of *VmFH1* (Figure 19). The characteristic proline-rich region (PPxP), which forms a hinge between the membrane-anchored N-terminal helix and other parts of the protein, as well as the (A/G)Gx(D/E)T(T/S) consensus, which is involved in oxygen activation and the transfer of protons to the active site, could also be found (Figure 19). The percentage identities of the deduced amino acid sequence of *VmFH1* to the F3'5'H genes of *Catharanthus roseus*, *Petunia hybrida Hf2*, *Nierembergia* sp., *Eustoma russellianum*, *Eustoma grandiflorum*, *Petunia hybrida Hf1*, *Lycianthes rantonnei*, *Solanum melongena*, *Gentiana triflora*, *Torenia hybrida*, *Campanula medium* and *Callistephus chinensis* were 83, 78, 77, 76, 76, 76, 75, 74, 74, 71, 67 and 51%, respectively. Figure 20 shows a phylogenetic tree based on F3'5'H amino acid sequences. The identity between F3'5'H of *Vinca major* and *Catharanthus roseus*, both belonging to the family Apocynaceae, was highest. The identity of the deduced amino acid sequence of *VmFH1* to other plant cytochrome P450 proteins listed in the database was less than 40%. Like the previously reported F3'5'H genes, *VmFH1* belongs to *CYP75*, a gene family encoding F3'5'H, in the cytochrome P450 gene superfamily.

To determine the complexity of the F3'5'H gene family in the genome of *Vinca major*, Southern hybridization was carried out using a 0.82-kbp DNA fragment from *VmFH1* as a probe (Figure 21). Three or four bands were observed for each restriction enzyme, indicating the presence of a small gene family (three to four copies) of F3'5'H in the genome of *Vinca major*. Similar observations have been demonstrated for *Petunia hybrida*, which has a small gene family composed of two different F3'5'H genes, *Hf1* and *Hf2* (Holton et al., 1993a).

Anthocyanidin accumulation and VmFH1 *expression in* Vinca major

Developmental stages of the flower of *Vinca major* are as follows (Figure 22A): stage 1, flower bud with non-pigmented (white) petals; stage 2, flower bud with pale blue petals; stage 3, flower bud with blue petals; stage 4, opening flower; and stage 5, fully opened flower. HPLC analysis indicated that young leaves and petals of the stage 1 flower did not contain detectable amounts of anthocyanidins. On the other hand, delphinidin was the only anthocyanidin detected in petals of the stage 2–5 flowers, with those of the stage 4 flowers accumulating the largest amount of delphinidin.

RT-PCR analysis using specific primers was carried out to analyze expression of the F3'5'H gene corresponding to *VmFH1* in petals in each developmental stage of the flower and in young leaves (Figure 22B). Strong signals were observed in petals of the stage 2–4 flowers, but petals of the stage 1 and 5 flowers showed only weak signals. No signal was detected in young leaves. These results indicate that both anthocyanidin accumulation and F3'5'H expression in petals of *Vinca major* are developmentally regulated, and that anthocyanin accumulation may be under transcriptional control. A similar expression pattern of the F3'5'H gene has been reported in petals of *Eustoma grandiflorum* (Nielsen and Podivinsky, 1997) and *Torenia hybrida* (Ueyama et al., 2002). Moreover, such an expression pattern is similar to that of the majority of genes involved in the flavonoid biosynthetic pathway.

Heterologous expression of VmFH1 *in transgenic* Petunia hybrida

In general, red and pink flowers mainly contain derivatives of cyanidin and pelargonidin (Forkmann, 1991). Pink-flowering genotypes of *Petunia hybrida*

have shown to have F3'H but generally lack F3'5'H (Wiering and Vlaming, 1984; Shimada et al., 1999; Shimada, 2000) (Figure 1). On the other hand, the DFR of *Petunia hybrida* has been demonstrated not to catalyze the reduction of dihydrokaempferol to leucopelargonidin (Forkmann and Ruhnau, 1987). Therefore, *Petunia hybrida* 'Polo Red Target', a red-flowering cultivar used in the present experiment (Figure 23A), was expected to accumulate mainly 3-hydroxylated anthocyanidins, cyanidin and peonidin, in their flower petals as in the case of pink-flowering genotypes of this species (Wiering and Vlaming, 1984; Shimada et al., 1999; Shimada, 2000). Indeed, only these two anthocyanidins could be detected by HPLC analysis in petals of 'Polo Red Target' (Figure 23B). Thus, ectopic expression of the F3'5'H gene in petals of this cultivar might lead to accumulation of 3',5'-hydroxylated anthocyanidins.

In order to examine the effect of chimeric F3'5'H genes of *Vinca major* on flower color, as well as anthocyanidin composition, in a heterologous plant species, *Petunia hybrida* 'Polo Red Target' was transformed with *VmFH1* using each of *A. tumefaciens* strains EHA101/pNAVFH (Figure 18A) and EHA101/pNA-VFH-35S (Figure 18B). More than 30 independent transgenic plants, as confirmed by PCR analysis, were obtained with each *A. tumefaciens* strains. There was a large variation in flower color phenotype among transgenic lines, and most of them showed minor or no flower color alteration. Five (VmFH-4, 8, 9, 12, and 15) and two (35SVmFH-1 and 5) transgenic lines obtained with EHA101/pNAVFH and EHA101/pNA-VFH-35S, respectively, showed apparent flower color alterations. Therefore, these seven lines were used for further experiments. All the seven lines exhibited normal phenotype except with respect to flower color. There are a few variations in flower color among selected transgenic lines derived from the same *Agrobacterium* strain, but little variation was observed among different flowers in a single transgenic line. Figure 23A shows typical flower color phenotypes of selected transgenic plants.

Transgenic plants with *A. tumefaciens* strain EHA101/pNAVFH generally had slightly light red flowers with a blue-purple rim. More drastic flower color alteration was observed in transgenic plants with *A. tumefaciens* strain EHA101/pNA-VFH-35S, which generally had deep red flowers with deep purple sectors. Figure 23B shows typical HPLC profiles for the accumulation of anthocyanidins in petals of non-transgenic and transgenic plants. All the analyzed transgenic plants accumulated 3',5'-hydroxylated anthocyanidins (delphinidin, petunidin and malvidin), which were never detected in the control, non-transgenic, plants. The mean percentages of 3',5'-hydroxylated anthocyanidins in the total anthocyanidins in petals of VmFH-4, 8, 9, 12 and 15 were 8, 37, 4, 14 and 7%, respectively, whereas the mean percentages in 35SvFH-1 and 5 were 66 and 61%, respectively. These results indicate that the F3'5'H gene of *Vinca major*, *VmFH1*, encodes F3'5'H, and that it is active in a heterologous plant species. They also indicate that the *VmFH1* promoter may be less active than the CaMV35S promoter in petals.

Shimada et al. (1999) reported that flower color of *Nicotiana tabacum* altered from pink to magenta, and 3',5'-hydroxylated anthocyanidins accumulated in petals after transformation with the F3'5'H gene of *Petunia hybrida* or *Eustoma russellianum*. In their study, 35 and 23% of total petal anthocyanidins were 3',5'-hydroxylated anthocyanidins in transgenic plants expressing the F3'5'H genes of *Petunia hybrida* and *Eustoma russellianum*, respectively, under the control of the CaMV35S promoter. On the other hand, over 60% were 3',5'-hydroxylated anthocyanidins in transgenic *Petunia hybrida* plants expressing *VmFH1* under the same promoter in the present experiment. This difference may result from differences in the intrinsic properties of the F3'5'H gene from each species and/or from the physiological condition of the host plant tissues.

In the present experiment, the F3'5'H gene was isolated from *Vinca major*

as the first step toward the production of blue-flowering cultivars in Liliaceous ornamentals, for which blue flower color is still lacking. The usefulness of this gene for flower color modification in a heterologous plant species was also confirmed. This gene may be useful for producing blue flower color by genetic transformation in Liliaceous ornamentals.

Experiment 3

Isolation and characterization of the DFR gene from *Agapanthus praecox* ssp. *orientalis*

Introduction

DFR, a pivotal enzyme of the flavonoid biosynthetic pathway, catalyzes the reduction of the dihydroflavonols, DHK, DHQ and DHM, to the leucoanthocyanidins, leucocyanidin, leucopelargonidin and leucodelphinidin, respectively (Figure 1). The DFR genes have already been isolated from some monocot as well as dicot species including *Zea mays* (O'Reilly et al., 1985; Schwarz-Sommer et al., 1987), *Antirrhinum majus* (Martin et al., 1985), *Petunia hybrida* (Beld et al., 1989; Huit et al., 1994), *Gerbera hybrida* (Helariutta et al., 1993), *Rosa hybrida* (Tanaka et al., 1995), *Torenia fournieri* (Aida et al., 2000a) and *Vaccinium macrocarpon* (Polashock et al., 2002). In the present experiment, a genomic clone for the DFR gene was isolated by a PCR-based strategy from *Agapanthus praecox* ssp. *orientalis*, a cultivar producing blue flowers. Spatial and temporal expression patterns of the isolated DFR gene were subsequently examined during flower development of *A. praecox* ssp. *orientalis*.

Materials and methods

Plant material

Plants of *A. praecox* ssp. *orientalis* 'Royal Purple Select' were cultivated in the field.

Isolation of plant DNA and RNA

Total genomic DNA was isolated from leaves according to Chapter 2, Experiment 3. Total RNA was isolated from tepals, stamens, pistils, bract, pedicles, scapes and leaves of *A. praecox* ssp. *orientalis* 'Royal Purple Select' according to Chapter 2, Experiment 4.

Isolation of a DFR cDNA clone from flower tepals and a genomic DFR clone from leaves

Isolation of a DFR cDNA clone and a genomic DFR clone were performed according to Chapter 3, Experiment 2. 3'-RACE was carried out using total RNA as a template isolated from tepals of stage 4 flowers (see Results and discussion) and a degenerate primer, 5'-CC(T/C/A/G) GA(A/G) AA(T/C) GA(A/G) GT(T/C/A/G) AT(T/C/A) AA(A/G) CC-3', which was designed from the conservative region of several published DFR genes. A complete genomic clone of the DFR gene was isolated by PCR using the primer set, 5'-AAA AAT GTC AGT AGC AAT CG-3' (DFR4) and 5'-TAG CAA AGC CAT AAT CAT CC-3' (cDFR2).

Southern hybridization

Southern hybridization was performed according to Chapter 2, Experiment 4. Genomic DNA (50 μg) was digested with *Hin*dIII, *Sac*I or *Sca*I. The genomic region of the DFR gene of *A. praecox* ssp. *orientalis* 'Royal Purple Select' has no restriction sites recognized by these enzymes. The 0.81-kbp DNA fragment used as a probe was generated by PCR (Suzuki and Nakano, 2002a) using p35S-ADFR (constructed in the present experiment) as a template and the

primer set, 5'-CGA AGT GAT TAA GCC TAC AAT AGA AGG AAT GTT AG-3' (DFR5L) and 5'-ATA GGA ACC CTC TCC TCT GAT CCG TCT CTA TTA AC-3' (cDFR5L) (Figure 24A). The amplified fragment was purified with a cartridge for DNA concentration and primer elimination, SUPREC-02 (Takara, Japan), before using as a probe.

RT-PCR analysis for the expression of the DFR gene

RT-PCR was carried out using the primer set, DFR5L and cDFR5L (Figure 24), according to Chapter 3, Experiment 2.

HPLC analysis of anthocyanidins

Anthocyanin extraction from tepals, hydrolyzation of the crude extracts, and anthocyanidin analysis by HPLC were performed according to Chapter 3, Experiment 1.

Results and discussion

Isolation of a genomic clone for the DFR gene

A genomic clone for the DFR gene of *A. praecox* ssp. *orientalis* 'Royal Purple Select' was isolated by a PCR-based strategy and designated *ApDFR1* (accession number AB099529 in the GenBank/EMBL/DDBJ databases). *ApDFR1* contained an ORF of 1,137 bp, consisting of five exons (142, 170, 355, 193 and 277 bp) (Figure 24A) encoding a polypeptide of 379 amino acid residues (Figure 25). The genomic structure and deduced amino acid sequence of *ApDFR1* were compared with those of the previously reported DFR genes.

ApDFR1 had a similar genomic structure to the DFR genes of both monocots and dicots species (Figure 24B). It should be noted that the intron III, which is generally observed in the previously reported DFR genes, was not found in *ApDFR1*. On the other hand, the intron IV and V, which have not yet been reported in monocot species, were found in *ApDFR1*.

DFR is a member of the short chain dehydrogenase/reductase (SDR) superfamily (Schwinn and Davies, 2004). The enzymes in the SDR superfamily contain an amino acid sequence motif that seems to be responsible for NADPH-binding and characterized by similar exon/intron pattern (Martens et al., 2002). A putative NADPH-binding motif at the N-terminal region was found in the deduced amino acid sequence of *ApDFR1* (Figure 25). The percentage identities of the deduced amino acid sequence of *ApDFR1* to DFR of *Lilium* sp., *Cymbidium hybrida*, *Hordeum vulgare*, *Zea mays*, *Rosa hybrida*, *Petunia hybrida* and *Gerbera hybrida* were 75, 69, 69, 68, 65, 64 and 64%, respectively (Figure 25). A phylogenetic tree based on DFR amino acid sequences is shown in Figure 26. *A. praecox* ssp. *orientalis* DFR belonged to the subfamily containing monocot DFR, and showed the highest homology to that of the Liliaceous plant *Lilium* sp.

To determine the complexity of the DFR gene family in the genome of *A. praecox* ssp. *orientalis*, Southern hybridization was carried out using a 0.81-kbp DNA fragment from *ApDFR1* as a probe (Figure 27). One or two bands were observed for each restriction enzyme, indicating the presence of a single gene copy or a small gene family of DFR in the genome of *A. praecox* ssp. *orientalis*. Similar observations have been demonstrated for some species, such as *Cymbidium hybrida* (Johnson et al., 1999), *Hordeum vulgare* (Kristiansen and Rohde, 1991), *Antirrhinum majus* (Martin et al., 1985) and *Zea mays* (Schwarz-Sommer et al., 1987), all of which have a single copy of the DFR gene in their genome.

Anthocyanidin accumulation and ApDFR1 *expression in* A. praecox *ssp.* orientalis

Developmental stages of the flower of *A. praecox* ssp. *orientalis* are as follows (Figure 28A): stage 1, flower bud with non-pigmented (light green) tepals; stage 2, flower bud with pale blue tepals; stage 3, flower bud with blue tepals; stage 4, flower bud just before anthesis; and stage 5, fully opened flower. HPLC analysis indicated that main anthocyanidin in tepals of the stage 2–5 flowers was delphinidin, followed by petunidin and cyanidin. The total amount of anthocyanidins increased with the development of flowers (Figure 28B). Young leaves and tepals of the stage 1 flower did not contain detectable amounts of anthocyanidins.

In order to analyze expression of *ApDFR1* in various organs of *A. praecox* ssp. *orientalis*, RT-PCR analysis using specific primers was carried out (Figure 29). Strong signals were observed in tepals of the stage 3–5 flowers, and stamens, pistils and bracts of the stage 4 flower, whereas no signal was detected in scapes and leaves, in which no pigmentation was observed. These results indicate that expression of *ApDFR1* is specific to flower organs, and developmentally parallel to the anthocyanidin accumulation in tepals. A similar expression pattern of the DFR gene has been reported in petals of *Petunia hybrida* (Beld et al., 1989), *Gerbera hybrida* (Helariutta et al., 1993), *Rosa hybrida* (Tanaka et al., 1995) and *Ipomoea nil* (Inagaki et al., 1999). Moreover, such an expression pattern is similar to that of the majority of genes involved in the flavonoid biosynthetic pathway, as described for the F3'5'H gene in Chapter 3, Experiment 2.

Possible inference for the substrate specificity of ApDFR1

Substrate specificity of DFR is one of the important factors in the determination of flower color (Johnson et al., 2001). DFR of most species, such as *Dianthus caryophyllus* (Stich et al., 1992), *Gerbera hybrida* (Helariutta et al., 1993), *Matthiola incana* (Heller et al., 1985), *Rosa hybrida* (Tanaka et al., 1995), *Zea mays* (Meyer et al., 1987), can utilize all the three dihydroflavonols, DHK, DHQ and DHM, as substrates. On the other hand, DFR of *Petunia hybrida* (Forkmann and Ruhnau, 1987) and *Cymbidium hybrida* (Johnson et al., 1999) can not catalyze DHK efficiently, and therefore, pelargonidin derivatives are scarcely synthesized in both species, leading to the lack of orange flowers. Johnson et al. (2001) identified a region (26 amino acid residue) that determines the substrate specificity of DFR by analyzing the chimeric DFR genes of *Petunia hybrida* (with substrate specificity) and *Gerbera hybrida* (without substrate specificity). They also showed that a single amino acid residue, asparagine 134 (residue 134 in Figure 25), in this presumed substrate-binding region directly influenced the substrate specificity. Since amino acid residue corresponding to the residue 134 in DFR of *A. praecox* ssp. *orientalis* is asparagine like *Gerbera hybrida*, the DFR may catalyze all the three dihydroflavonols. However, in Chapter 3, Experiment1, no pelargonidin was detected in flower organs of *Agapanthus* spp. Further experiments are necessary to identify the substrate specificity of DFR of *Agapanthus* spp.

In conclusion, the DFR gene (*ApDFR1*) was isolated from *A. praecox* ssp. *orientalis* 'Royal Purple Select'. The genomic structure of *ApDFR1* was similar to that of the previously reported DFR, and the deduced amino acid sequence of *ApDFR1* showed high identity to that of other DFR. In addition, expression of *ApDFR1* was specific to flower organs, and developmentally parallel to the anthocyanidin accumulation in tepals. Further experiments are necessary to examine the usefulness of this gene for flower color modification in a

heterologous plant species.

Experiment 4

Isolation and characterization of the cytochrome P450 gene from *Muscari armeniacum*

Introduction

Although the F3'5'H genes have already been cloned from several dicot species as described in Chapter 3, Experiment 2, its counterparts have not yet been isolated from monocot species. In the present experiment, isolation of cDNA and genomic clones for the F3'5'H gene was examined in the Liliaceous monocot *Muscari armeniacum*, in which an efficient transformation system has already been established (Suzuki and Nakano, 2002a).

Materials and methods

Plant materials

Plants of *M. armeniacum* 'Blue Pearl', *M. armeniacum* 'Valerie Finnis', *M. comosum* var. *plumosum*, *M. botryoides* 'Album', *M. macrocarpum, Muscari* 'Sky Blue' and *Muscari* 'White Beauty' were cultivated in the field.

Isolation of plant DNA and RNA

Total genomic DNA was isolated from leaves of *M. armeniacum* 'Blue Pearl' according to Chapter 2, Experiment 3. Total RNA was isolated from tepals, stamens, pistils, scapes and leaves of all the seven *Muscari* genotypes according to Chapter 2, Experiment 4.

Isolation of a cDNA clone from flower tepals and a genomic clone from leaves

Isolation of a F3'5'H cDNA clone and a genomic F3'5'H clone of *M. armeniacum* 'Blue Pearl' were examined according to Chapter 3, Experiment 2. 3'-RACE was carried out using total RNA isolated from tepals of stage 4 flowers (see Results and discussion) as a template, and a degenerate primer, 5'-TA(T/C) TT(T/C) AA(T/C) AT(T/C/A) GG(T/C/A/G) GA(T/C) TT(T/C) AT(T/C/A/G) CC-3', which was designed from the conservative region of several published F3'5'H genes. A complete genomic clone was isolated by PCR using the primer set, 5'-GCT CAC TAT GTC CTT CAC AGA TCA TCA CTA TTT GC-3' (MKFH1L) and 5'-CAT ATA ACT CCA CAG CTA CAT GCA CAT CAC CAG AG-3' (cMKFH1L).

Southern hybridization

Southern hybridization was performed according to Chapter 2, Experiment 4. Twenty of Genomic DNA of *M. armeniacum* 'Blue Pearl' (20 μg) was digested with *Eco*RV, *Bgl*II, *Sca*I, *Xba*I or *Eco*81I. The genomic region of *MaP450* (isolated in the present experiment) has no restriction sites of these enzymes except for *Eco*RV. A 1.67-kbp DNA fragment used as a probe was generated by PCR (Suzuki and Nakano, 2002a) using pCR2.1-MaP450 (constructed in the present experiment) as a template and the primer set, MKFH1L and cMKFH1L (Figure 32). The amplified fragment was refined with the Geneclean Spin kit (BIO101, USA) for primer elimination before using as a probe.

RT-PCR analysis for the expression of the isolated gene

RT-PCR was carried out using the primer set, 5'-CAA AGA TTT CAG AGG TAA G-3' (MFH2) and 5'-ACA GAT TCT ACG ACC AGC AC-3' (MFHR1) (Figure 32), according to Chapter 3, Experiment 2.

HPLC analysis of anthocyanidins

Anthocyanin extraction from tepals, scapes and young leaves, hydrolyzation of the crude extracts, and anthocyanidin analysis by HPLC were performed according to Chapter 3, Experiment 1.

Results and discussion

Isolation of a genomic clone

By a PCR-based strategy using a degenerate primer designed from the conservative region of several published F3'5'H genes, a full-length cDNA clone (accession number AB127340) and its genomic clone, designated *MaP450*, (accession number AB127341) were isolated from *M. armeniacum* 'Blue Pearl', a blue-flowering cultivar. Nucleotide sequence analysis revealed that *MaP450* contained an ORF of 1,512 bp consisting of 2 exons (888 and 624 bp) encoding a polypeptide of 503 amino acid residues (Figure 30). The deduced amino acid sequence of *MaP450* had several characteristic conserved regions of the cytochrome P450 proteins such as heme-binding region, FxxGxxxCxG; proline-rich region, PPxP; oxygen-binding pocket, (A/G)Gx(D/E)T(T/S); K-region, ExxR; and aromatic region, (F/Y/W)(G)xxPxx(F/Y/W)xPx(R/H)(F/Y/W) (Chapple, 1998; Omura et al., 2003) (Figure 30). Figure 31 shows a phylogenic tree based on the deduced amino acid sequences of *MaP450*, the

cytochrome P450 genes involved in the flavonoid biosynthetic pathway, and the cytochrome P450 genes not involved in this pathway but showing relatively high homology to *MaP450*. The identities between *MaP450* and the cytochrome P450 genes involved in the flavonoid biosynthetic pathway, such as cinnamate 4-hydroxylase (CYP73A; 35–36%), flavone synthase II (CYP93B; 37–39%), F3'5'H (CYP75A; 34–37%), and F3'H (CYP75B; 38–42%), were low. However, the percentage identities of *MaP450* to genes in the subfamilies of the CYP71 family, CYP71A9 (*Glycine max*), CYP 71D10 (*Glycine max*), CYP71N1 (*Musa acuminata*), CYP71C1 (*Zea mays*), CYP71C4 (*Zea mays*), CYP71G1v2 (*Asparagus officinalis*), were 49, 45, 45, 43, 43 and 43%, respectively. *MaP450* was recently named CYP71J2 (D. Nelson, personal communication).

To determine the complexity of *MaP450* in the genome of *M. armeniacum* 'Blue Pearl', Southern hybridization was carried out using a 1.67-kbp DNA fragment from *MaP450* as a probe (Figure 32). Five or six bands were observed for each restriction enzyme, indicating the presence of a gene family (five to six copies) of *MaP450* in the genome of *M. armeniacum* 'Blue Pearl'.

Anthocyanidin accumulation and MaP450 *expression in* Muscari *spp.*

Accumulation of anthocyanidins and expression *MaP450* were initially examined in various organs of *M. armeniacum* 'Blue Pearl'. Developmental stages of the flower of this cultivar are as follows (Figure 33A): stage 1, flower bud with non-pigmented (light green) tepals; stage 2, flower bud with pale blue tepals; stage 3, flower bud with blue tepals; stage 4, flower bud just before anthesis; and stage 5, fully opened flower. HPLC analysis indicated that tepals of stage 2–5 flowers mainly contained delphinidin, followed by petunidin and cyanidin, and the total anthocyanidins per flower increased with the

development of flowers (Figure 33B). These 3 anthocyanidins were also slightly detected in scapes and young leaves. RT-PCR analysis using specific primers was carried out to analyze the expression of *MaP450* in various organs (Figure 34). Strong signals were observed in tepals of the stage 3–5 flowers, whereas stamens and pistils of the stage 4 flower showed only weak signals. Signals were also detected in scapes and young leaves, both of which slightly accumulated anthocyanidins. These results indicate that *MaP450* is expressed developmentally paralleling anthocyanidin accumulation in tepals. The expression pattern of *MaP450* is similar to that of the majority of genes involved in the flavonoid biosynthetic pathway (Mol et al., 1998).

RT-PCR analysis using specific primers also carried out using tepals of the stage 4 flower of various *Muscari* genotypes with different flower colors (Figure 35). Strong signals for *MaP450* expression were detected in tepals of *Muscari* genotypes with colored flowers, such as *M. armeniacum* 'Blue Pearl' (blue) and 'Valerie Finnis' (pale blue), *M. comosum* var. *plumosum* (reddish purple), *M. macrocarpum* (yellow), and *Muscari* 'Sky Blue' (deep blue). On the other hand, only weak signals were observed in tepals of *Muscari* genotypes with white flowers, such as *M. botryoides* 'Album' and *Muscari* 'White Beauty'.

In the present experiment, isolation of the F3'5'H gene from *M. armeniacum* was examined. Although the deduced amino acid sequence of the isolated gene *MaP450* had several characteristic conserved regions of the cytochrome P450 proteins, it showed only low identities to that of the previously reported cytochrome P450 genes involved in the flavonoid biosynthetic pathway including the F3'5'H genes. However, a close correlation between *MaP450* expression and anthocyanidin accumulation as well as flower color indicates that this gene may be involved in the flavonoid biosynthetic pathway in *Muscari* species. Further experiments are necessary to elucidate the biological function and physiological role of *MaP450* in *Muscari* species.

Chapter 4

Attempts at flower color modification via genetic transformation

Experiment 1

Agrobacterium-mediated transformation of *Tricyrtis hirta* with genes for the flavonoid biosynthetic pathway

Introduction

Tricyrtis hirta has recently become popular as an ornamental plant for pot and garden uses because of its attractive arching stems, beautiful foliage, exotic starry flowers, and the ability to grow in the shade. However, *T. hirta* has only a limited range of flower color, probably due to the accumulation only cyanidin-derivative anthocyanins in tepals (Chapter 3, Experiment 1), and therefore, induction of novel flower colors is desired for this species. Toward the application of molecular breeding for flower color modification to *T. hirta*, an efficient system for producing transgenic plants of this species was established in Chapter 2, Experiment 3. In addition, two genes involved in the flavonoid biosynthetic pathway, the F3'5'H gene of *Vinca major* and the DFR gene of *Agapanthus praecox* ssp. *orientalis*, were isolated in Chapter 3, Experiment 2 and Experiment 3, respectively. In the present experiment, flower color modification was examined in *T. hirta* by utilizing the established transformation system and these isolated genes.

Materials and methods

Plant materials

Tepal-derived embryogenic calluses of *T. hirta* (Nakano et al., 2004) were used as a target material for *Agrobacterium*-mediated transformation as in

Chapter 2, Experiment 3. The non-transgenic plant C1, a regenerant from the embryogenic calluses, was potted and cultivated in a growth chamber.

Agrobacterium *strains*

Three *Agrobacterium tumefaciens* strains, EHA101/pNA-VFH-35S (Figure 18B), EHA101/pNA-ApDFR1-35S (Figure 36A) and EHA101/ pNA-VmFH1-ApDFR1 (Figure 36B), were used. The T-DNA region of each binary vector contains the following genes: pNA-VFH-35S, the NPTII gene under the control of the NOS promoter, the F3'5'H gene of *Vinca major* (*VmFH1*) under the control of the CaMV35S promoter (35S-*VmFH1*), and the HPT gene under the control of the CaMV35S promoter; pNA-ApDFR1-35S, the NPTII gene under the control of the NOS promoter, the DFR gene of *A. praecox* spp. *orientalis* (*ApDFR1*) under the control of the CaMV35S promoter (35S-*ApDFR1*), and the HPT gene under the control of the CaMV35S promoter; pNA-VmFH1-ApDFR1, 35S-*ApDFR1*, 35S-*VmFH1*, and the HPT gene under the control of the CaMV35S promoter. *A. tumefaciens* strain EHA101/pNA-VFH-35S was produced in Chapter 3, Experiment 2. *A. tumefaciens* strains EHA101/pNA-ApDFR1-35S and EHA101/pNA-VmFH1-ApDFR1 were produced as follows. pBI221 (Jefferson et al. 1987) was treated with *Sma*I and *Sac*I to remove the GUS gene and the 3' and 5' terminal regions blunted using the DNA Blunting Kit (Takara, Japan), followed by ligation with DNA Ligation Kit (Takara, Japan), yielding p35S-N. A cDNA clone for *ApDFR1* (Chapter 3, Experiment 3) was amplified by RT-PCR using the Takara RNA LA PCR Kit (Takara, Japan), total RNA extracted from tepals of *A. praecox* spp. *orientalis* 'Royal Purple Select' according to Chapter 2, Experiment 4 as a template, and the primer set, 5'-AAA AAT GTC AGT AGC AAT CG-3' (DFR4) and 5'-TAG CAA AGC CAT AAT

CAT CC-3' (cDFR2). The amplified product was inserted into the *Bam*HI site of p35S-N, that was blunted using the DNA Blunting Kit (Takara, Japan) followed by treating the 3' terminal region with the ΔTth DNA Polymerase (Toyobo, Japan), yielding p35S-ADFR. Subsequently, the DNA fragment containing the CaMV35S promoter, *ApDFR1* and the NOS terminator was excised from p35S-ADFR with *Pvu*II, and inserted between the blunted *Sal*I and *Hin*dIII sites of the plant expression vector pIG121Hm (Ohta et al. 1990) in exchange for the CaMV35S promoter, the intron-containing GUS gene and the NOS terminator, yielding pNA-ApDFR1-35S (Figure 36A). On the other hand, the DNA fragment containing the CaMV35S promoter, *ApDFR1* and the NOS terminator was excised from p35S-ADFR with *Pvu*II, inserted between the blunted *Hin*dIII and *Avi*II sites of pNA-VFH-35S (Figure 18; Chapter 3, Experiment 2) in exchange for the NOS promoter, the NPTII gene and the NOS terminator, yielding pNA-VmFH1-ApDFR1 (Figure 36B). pNA-ApDFR1-35S and pNA-VmFH1-ApDFR1 were transferred to *A. tumefaciens* strain EHA101, resulting in strains EHA101/pNA-35SApDFR1 and EHA101/ pNA-VmFH1-ApDFR1, respectively.

Transformation of T. hirta

Transgenic *T. hirta* plants were produced by co-cultivation of embryogenic calluses with *A. tumefaciens* strains EHA101/pNA-VFH-35S, EHA101/pNA-35SApDFR1 or EHA101/pNA-VmFH1-ApDFR1, according to Chapter 2, Experiment 3. Integration of the transgene was confirmed by PCR using genomic DNA isolated from leaves of putative transgenic plants according to Chapter 2, Experiment 3 as a template, and the primer set, VFH2L and cVFH1L (Figure 18A) or the set, DFR5L and cDFR5L (Figure 36A) according to Chapter 3, Experiment 2. Following acclimatization, transgenic plants were

transplanted to pots and cultivated in a growth chamber according to Chapter 2, Experiment 3.

HPLC analysis of anthocyanins

Anthocyanin extraction from tepals and anthocyanin analysis by HPLC were performed according to Chapter 3, Experiment 1.

Results and discussion

Production of transgenic plants

Co-cultivation of embryogenic calluses with each *Agrobacterium* strain, and selection and germination of Hyg^r somatic embryos were performed according to Chapter 2, Experiment 3. Plantlets developed from Hyg^r somatic embryos were subjected to PCR analysis for confirmation of their transgenic nature (data not shown). Totally 29, 27 and 10 independent transgenic plants were obtained with *A. tumefaciens* strains, EHA101/pNA-VFH-35S, EHA101/pNA-ApDFR1-35S and EHA101/ pNA-VFH-ApDFR1, respectively.

Anthocyanin analysis in transgenic T. hirta

One year after cultivation in a growth chamber, eight (F2, F4, F5, F9, F11, F12, F32 and F34), three (D4, D15 and D17) and one (FD10) independent transgenic plants, obtained with *A. tumefaciens* strains, EHA101/pNA-VFH-35S, EHA101/pNA-ApDFR1-35S and EHA101/pNA-VFH-ApDFR1, respectively, produced flowers. Flowers of F2 and FD10 did not open fully. Irrespective of the transgene, no apparent morphological alterations were observed in

vegetative organs of transgenic plants compared with the control, non-transgenic plant C1. Anthocyanin analysis was carried out on all the 12 independent transgenic plants described above. Figure 37 shows typical flower color phenotypes and typical HPLC profiles for the accumulation of anthocyanins in tepals of the control, non-transgenic plant C1 and several transgenic plants. No qualitative alterations in both flower color and anthocyanin composition were observed in these transgenic plants. As in the case of non-transgenic *T. hirta* plants analyzed in Chapter 3, Experiment 1, C1 produced light reddish-purple flowers with dark reddish-purple spots, and accumulated six anthocyanins, 8-*C*-glucosylcyanidin 3-*O*-malonylglucoside (Peak No. 1), cyanidin 3- glucoside (Peak No. 2), cyanidin 3-rutinoside (Peak No. 3), cyanidin 3-*O*-malonylglucoside (Peak No. 4), 8-*C*-sinapylglucosylcyanidin 3-*O*-malonylglucoside (Peak No. 5) and an unidentified anthocyanin (Peak No. 6) in tepals. All the transgenic plants contained the same six anthocyanins in tepals as those C1 did, irrespective of the transgene. Since, in Chapter 3, Experiment 2, heterologous expression of *VmFH1* in transgenic plants of *Petunia hybrida* led to newly accumulation of 3',5'-hydroxylated anthocyanidins (delphinidin, petunidin and malvidin) in tepals, similar results were initially expected to be obtained by transformation of *T. hirta* with *VmFH1*. However, no derivatives of 3',5'-hydroxylated anthocyanidins were detected in any transgenic plants produced with *A. bacterium* strains EHA101/pNA-VFH-35S and EHA101/pNA-VFH-ApDFR1. Although the cause of no accumulation of 3',5'-hydroxylated anthocyanidin derivatives in transgenic *T. hirta* is not identified at present, there are at least four possible explanations for this result. First, transgene silencing may occur in the transgenic plants of *T. hirta*. However, this is less probable because long-term stability of transgene (GUS reporter gene) expression was confirmed in transgenic *T. hirta* plants in Chapter 2, Experiment 5. The second possible

explanation is that F3'5'H derived from *VmFH1* may have only very weak activity in transgenic *T. hirta*. In *Lilium* sp., transgenic plants containing the F3'5'H gene from *Campanula medium* newly accumulated 3',5'-hydroxylated anthocyanidins in leaves, whereas no 3',5'-hydroxylated anthocyanidins were detected in leaves of transgenic plants containing *VmFH1* (H. Kobayashi, personal communication). The third possible explanation is that strong competition may occur among DFR, FLS, F3'H and F3'5'H for dihydroflavonol substrates (Figure 1). For example, if F3'H activity is much stronger than F3'5H activity, 3'-hydroxylated anthocyanidin (cyanidin and peonidin) derivatives will be preferentially accumulated but 3',5'-hydroxylated anthocyanidin derivatives will be scarcely synthesized, even when F3'5'H derived from *VmFH1* has some activities in transgenic *T. hirta*. Enzyme competition for dihydroflavonol substrates has already been reported to influence flower color in several species such as *Petunia hybrida* (Davies et al., 2003; Shimada et al., 2001), *Nicotiana tabacum* (Holton et al., 1993b), *Eustoma grandiflorum* (Davies et al., 1997) and *Forsythia* × *intermedia* (Rosati et al., 1997). The fourth possible explanation is that downstream enzyme(s) in the flavonoid biosynthesis pathway, such as ANS and UFGT, may have substrate specificity like DFR (Zufall and Rausher, 2004). For example, if endogenous ANS and/or UFGT of *T. hirta* dose not catalyze leucodelphinidin due to substrate specificity, neither 3',5'-hydroxylated anthocyanidins nor their derivatives will be accumulated, even when dihydromyricetin and subsequent leucodelphinidin are synthesized by transformation with *VmFH1*. Further experiments are necessary to clarify the cause of no accumulation of 3',5'-hydroxylated anthocyanidin derivatives in transgenic *T. hirta* containing *VmFH1*.

In conclusion, transgenic plants of *T. hirta* containing the F3'5'H gene of *Vinca major* and/or the DFR gene of *Agapanthus praecox* ssp. *orientalis* were successfully produced. However, no qualitative alterations in both flower color

and anthocyanin composition were observed in these transgenic plants. For flower color modification of *T. hirta*, especially for producing blue-flowering strains and cultivars, by engineering the flavonoid biosynthetic pathway, further accumulation of biochemical and molecular biological information of the flavonoid biosynthetic pathway is necessary in this species.

Experiment 2

Agrobacterium-mediated transformation of *Muscari armeniacum* with the F3'5'H gene from *Vinca major*

Introduction

The genus *Muscari* consists of about 40 species, among which *M. armeniacum* is most popular and widely cultivated for pot and garden uses throughout the temperate regions of the world. However, flower color of *M. armeniacum* is limited to blue or pale blue, and therefore, it is desired to introduce novel flower colors into this species. In order to apply molecular breeding for flower color modification to *M. armeniacum*, Suzuki and Nakano (2002a) established an *Agrobacterium*-mediated transformation system in the cultivar 'Blue Pearl' of this species. In addition, anthocyanidin and anthocyanin analyses in tepals of *M. armeniacum* 'Blue Pearl' and isolation of the F3'5'H gene from *Vinca major* were carried out in this cultivar in Chapter 3, Experiment 1 and Experiment 3, respectively. In the present experiment, flower color modification was examined in *M. armeniacum* 'Blue Pearl' by utilizing the previously established transformation system and this isolated genes.

Materials and methods

Plant material

Embryogenic calluses of *M. armeniacum* 'Blue Pearl' was induced from leaf explants and maintained as previously described (Suzuki and Nakano, 2001). About 2-year-old callus cultures were used as a target material for

Agrobacterium-mediated transformation.

Agrobacterium *strain*

Agrobacterium tumefaciens strain EHA101/pNAVFH produced in Chapter 3, Experiment 2 was used. The T-DNA region of pNAVFH contains the NPTII gene under the control of the NOS promoter, the F3'5'H gene of *V. major* (*VmFH1*) under the control of the original promoter of *VmFH1* (VFH-*VmFH1*), and the HPT gene under the control of the CaMV35S promoter (Figure 18A).

Transformation of M. armeniacum

Transgenic plants of *M. armeniacum* 'Blue Pearl' were produced by co-cultivation of embryogenic calluses with *A. tumefaciens* strain EHA101/pNAVFH, according to Suzuki and Nakano (2002a). Integration of the transgene was confirmed by PCR using total DNA isolated from leaf tissues according to Chapter 2, Experiment 3 as a template, and the primer set, VFH2L and cVFH1L (Figure 18A), as described in Chapter 3, Experiment 2. Following acclimatization, transgenic plants were transplanted to pots and cultivated in a growth chamber as described for transgenic *Tricyrtis hirta* plants in Chapter 2, Experiment 3.

Results and discussion

Two independent transgenic plants of *M. armeniacum* 'Blue Peal' (FL21 and FL40) have so far been obtained, and these plants have been cultivated in a growth chamber (Figure 38). The transgenic nature of these plants was confirmed by PCR analysis (data not shown). At least early stages of growth,

they showed no apparent morphological alterations.

In the present experiment, the transformation system previously established by Suzuki and Nakano (2002a) was utilized. According to Suzuki and Nakano (2002b), about 30 independent transgenic plants could be obtained from 5 g FW of co-cultivated calluses for *M. armeniacum* 'Blue Pearl' by this system under the optimum condition. On the other hand, only two independent transgenic plants have so far been obtained in the present experiment from more than 5 g FW of co-cultivated calluses (Figure 38). Following co-cultivation, a number of independent Hyg^r embryogenic cultures were obtained on a selection medium, and shoots were successfully regenerated from most of these cultures in the present experiment. However, these shoots hardly produced roots, and only a few plantlets were obtained. Treatment with PGRs and cold treatments had no promotive effects on rooting of the shoots (data not shown). The failure in the regeneration of transgenic plants in the present experiment may be due to the decline of the regeneration potential of embryogenic callus cultures used as a target material for transformation during long-term subculture (about 2 years). The decline with time of the regeneration potential of callus and suspension cultures has already been demonstrated for numerous plants species (Murashige and Nakano, 1967; Vasil and Vasil, 1985; Zaghmout and Torello, 1992; Nakano et al., 2000; Nakano et al., 2005).

In the present experiment, two independent transgenic plants of *M. armeniacum* 'Blue Pearl' containing *VmFH1* under the control of *VmFH1* original promoter were produced. Because *M. armeniacum* plants generally require 2–3 years from acclimatization to flowering, the transgenic plants obtained in the present experiments will produce flowers 2–3 years later. Analyses of anthocyanidins and anthocyanins in flower organs as well as detailed morphological characterization should be carried out on the transgenic plants at the flowering stage. These transgenic plants may be important not only

as plants with a novel flower color but also as research materials to develop the flower organ specific promoter in Liliaceous ornamentals. In addition, genetic transformation of *M. armeniacum* 'Blue Pearl' should be carried out again by using embryogenic calluses immediately after establishment of callus cultures.

Chapter 5
General discussion

The present study aims at practical application of molecular breeding for flower color modification to Liliaceous ornamentals. In order to achieve the aim, several experiments were carried out on the establishment of efficient and reliable genetic transformation systems and accumulation of biochemical and molecular biological information on the flavonoid biosynthesis. Furthermore, flower color modification via genetic engineering was examined by using the established transformation systems and the insolated genes for the flavonoid biosynthetic pathway.

1. Establishment of genetic transformation systems

There are at least three important factors for establishing genetic transformation systems applicable for molecular breeding: preparation of target materials for transformation, efficient regeneration of transgenic plants, and stable expression of transgene(s) in transgenic plants. With regard to target materials for transformation, fast growing and highly regenerable callus cultures have been shown to be most suitable in Liliaceous ornamentals (Suzuki and Nakano, 2002b). In Chapter 2, Experiment 1 and Experiment 2, such cultures were newly established in five *Muscari* genotypes and 28 *Lilium* genotypes. Toward wide application of genetic transformation to breeding of *Lilium* spp. and *Muscari* spp., further studies should be directed to develop genetic transformation systems by using the established callus cultures for each genotype. In addition, it is also necessary to develop fast growing and highly regenerable callus cultures in some Liliaceous ornamentals, commercially important but recalcitrant in tissue culture, such as *Tulipa* spp.

In Chapter 2, Experiment 3, a system for producing transgenic plants was newly established in *Tricyrtis hirta* via *Agrobacterium*-mediated transformation. On average, about ten independent transgenic plants could be obtained per 1 g

FW of co-cultivated embryogenic calluses by using this system. The transformation efficiency of *Tricyrtis hirta* was much higher than that of other Liliaceous ornamentals, such as *Agapanthus praecox* ssp. *orientalis* (about 15 independent transgenic plants per 3 g FW of calluses; Suzuki et al., 2001), *Muscari armeniacum* (about 30 independent transgenic plants per 5 g FW of calluses; Suzuki and Nakano, 2002a) and *Lilium* sp. (five to ten independent transgenic plants per 50–80 g FW of calluses; Hoshi et al., 2004). In addition, *Tricyrtis hirta* required only one year from *in vitro* regeneration to flowering differently from most Liliaceous ornamentals. Therefore, this species seems to be suitable as a model for molecular breeding as well as molecular biological studies in Liliaceous ornamentals.

For perennial and vegetatively propagated crops including almost all the Liliaceous ornamentals, long-term and stable expression of transgene(s) is one of the indispensable requisites for their successful improvement by genetic transformation. In Chapter 2, Experiment 4 and Experiment 5, stability of transgene expression was examined in two Liliaceous ornamentals, *Agapanthus praecox* ssp. *orientalis* and *Tricyrtis hirta*, and all the transgenic plants of both species showed stable expression of the transgene (GUS gene) even after long-term cultivation. Although there have been many reports on the occurrence of transgene silencing in monocot crops including *Avena sativa* (Cho et al., 1999), *Oryza sativa* (Oard et al., 1996; Kohli et al., 1999), *Triticum aestivum* (Chen et al., 1998) and *Zea mays* (Register et al., 1994), no apparent transgene silencing was observed in these two Liliaceous ornamentals. These results indicate that transgene silencing may not be a common occurrence in transgenic plants of Liliaceous ornamentals. Further experiments are necessary to examine long-term stability of transgene expression in other Liliaceous ornamentals.

2. Biochemical and molecular biological information on the flavonoid

biosynthetic pathway

Biochemical information on the flavonoid biosynthesis in target plants for molecular breeding provides a strong foundation for producing transgenic plants with novel flower colors. In Chapter 3, Experiment 1, anthocyanidins and/or anthocyanins in flower organs were analyzed in several Liliaceous ornamentals, *Muscari* spp., *Agapanthus* spp. and *Tricyrtis* spp. In tepals and/or stamens of *Agapanthus* spp. and *Muscari* spp., the main anthocyanidin in blue and pale blue organs was delphinidin, and cyanidin was mainly detected in reddish purple organs. Thus a general correlation between flower color and anthocyanidin composition reported in a wide range of plant species (Forkmann, 1991) was also observed in Liliaceous ornamentals. Since pelargonidin was never detected in flower organs of *Agapanthus* spp. and *Muscari* spp., DFR of these species may not catalyze the reduction of DHK to leucopelargonidin due to the substrate specificity, as in the cases of *Petunia hybrida* (Forkmann and Ruhnau, 1987) and *Cymbidium hybrida* (Johnson et al., 1999). On the other hand, all the five cyanidin derivatives, previously identified in tepals of *Tricyrtis formosana*, were also detected in tepals of *Tricyrtis hirta* by anthocyanin analysis. Since *Tricyrtis hirta* and *Tricyrtis formosana* showed a similar anthocyanin composition but different flower colors, further experiments are needed to clarify the difference in the mechanism of flower coloration between these species. Although only anthocyanidins and anthocyanins were analyzed in the present study, other flavonoids such as flavones and flavonols should also be analyzed in Liliaceous ornamentals, since these pigments generally affect flower color indirectly (Mol et al., 1998; Aida et al., 2000b; Ben-Meir et al., 2002).

To collect molecular biological information on the flavonoid biosynthetic pathway, isolation and characterization of the genes in this pathway was examined. Initially, isolation of genes for F3'5'H, a key enzyme for the

expression of blue or purple flower color, was examined. In preliminary experiments, although isolation of the F3'5'H gene from *Agapanthus praecox* ssp. *orientalis* was attempted by a PCR-based strategy, no F3'5'H gene was obtained. In addition, an attempt at isolation of the F3'5'H gene from *Muscari armeniacum* in Chapter 3, Experiment 4 resulted in the isolation of *MaP450*, which has only 34–37% identities with those of the previously reported F3'5'H genes. Since the F3'5'H genes have so far been isolated from dicot species, monocot F3'5'H genes may have only poor identities of the DNA sequence to the previously reported dicot F3'5'H genes and these could not be isolated by PCR-based strategies. On the other hand, in Chapter 3, Experiment 2, a genomic clone for the F3'5H gene, designated *VmFH1*, was successfully isolated from petals of *Vinca major*. When *VmFH1* was expressed in transgenic *Petunia hybrida* under the control of the CaMV35S promoter, transgenic plants newly accumulated 3',5'-hydroxylated anthocyanins in their petals. These results indicate that *VmFH1* encodes F3'5'H and is active in a heterologous plant species. Although flower color alteration was not observed in transgenic *Tricyrtis hirta* containing *VmFH1* under the control of the CaMV35S promoter (Chapter 4, Experiment 1), the applicability of *VmFH1* to molecular flower color modification should be examined in a wide range of Liliaceous ornamentals.

DFR is a pivotal enzyme of the flavonoid biosynthetic pathway, because the substrate specificity of this enzyme is one of the major factors in flower pigmentation (Johnson et al., 1999). Lack of orange flower color in *Agapanthus* spp. and *Muscari* spp. may be due to the substrate specificity of DFR of each species as described above. Since a genomic clone for the DFR gene, designated *ApDFR1*, was successfully isolated from *Agapanthus praecox* ssp. *orientalis* in Chapter 3, Experiment 3, it is hereafter necessary to clarify the substrate specificity of the *Agapanthus praecox* ssp. *orientalis* DFR by transformation of

a white-flowering *Petunia hybrida* mutant, W80, which is lacking in DFR, F3'H and F3'5'H activities and accumulates DHK (Johnson et al., 1999), with *ApDFR1*.

3. Attempts at flower color modification by genetic transformation

The present study aims at application of molecular breeding for flower color modification to Liliaceous ornamentals. One of the breeding objectives is producing novel cultivars with blue flowers in species lacking blue flower color, such as *Lilium* spp. and *Tricyrtis hirta*. In Chapter 4, Experiment 1, transgenic plants of *Tricyrtis hirta* containing the F3'5'H gene from *Vinca major*, *VmFH1*, and/or the DFR gene from *Agapanthus praecox* spp. *orientalis*, *ApDFR1*, were successfully obtained. Several transgenic plants subsequently produced flowers, but no apparent alterations in flower color and composition of tepal anthocyanins were observed in these plants. To date, molecular breeding for blue flower color has been performed in some dicot ornamentals (Holton et al., 1993a; Tanaka et al., 1998; Shimada et al., 1999). Violet-flowering cultivars of *Dianthus caryophyllus* have already been produced by transformation of a DFR-deficient strain with the F3'5'H and DFR genes of *Petunia hybrida* by Florigene, Australia and Suntory, Japan. Petals of these transgenic *Dianthus caryophyllus* predominantly contained delphinidin (reviewed in Tanaka et al., 1998). On the other hand, Vetten et al. (1999) reported that a flower-specific cytochrome *b5* was required for full activity of the F3'5'H gene in *Petunia hybrida*. In addition, introduction and expression of the gene encoding cytochrome *b5*, *difF*, in *Dianthus caryophyllus* together with the F3'5'H gene of *Petunia hybrida* resulted in the generation of nearly black flowers, owing to a strong accumulation of delphinidin derivatives (reviewed in Forkmann and Martens, 2001). Manipulation of *difF*, together with the DFR and F3'5'H gene,

may be helpful in realizing the development of blue-flowering cultivars in Liliaceous ornamentals lacking blue flower color. Recently aureusidin synthase, an enzyme responsible for the synthesis of aurones, which provide yellow color to flowers in some plants, was identified in *Antirrhinum majus* (Nakayama et al., 2000). Manipulation of the gene for aureusidin synthase may realize the development of yellow-flowering cultivars in Liliaceous ornamentals lacking yellow flower color, such as *Agapanthus* spp., *Muscari armeniacum* and *Tricyrtis hirta.*

As mentioned in Chapter 1, two main strategies have successfully been employed for modifying flower color via genetic transformation, and one strategy is based on the introduction of the exogenous gene(s) (Ben-Meir et al., 2002), which was applied to Liliaceous ornamentals in Chapter 4. The other is based on the up- or down-regulation of expression of the endogenous gene(s) (Ben-Meir et al., 2002; Schwinn and Davies, 2004). Shimada et al. (2001) reported that when the sense F3'5'H gene constructs of *Petunia hybrida* were introduced into a blue-flowering cultivar of this species, transgenic plants accumulated an elevated amount of 3'hydroxylated anthocyanins in petals and their flower color changed from deep blue to pale pink. This phenomenon may be resulted from the switching the anthocyanin biosynthesis from 3'5'-hydroxylation to 3'-hydroxylation by inhibition of the F3'5'H activity via co-suppression (Figure 1). In the present study, a plan to produce red-flowering transgenic plants of *Agapanthus* spp. and *Muscari* spp. was also initially made by employing the same strategy as described above for *Petunia hybrida.* However this plan has not yet been carried out, because the F3'5'H gene has not yet been isolated from target plants. Further studies are necessary to isolate the F3'5'H gene from Liliaceous ornamentals. On the other hand, production of white-flowering transgenic plants by suppressing expression of the endogenous CHS gene has so far been demonstrated for several dicot ornamentals, such as

Dendranthema grandiflorum (Courtney-Gutterson et al., 1994), *Dianthus caryophyllus* (Gutterson, 1995), *Eustoma grandiflorum* (Deroles et al., 1998) and *Petunia hybrida* (van der Krol et al., 1988). White-flowering transgenic plants have also been produced by suppressing expression of the endogenous DFR gene in *Torenia hybrida* (Suzuki et al., 2000). Thus, white-flowering cultivars could be produced by suppressing expression of the endogenous CHS or DFR gene in Liliaceous ornamentals lacking white flower color, such as *Gloriosa* spp., *Muscari armeniacum* and *Tricyrtis hirta.*

In conclusion, fundamental information obtained in the present study may be significant for applying molecular breeding for flower color modification to Liliaceous ornamentals. In the near future, cultivars with novel and desired flower colors may be produced by molecular breeding in Liliaceous ornamentals, i.e. blue-flowering *Lilium* and *Tricyrtis* cultivars, red-flowering *Muscari* and *Agapanthus* cultivars and so on.

Tables and figures

Table 1.

Comparison of the percentage of leaf or flower bud explants forming calluses and growth of leaf- or flower bud-derived calluses among various *Muscari* species and cultivars.

Species and cultivars	Leaf explants		Flower bud explants	
	Callus (%) [a]	Callus growth [b]	Callus (%) [a]	Callus growth [b]
M. armeniacum	82.3 b	+++	100 a	+++
M. armeniacum 'Blue Pearl'	90.0 ab	+++	100 a	+++
M. armeniacum 'Blue Spike'	83.3 b	+++	96.7 ab	+++
M. armeniacum 'Fantasy Creation'	80.0 b	+++	91.7 ab	+++
M. armeniacum 'Valerie Finnis'	83.3 b	+++	100 ab	+++
M. azureum	0 g	-	73.3 cd	+
M. azureum 'Album'	10.0 fg	+	53.3 ef	+
M. botryoides 'Album'	0 g	-	66.7 de	+
M. botryoides 'Superstar'	26.7 de	+	100 a	++
M. comosum var. *plumosum*	63.3 c	++		
M. latifolium	16.7 ef	+	41.0 f	+
M. macrocarpum	36.7 d	+	43.3 f	+
M. moschatum	10.0 fg	+		
M. moschatum var. *flavum*	3.3 fg	+		
M. neglectum 'Dark Eyes'	100 a	+++	93.3 ab	++
M. paradoxum	0 g	-	86.7 abc	++
M. tubergenianum	83.3 b	+++	83.3 bc	+++
Muscari 'Sky Blue'	3.3 fg	+	96.7 ab	++
Muscari 'White Beauty'	56.7 c	++	73.3 cd	+++

[a] Data were recorded 8 weeks after culture initiation. Values represent the mean of three independent experiments, each of which consisted of ten explants. Means in the same column followed by same letter are not significantly different at $P = 0.05$ with LSD test.

[b] Data were recorded at the fourth subculture. Increase in callus mass in 4 weeks is expressed as follows: -, no growth; +, slight increases; ++, less than twofold increases; and +++, twofold or more increases.

Table 2.

Comparison of the percentage of leaf- or flower bud-derived calluses producing somatic embryos and the number of somatic embryos per somatic embryo-producing callus among various *Muscari* species and cultivars [a].

Species and cultivars	Leaf-derived calluses		Flower bud-derived calluses	
	Somatic embryos (%) [b]	No. of somatic embryos [c]	Somatic embryos (%) [b]	No. of somatic embryos [c]
M. armeniacum	10.0 b	+	73.3 ab	++
M. armeniacum 'Blue Pearl'	66.7 a	++	66.7 b	+++
M. armeniacum 'Blue Spike'	46.7 a	++	63.3 b	++
M. armeniacum 'Fantasy Creation'	6.7 b	+	83.3 a	++
M. armeniacum 'Valerie Finnis'	0 b	-	0 d	-
M. botryoides 'Album'			0 d	-
M. comosum var. *plumosum*	0 b	-		
M. latifolium			0 d	-
M. macrocarpum			0 d	-
M. neglectum 'Dark Eyes'	43.3 a	++	0 d	-
M. paradoxum			0 d	-
M. tubergenianum	0 b	-	16.7 c	++
Muscari 'White Beauty'	0 b	-	0 d	-

[a] Data were recorded 4 weeks after transfer of calluses onto a regeneration medium.

[b] Values represent the mean of three independent experiments, each of which consisted of ten calluses. Means in the same column followed by same letter are not significantly different at $P = 0.05$ with LSD test.

[c] No. of somatic embryos per somatic embryo-producing callus is expressed as follows: -, no formation; +, less than ten embryos; ++, 10–50 embryos; and +++, more than 50 embryos.

Table 3.

Percentage of callus-forming explants, callus type and callus growth in *Lilium* spp. as affected by genotype and explant type [a].

Section and hybrid group [a]	Species and cultivars	Explant type [b]	% of explants producing calluses [c]	Callus type [d]	Callus growth [e]
Martagon	*L. martagon*	seed	33.3 ± 24.0	YN	++
	L. tsingtauense	seed	13.3 ± 13.3	BN	-
Pseudolirium	*L. pardalinum* 'Red Sunset'	seed	6.7 ± 6.7	YN + YF	++
	L. canadense	seed	0		
	L. michiganense	seed	0		
Liriotypus	*L. monadelphum*	seed	18.3 ± 11.7 [f]	YN	++
Archelirion	*L. auratum*	bulb scale	100	YN	++
		leaf	78.9 ± 14.3	YN	++
	L. auratum var. *platyphyllum*	seed	6.7 ± 6.7	YN	++
	L. japonicum	seed	6.7 ± 6.7	YN	+
		bulb scale	100	YN	++
		leaf	43.6 ± 13.5	YN	+
	L. nobilissimum	leaf	58.6 ± 12.8	YN	++
	L. rubellum	seed	49.0 ± 22.6	YN	++
		bulb scale	91.5 ± 8.9	YN	++
		leaf	78.5 ± 18.6	YN	++
	L. speciosum	seed	70.4 ± 17.3	YN	++

(continued on the next page)

Section and hybrid group [a]	Species and cultivars	Explant type [b]	% of explants producing calluses [c]	Callus type [d]	Callus growth [e]
Sinomartagon	*L. concolor*	seed	70.0 ± 15.8	YN	++
	L. henryi	seed	53.3 ± 6.7	YN	++
	L. lancifolium	bulb scale	83.0 ± 13.6	YN	+++
	L. pumilum	seed	0		
Leucolirion	*L. formosanum*	seed	67.8 ± 15.0	YN + YF	+++
		bulb scale	100	YN + YF	+++
		leaf	100	YN + YF	+++
	L. longiflorum 'Georgia'	bulb scale	100	YN	++
		leaf	100	YN	++
		filament	98.6 ± 1.3	YN + YF	+++
	L. sargentiae	seed	40.0 ± 11.5	YN	++
	L. wallichianum	seed	60.3 ± 21.6	YN + YF	+++
Daurolirion	*L. dauricum*	seed	53.3 ± 24.0	YN	+

(continued on the next page)

Section and hybrid group [a]	Species and cultivars	Explant type [b]	% of explants producing calluses [c]	Callus type [d]	Callus growth [e]
Asiatic hybrid	'Amarone'	leaf	76.3 ± 11.3	YN	++
		filament	85.6 ± 12.3	YN + YF	+++
	'Connecticut King'	bulb scale	100	YN	++
		leaf	84.3 ± 8.9	YN	+++
		filament	88.6 ± 7.3	YN + YF	+++
	'Montenegro'	leaf	77.6 ± 6.8	YN	++
		filament	82.1 ± 9.7	YN + YF	+++
LA hybrid	'Rotehorn'	bulb scale	100	YN	++
		leaf	76.3 ± 15.6	YN	+++
	'Royal Parade'	bulb scale	96.0 ± 1.7	YN	+++
		leaf	64.2 ± 16.5	YN	+++
Longiflorum hybrid	*L.* × *formolongi* 'White Aga'	bulb scale	100	YN + YF	+++
		leaf	88.3 ± 6.7	YN + YF	+++
	L. × *formolongi* 'White Lancer'	bulb scale	100	YN + YF	+++
		leaf	83.2 ± 3.6	YN + YF	+++

(continued on the next page)

Section and hybrid group [a]	Species and cultivars	Explant type [b]	% of explants producing calluses [c]	Callus type [d]	Callus growth [e]
Oriental hybrid	'Casa Blanca'	bulb scale	98.3 ± 1.3	YN	+++
		leaf	72.3 ± 13.7	YN	++
		filament	86.3 ± 9.6	YN	++
	'Mona Lisa'	bulb scale	100	YN	+++
		leaf	63.0 ± 16.4	YN	+++
	'Sweet Memory'	bulb scale	89.3 ± 6.5	YN	++
		leaf	64.2 ± 15.0	YN	++
Trumpet hybrid	'Golden Splendor'	bulb scale	100	YN	+++
		leaf	78.6 ± 9.6	YN	+++
	'Pink Perfection'	bulb scale	100	YN	+++
		leaf	83.2 ± 12.6	YN	+++

[a] Sections are according to Comber (1949).
[b] Seeds, bulb scales of *in vitro* plantlets, expanding leaves, and filaments of flower buds 5 to 7 days before anthesis were used as explants.
[c] Data were recorded 2 months after culture initiation. Values represent the mean ± SE of three independent experiments, each of which consisted of at least ten explants.
[d] Callus type was determined at the third subculture. BN, brown and nodular; YF, yellow and friable; YN, yellow and nodular.
[e] Callus growth was determined at the third subculture. Increase in callus mass in 1 month is expressed as follows: -, no growth; +, slight increases; ++, less than twofold increases; +++, twofold or more increases.
[f] For seed explants of *L. monadelphum*, data were recorded 6 months after culture initiation.

Table 4.

Percentage of callus clusters producing shoots in *Lilium* spp. as affected by genotype, origin of callus, callus type, and BA in shoot induction medium [a].

Section and hybrid group [b]	Species and cultivars	Origin of calluses	Callus type [c]	Shoot induction medium	
				PGR-free	22 μM BA
Martagon	*L. martagon*	seed	YN	66.7 ± 13.3	33.4 ± 8.6
Pseudolirium	*L. pardalinum* 'Red Sunset'	seed	YN	56.8 ± 12.6	49.3 ± 8.5
			YF	69.3 ± 9.4	56.5 ± 11.3
Liriotypus	*L. monadelphum*	seed	YN	76.0 ± 6.7	33.3 ± 3.3
Archelirion	*L. auratum*	bulb scale	YN	100	87.2 ± 11.8
		leaf	YN	86.5 ± 9.6	68.5 ± 12.1
	L. auratum var. *platyphyllum*	seed	YN	100	86.7 ± 3.3
	L. japonicum	bulb scale	YN	56.7 ± 9.6	13.3 ± 6.7
	L. nobilissimum	leaf	YN	83.6 ± 14.4	73.2 ± 11.0
	L. rubellum	seed	YN	48.6 ± 18.7	29.3 ± 7.7
		bulb scale	YN	56.3 ± 6.6	55.4 ± 8.3
		leaf	YN	78.5 ± 15.3	69.4 ± 13.2
	L. speciosum	seed	YN	36.7 ± 14.5	43.3 ± 17.6
Sinomartagon	*L. concolor*	seed	YN	78.0 ± 8.7	73.3 ± 12.6
	L. henryi	seed	YN	89.4 ± 8.1	63.7 ± 14.4
	L. lancifolium	bulb scale	YN	100	100

(continued on the next page)

Section and hybrid group [b]	Species and cultivars	Origin of calluses	Callus type [c]	Shoot induction medium	
				PGR-free	22 μM BA
Leucolirion	*L. formosanum*	seed	YN	100	100
			YF	100	100
		bulb scale	YN	100	100
			YF	100	100
		leaf	YN	100	100
			YF	100	86.3 ± 6.5
	L. longiflorum 'Georgia'	bulb scale	YN	88.9 ± 7.1	63.7 ± 14.0
		leaf	YN	95.6 ± 3.1	55.7 ± 12.6
		filament	YN	86.3 ± 9.3	77.9 ± 16.5
			YF	100	48.3 ± 16.2
	L. sargentiae	seed	YN	66.7 ± 8.8	43.3 ± 8.5
	L. wallichianum	seed	YN	98.6 ± 1.2	63.7 ± 14.4
			YF	100	16.3 ± 8.4
Asiatic hybrid	'Amarone'	leaf	YN	78.9 ± 3.2	55.6 ± 8.9
		filament	YN	77.0 ± 8.3	53.2 ± 12.1
			YF	86.5 ± 9.6	73.8 ± 14.9

(continued on the next page)

Section and hybrid group [b]	Species and cultivars	Origin of calluses	Callus type [c]	Shoot induction medium	
				PGR-free	22 μM BA
Asiatic hybrid	'Connecticut King'	bulb scale	YN	100	72.5 ± 10.4
		leaf	YN	95.6 ± 1.3	77.8 ± 6.3
		filament	YN	100	68.3 ± 15.3
			YF	100	71.3 ± 9.6
	'Montenegro'	leaf	YN	85.4 ± 6.3	54.8 ± 12.1
		filament	YN	88.4 ± 6.4	66.4 ± 8.9
			YF	86.3 ± 9.4	56.3 ± 9.1
LA hybrid	'Rotehorn'	bulb scale	YN	85.4 ± 6.3	78.0 ± 11.4
		leaf	YN	77.4 ± 11.0	68.9 ± 14.3
	'Royal Parade'	bulb scale	YN	100	88.7 ± 2.3
		leaf	YN	92.3 ± 3.3	55.7 ± 12.6
Longiflorum hybrid	*L.* × *formolongi* 'White Aga'	bulb scale	YN	100	100
			YF	100	100
		leaf	YN	100	100
			YF	96.2 ± 3.3	100
	L. × *formolongi* 'White Lancer'	bulb scale	YN	100	100
			YF	100	88.3 ± 6.2
		leaf	YN	100	100
			YF	100	100

(continued on the next page)

Section and hybrid group [b]	Species and cultivars	Origin of calluses	Callus type [c]	Shoot induction medium	
				PGR-free	22 μM BA
Oriental hybrid	'Casa Blanca'	bulb scale	YN	100	88.3 ± 7.5
		leaf	YN	94.5 ± 2.3	90.2 ± 6.7
		filament	YN	100	78.5 ± 12.4
	'Mona Lisa'	bulb scale	YN	100	70.3 ± 13.3
		leaf	YN	100	84.6 ± 2.6
	'Sweet Memory'	bulb scale	YN	78.9 ± 8.8	77.6 ± 14.9
		leaf	YN	68.3 ± 14.6	82.0 ± 6.7
Trumpet hybrid	'Golden Splendor'	bulb scale	YN	100	72.1 ± 3.5
		leaf	YN	100	68.1 ± 12.5
	'Pink Perfection'	bulb scale	YN	92.3 ± 1.7	77.6 ± 6.7
		leaf	YN	94.5 ± 0.7	68.9 ± 14.3

[a] Data were recorded 2 months after transfer of callus clusters onto shoot induction media without PGRs or containing 22 μM BA. Values represent the mean ± SE of three independent experiments, each of which consisted of at least ten callus clusters.
[b] Sections are according to Comber (1949).
[c] YF, yellow and friable; YN, yellow and nodular.

Table 5.

Effects of the co-cultivation period and AS treatment on the number of blue spots resulting from transient expression of the GUS gene per 0.5 g FW embryogenic calluses of *Tricyrtis hirta* [a].

AS [b]	Co-cultivation period (days)			
	3	5	7	14
-	0	0	0	0
+	0	36.4± 8.7	359.6 ± 32.3	292.5 ± 53.6

[a] Values represent the mean ± SE of at least three independent co-cultivation experiments.

[b] + and - represent the presence and absence of 50 mg l^{-1} AS, respectively, in both inoculation and co-cultivation media.

Table 6.

Ploidy level and morphological characteristics of vegetative organs in transgenic plants of *Agapanthus praecox* ssp. *orientalis* 'ES-1'.

Line	Plant	Ploidy level (2*n*)	Morphology of vegetative organs [a]			
			Examined year	No. of leaves per inflorescence	Leaf length (cm) [b]	Leaf width (mm) [b]
C1	C1-1	2*x*	2005	16	55.7 ± 0.3	33.3 ± 0.3
L7	L7-2	2*x*	2005	8	44.7 ± 0.3	12.3 ± 0.7
	L7-3	2*x*	2005	17	53.3 ± 1.9	17.0 ± 1.2
L8	L8-2	4*x*	2004	12	56.3 ± 1.8	17.3 ± 1.3
	L8-3	4*x*	2005	10	41.7 ± 0.3	13.7 ± 0.3
L9	L9-1	2*x*	2004	13	33.0 ± 1.5	16.0 ± 0.6
	L9-2	2*x*	2004	10	39.7 ± 2.7	13.3 ± 0.3
	L9-3	2*x*	2004	15	44.0 ± 1.5	15.7 ± 0.3
L10	L10-1	2*x*	2004	14	40.0 ± 1.2	19.7 ± 1.2
	L10-2	2*x*	2004	12	42.3 ± 1.5	13.3 ± 0.7
E1	E1-1	2*x*	2005	11	39.3 ± 0.3	22.0 ± 0.6
	E1-4	2*x*	2005	8	35.7 ± 0.3	18.7 ± 0.7
	E1-5	2*x*	2004	18	43.3 ± 0.3	17.3 ± 0.3

[a] The earliest-flowering inflorescence was examined for each plant in each year.

[b] Values represent the mean ± SE of randomly selected five leaves.

Table 7.

Morphological characteristics of floral organs in transgenic plants of *Agapanthus praecox* ssp. *orientalis* 'ES-1' [a].

Line	Plant	Examined year	Length of inflorescence stalks (cm)	No. of flowers per inflorescence	Flower length (mm) [b]	Flower width (mm) [b]
C1	C1-1	2004	171	126	39.3 ± 1.3	34.7 ± 1.8
		2005	183	61	42.0 ± 2.0	36.7 ± 1.7
L7	L7-2	2005	97	32	27.0 ± 1.0	21.7 ± 3.3
	L7-3	2004	131	72	31.3 ± 0.7	32.7 ± 0.7
		2005	79	25	28.3 ± 1.5	23.3 ± 2.9
L8	L8-2	2004	84	23	32.7 ± 1.8	33.0 ± 6.1
	L8-3	2003	83	24	35.7 ± 0.3	35.1 ± 4.6
		2005	77	20	31.3 ± 4.8	36.7 ± 3.5
L9	L9-1	2004	72	50	26.3 ± 0.9	22.7 ± 0.9
	L9-2	2003	68	45	31.0 ± 1.0	18.7 ± 6.2
		2004	79	43	25.3 ± 0.9	19.3 ± 2.2
	L9-3	2004	68	36	25.0 ± 1.5	27.0 ± 1.5
L10	L10-1	2003	62	23	29.3 ± 1.2	28.7 ± 1.9
		2004	79	61	22.0 ± 0.6	nd [c]
	L10-2	2003	60	43	27.7 ± 1.2	26.0 ± 1.0
		2004	50	12	26.0 ± 1.5	36.7 ± 2.4
E1	E1-1	2004	143	52	34.7 ± 1.8	36.0 ± 2.1
		2005	125	30	34.0 ± 3.1	36.7 ± 2.4
	E1-4	2004	133	43	33.0 ± 1.5	nd [c]
		2005	134	46	34.0 ± 0.1	35.7 ± 1.2
	E1-5	2004	128	35	33.0 ± 0.6	35.3 ± 2.3

[a] The earliest-flowering inflorescence was examined for each plant in each year.

[b] Values represent the mean ± SE of randomly selected ten flowers.

[c] Not examined because of occasional development of strongly twisted tepals.

Table 8.

GUS histochemical assay of various organs in transgenic plants of *Agapanthus praecox* ssp. *orientalis* 'ES-1' that produced flowers.

Line	Plant	Examined year	Leaf	Root	Tepal	Pistil	Stamen
C1	C1-1	2004	-	-	-	-	-
		2005	-	-	-	-	-
L7	L7-2	2005	+	+	±	+	±
	L7-3	2004	+	+	±	+	±
		2005	+	±	±	+	±
L8	L8-2	2004	+	+	±	+	+
	L8-3	2003	+	+	±	+	±
		2005	+	+	±	+	±
L9	L9-1	2004	+	+	+	+	+
	L9-2	2004	+	+	+	+	+
	L9-3	2004	+	+	+	+	+
L10	L10-1	2003	+	+	+	+	+
		2004	+	+	+	+	+
	L10-2	2004	+	+	+	+	+
E1	E1-1	2003	+	+	+	+	+
		2004	+	+	+	+	+
		2005	+	+	+	+	+
	E1-4	2003	+	+	+	+	+
		2004	+	+	+	+	+
		2005	+	+	+	+	+
	E1-5	2003	+	+	+	+	+
		2004	+	+	+	+	+

+, strong GUS activity; ±, weak GUS activity; -, no GUS activity.

Table 9.

GUS histochemical assay of various organs in transgenic plant of *Tricyrtis hirta*.

Plant	Examined year	Leaf	Stem	Root	Tepal	Pistil	Stamen
C1	2004	-	-	-	-	-	-
	2005	-	-	-	-	-	-
T1	2004	+	+	+	+	+	±
	2005	+	+	+	+	+	+
T2	2004	+	+	+	+	+	±
	2005	+	+	+	+	+	+
T3	2004	+	+	+	+	+	±
	2005	+	+	+	+	+	+
T4	2004	+	+	+	nd	nd	nd
	2005	+	+	+	+	±	±

+, strong GUS activity; ±, weak GUS activity; -, no GUS activity; nd, not determined.

Table 10.

Relative anthocyanidin composition in tepals of various *Muscari* species and cultivars by HPLC analysis. Data represent the relative level of anthocyanidin (area % at 530 nm).

Flower color and genotype	Anthocyanidin					
	Pelargonidin	Cyanidin	Peonidin	Delphinidin	Petunidin	Malvidin
Blue flower						
M. armeniacum		2.6		81.1	13.1	3.2
M. armeniacum 'Blue Pearl'		6.7		88.9	4.4	
M. armeniacum 'Blue Spike'		3.0		89.3	6.5	1.2
M. armeniacum 'Fantasy Creation'		2.5		96.3	1.2	
M. paradoxum		6.2		92.3	1.5	
M. tubergenianum		4.2		83.0	10.4	2.4
M. latifolium (upper) [a]		3.2		88.1	7.9	0.8
Deep blue flower						
M. latifolium (middle and lower) [a]		1.2	1.4	21.2	55.0	21.2
Pale blue flower						
M. armeniacum 'Valerie Finnis'		3.9		89.1	7.0	
M. azureum		28.8		61.1	10.1	
Reddish purple flower						
M. comosum var. *plumosum*		52.3		47.7		
Pale purple flower						
M. moschatum		54.2		45.8		
Creamy yellow flower						
M. moschatum var. *flavum* [b]		+		+		
White flower						
M. botryoides 'Album'						

[a] For *M. latifolium*, the color of flowers in the upper part of inflorescences is blue while that of flowers in the middle and lower parts is deep blue.

[b] In *M. moschatum* var. *flavum*, only cyanidin and delphinidin were barely detected.

Table 11.

Relative anthocyanin composition in tepals of various *Muscari* species and cultivars by HPLC analysis. Data represent the relative level of anthocyanin (area % at 530 nm).

Flower color and genotype	Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13
	Rt (min.)	20.3	27.6	31.6	33.0	34.2	35.2	36.0	37.2	38.7	39.1	41.9	43.7	44.6
Blue flower														
M. armeniacum			1.1			24.7				67.6			6.6	
M. armeniacum 'Blue Pearl'						23.8				69.5			6.7	
M. armeniacum 'Blue Spike'			1.2			17.8				72.7			8.3	
M. armeniacum 'Fantasy Creation'						15.6				78.6			5.8	
M. paradoxum								5.8		77.7	10.4		6.1	
M. tubergenianum			2.3			19.4				69.2			9.1	
M. latifolium (upper) [a]						17.3				72.0			10.7	
Deep blue flower														
M. latifolium (middle and lower) [a]		5.8	44.0	18.9	7.7	5.6				18.0				
Pale blue flower														
M. armeniacum 'Valerie Finnis'						26.8				61.5			11.7	
M. azureum							15.5			9.7	62.1			12.7
Reddish purple flower														
M. comosum var. *plumosum*						14.2			9.6	49.1		27.1		
Pale purple flower														
M. moschatum						26.0				74.0				

[a] For *M. latifolium*, the color of flowers in the upper part of inflorescences is blue while that of flowers in the middle and lower parts is deep blue.

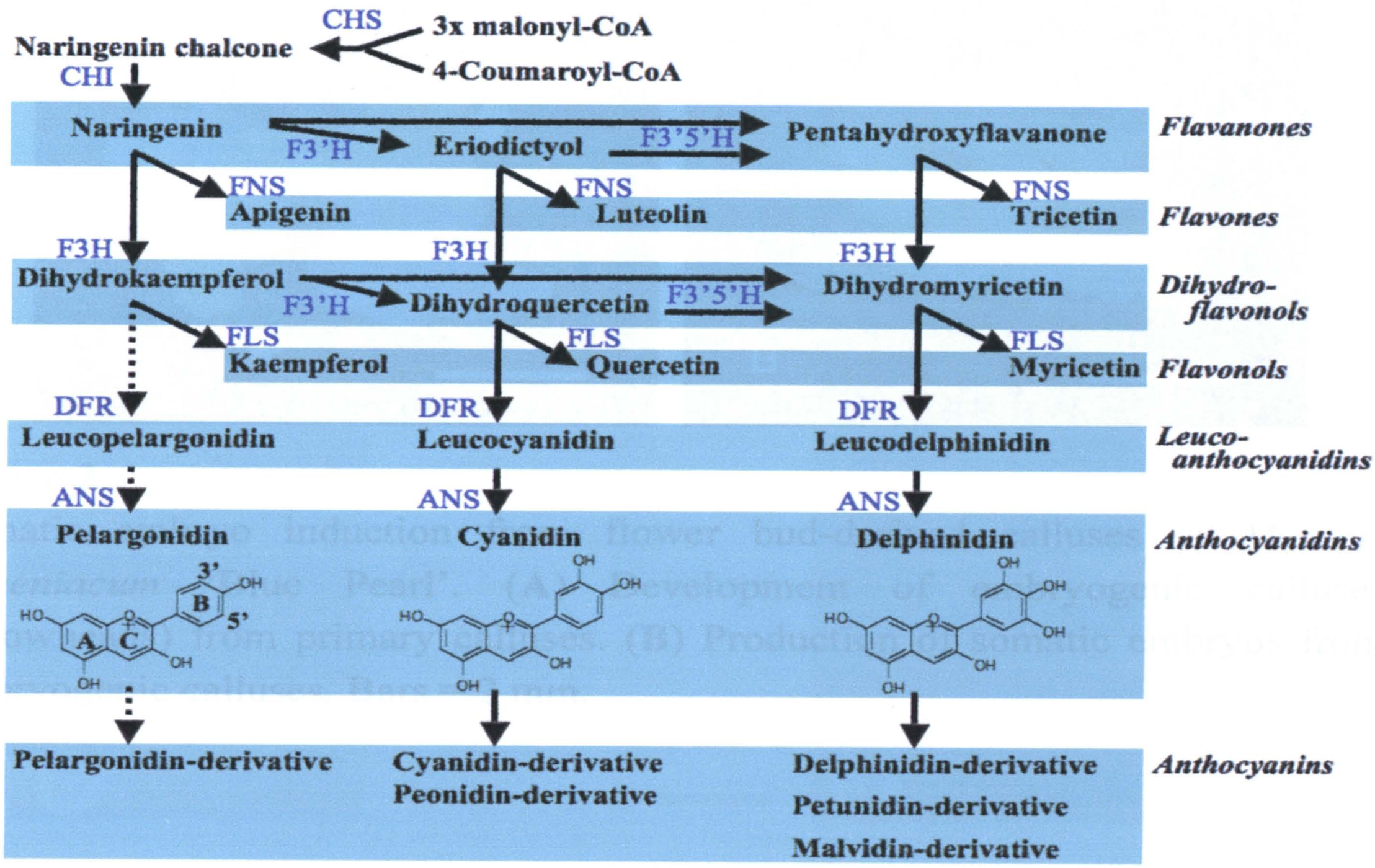


Figure 1.
A simplified representation of the flavonoid biosynthetic pathway. In *Petunia hybrida* and *Cymbidium hybrida*, the reactions shown by dotted arrows do not naturally occur because DFR of these species do not catalyze the reduction of dihydrokaempferol to leucopelargonidin (Forkmann and Ruhnau, 1987; Johnson et al., 1999). Generally, pelargonidin-derivatives are responsible for orange and salmon flower colors, cyanidin- and peonidin-derivatives provide magenta and crimson flower colors, and delphinidin-, petunidin- and malvidin-derivatives are the source of purple, mauve and blue flower colors (Forkmann, 1991). Capital letters in blue indicate abbreviations of the enzymes involved in the flavonoid biosynthetic pathway.

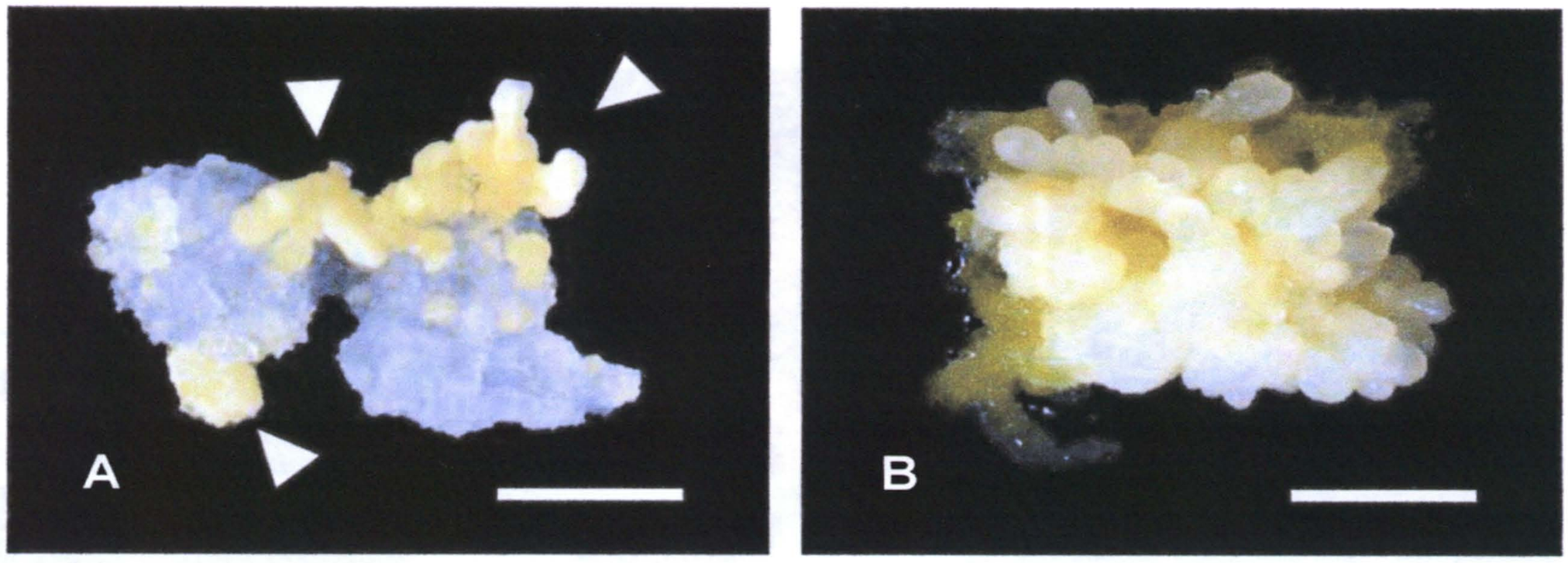


Figure 2.
Somatic embryo induction from flower bud-derived calluses in *Muscari armeniacum* 'Blue Pearl'. (**A**) Development of embryogenic calluses (arrowheads) from primary calluses. (**B**) Production of somatic embryos from embryogenic calluses. Bars = 2 mm.

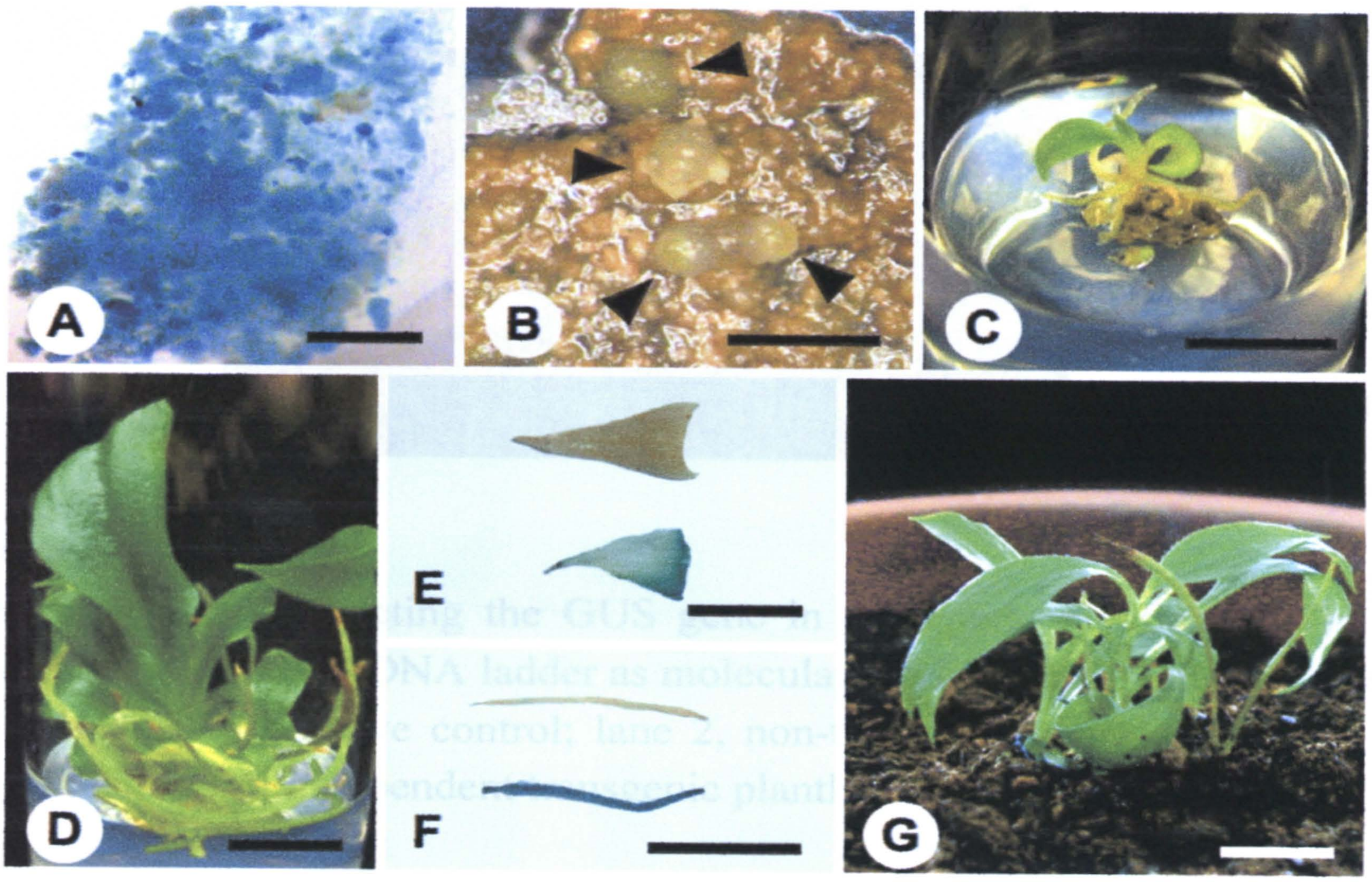


Figure 3.
A system for producing transgenic plants of *Tricyrtis hirta* via *Agrobacterium*-mediated transformation of embryogenic calluses. (**A**) Embryogenic calluses showing transient expression of the GUS gene after 7 days of co-cultivation in the presence of AS. Bar = 2 mm. (**B**) Hyg^r somatic embryos (arrowheads) developed on a medium containing 40 mg l^{-1} hygromycin. Bar = 2 mm. (**C**) Germination of Hyg^r somatic embryos on a medium containing 20 mg l^{-1} hygromycin. Bar = 1 cm. (**D**) A plantlet regenerated from a Hyg^r somatic embryo. Bar = 1 cm. (**E, F**) GUS histochemical assay of leaf (**E**) and root (**F**) segments of the control, non-transgenic (upper) and transgenic (lower) plantlets. Bars = 5 mm. (**G**) A transgenic plantlet established in a growth chamber. Bar = 1 cm.

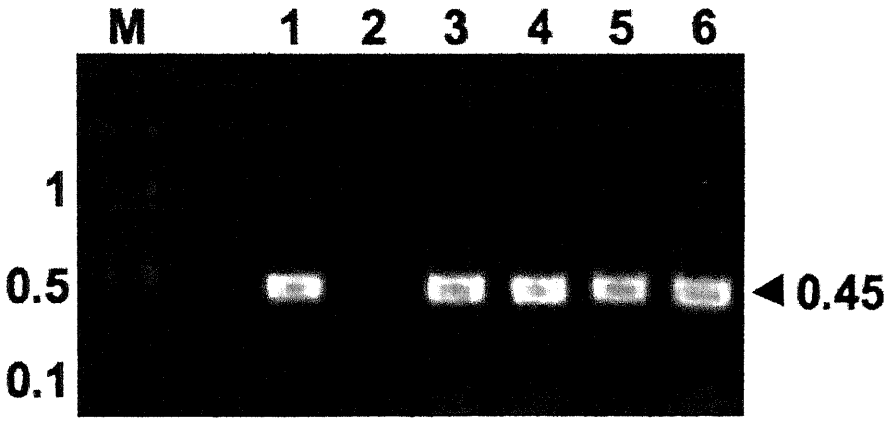


Figure 4.
PCR analysis for detecting the GUS gene in transgenic plantlets of *Tricyrtis hirta*. Lane M, 100-bp DNA ladder as molecular markers; lane 1, binary plasmid pIG121Hm as a positive control; lane 2, non-transgenic plantlet as a negative control; lanes 3-6, independent transgenic plantlets. Numerals indicate kbp.

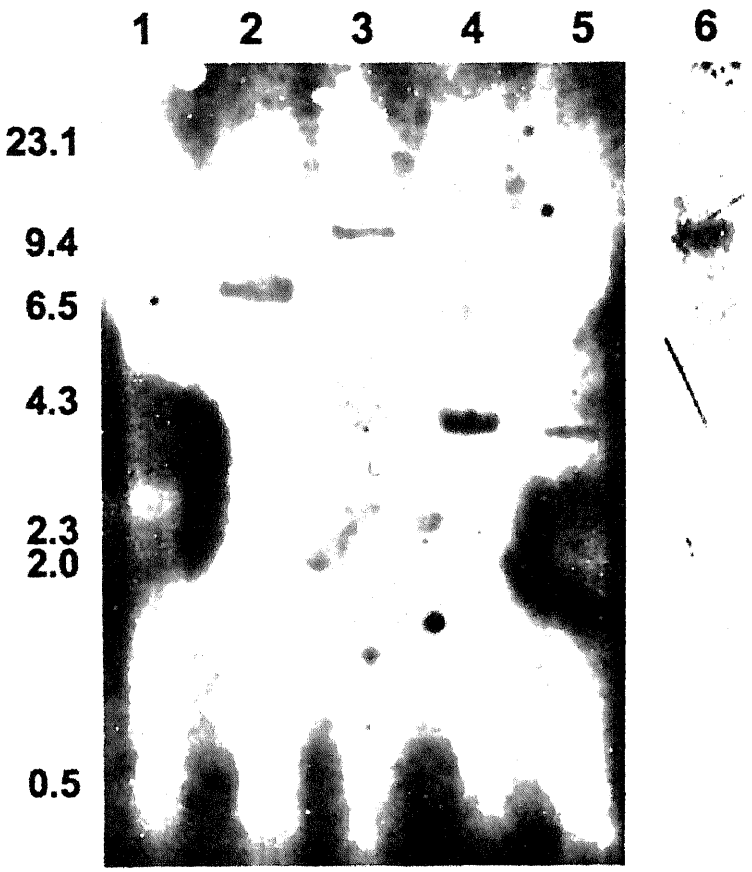


Figure 5.
Southern blot analysis of transgenic plants of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. *Hind*III-digested DNA samples were hybridized with the HPT gene probe for the control, non-transgenic plant (lane 1) and transgenic plants obtained with *Agrobacterium tumefaciens* strain LBA4404/pTOK233 (lane 2–5), or with the GUS gene probe for a transgenic plant obtained with strain EHA101/pIG121Hm (lane 6). Lane 1, C1-1; lane 2, L7-3; lane 3, L8-2; lane 4, L9-1; lane 5, L10-2; lane 6, E1-1. Numerals indicate kbp.

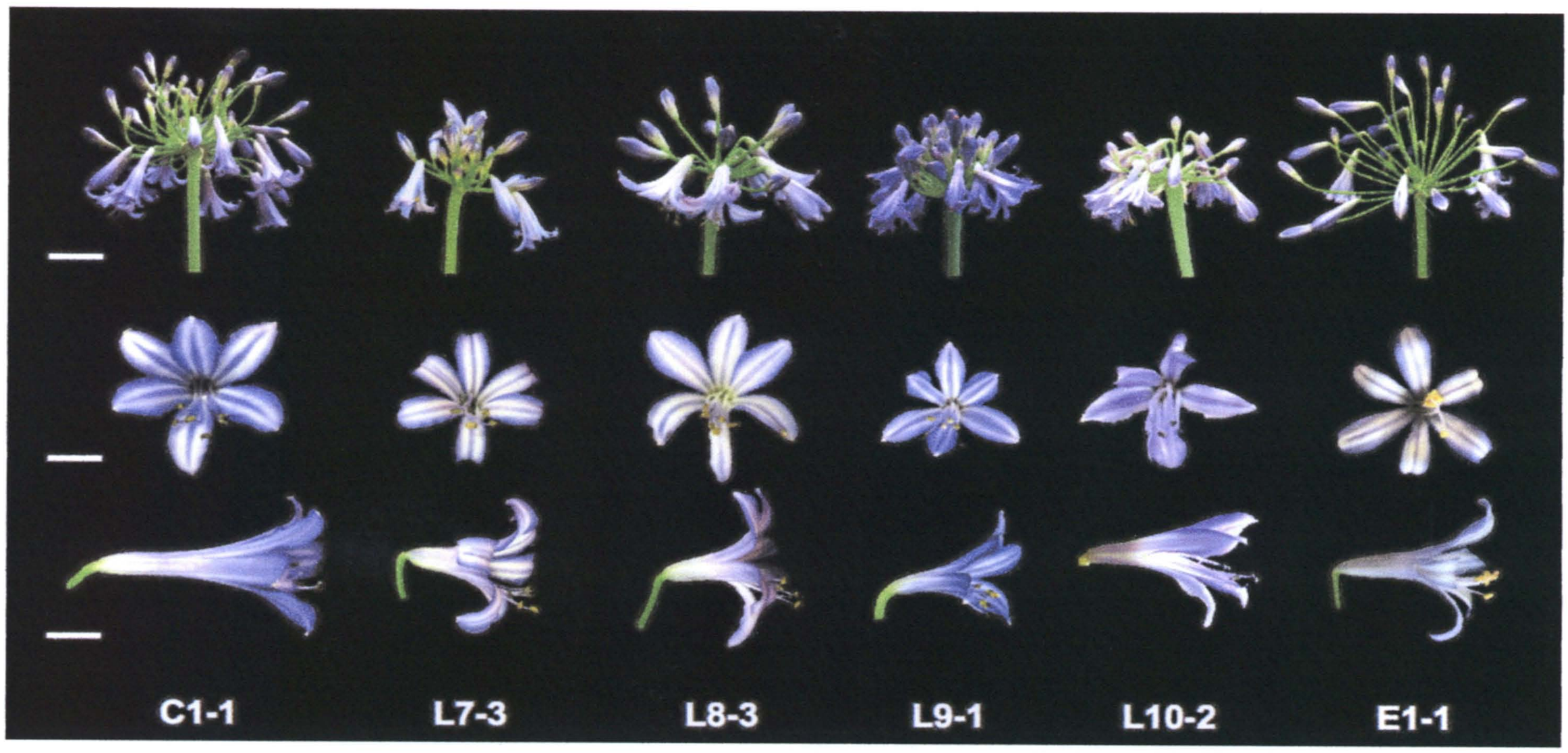


Figure 6.

Morphology of inflorescences (upper) and flowers (middle and lower) of the control, non transgenic plant C1-1 and the transgenic plants L7-3, L8-3, L9-1, L10-2 and E1-1 of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. Floral organs produced in 2004 are shown for L9-1 and L10-2, whereas those produced in 2005 are shown for the other plants. Upper bar = 5 cm. Middle and lower bars = 1 cm.

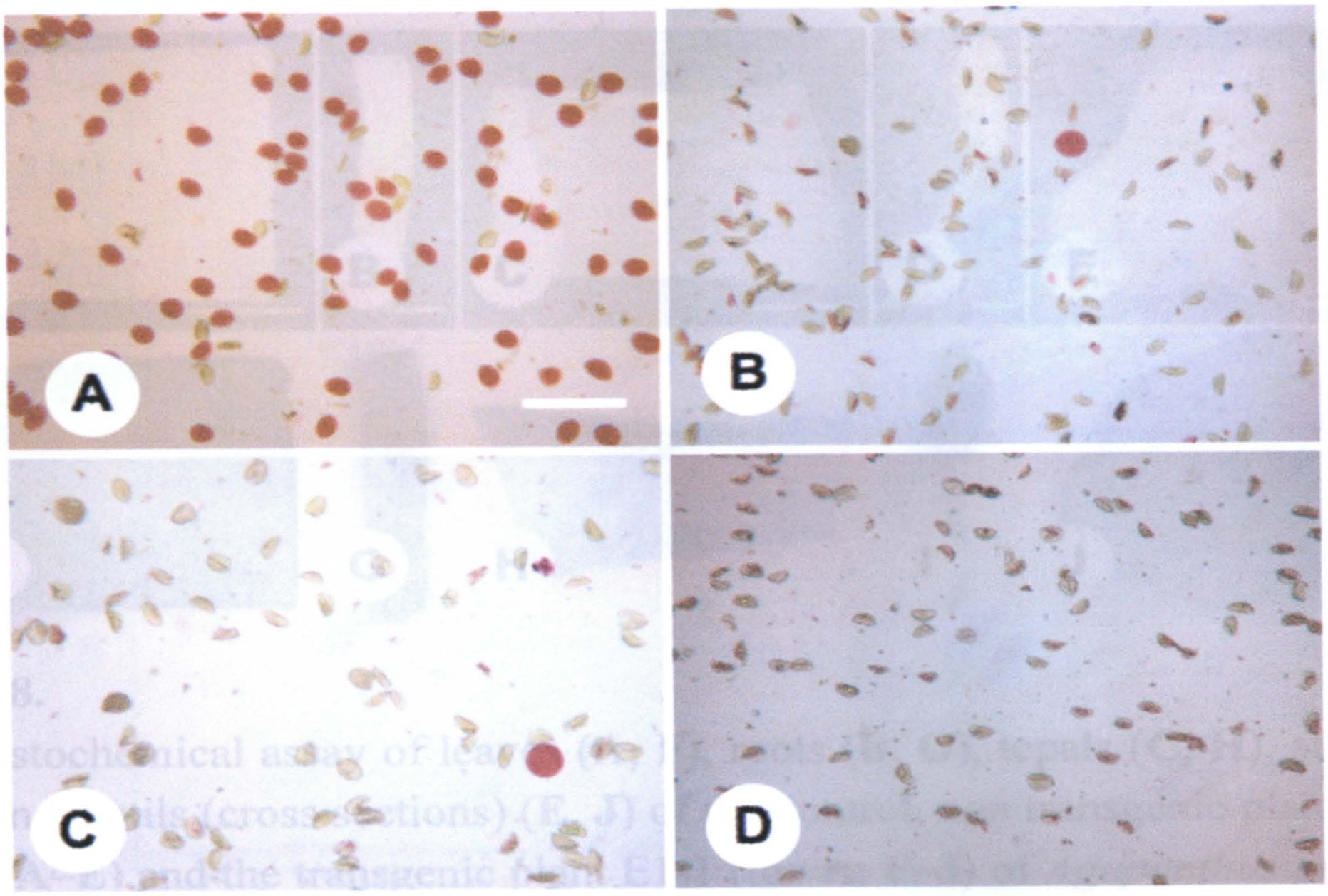


Figure 7.
Acetocarmine staining of pollen grains of the control, non transgenic plant C1-1 (**A**) and the transgenic plants L7-3 (**B**), L8-3 (**C**) and E1-1 (**D**) of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. The investigation was performed in 2005. Bar = 0.2 mm.

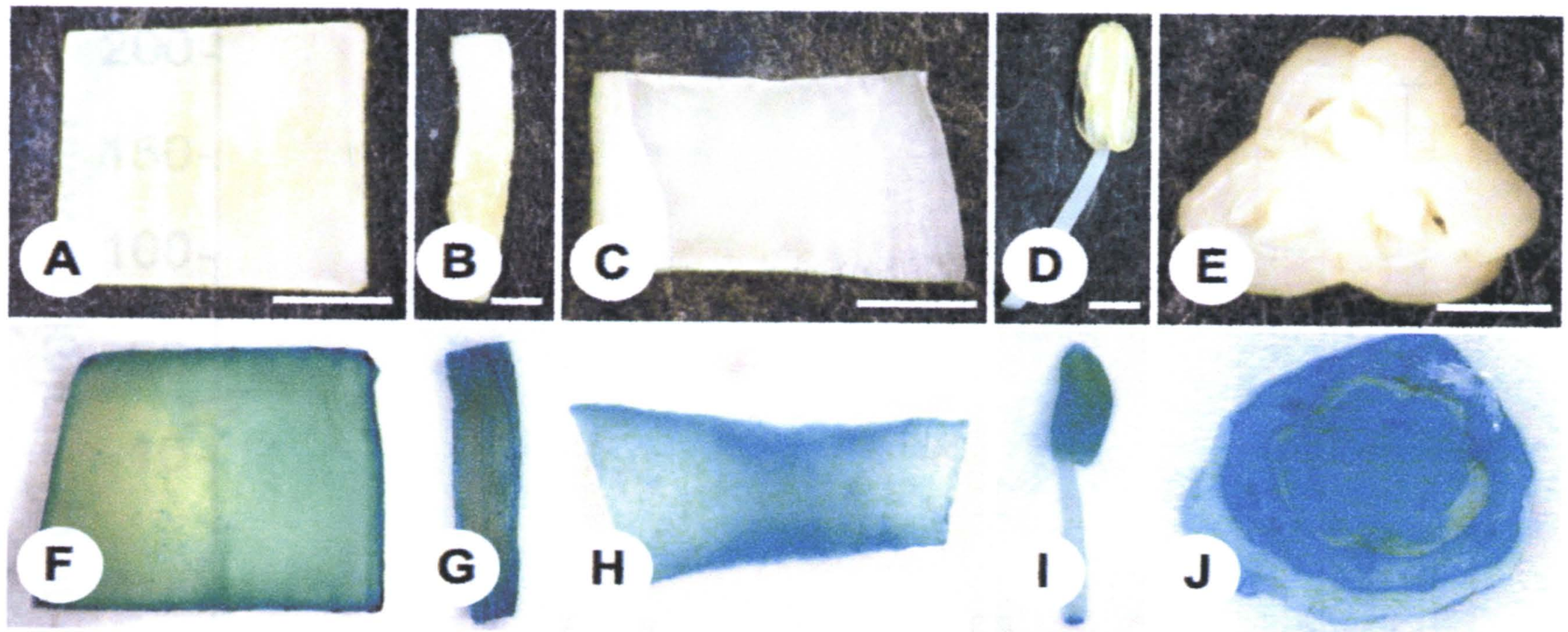


Figure 8.

GUS histochemical assay of leaves (**A**, **F**), roots (**B**, **G**), tepals (**C**, **H**), stamens (**D**, **I**) and pistils (cross sections) (**E**, **J**) of the control, non transgenic plant C1-1 (upper; **A–E**) and the transgenic plant E1-1 (lower; **F–J**) of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. The investigation was performed in 2005. Bars = 2 mm.

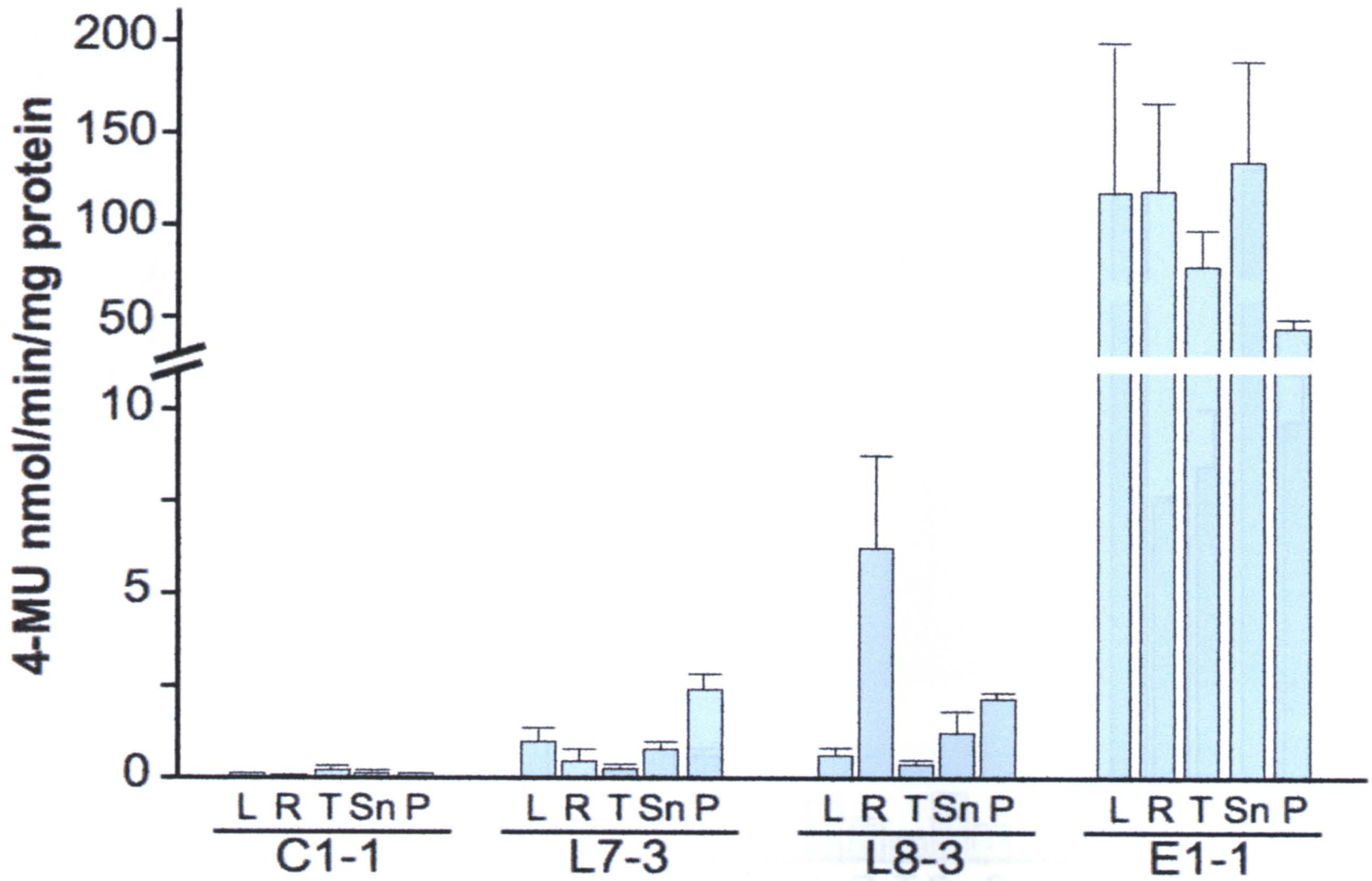


Figure 9.

Fluorometric quantification of GUS activity in various organs of the control, non transgenic plant C1-1 and the transgenic plants L7-3, L8-3 and E1-1 of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. The investigation was performed in 2005. Values represent the mean ± SE of triplicates. L, leaves; R, roots; T, tepals; Sn, stamens; P, pistils.

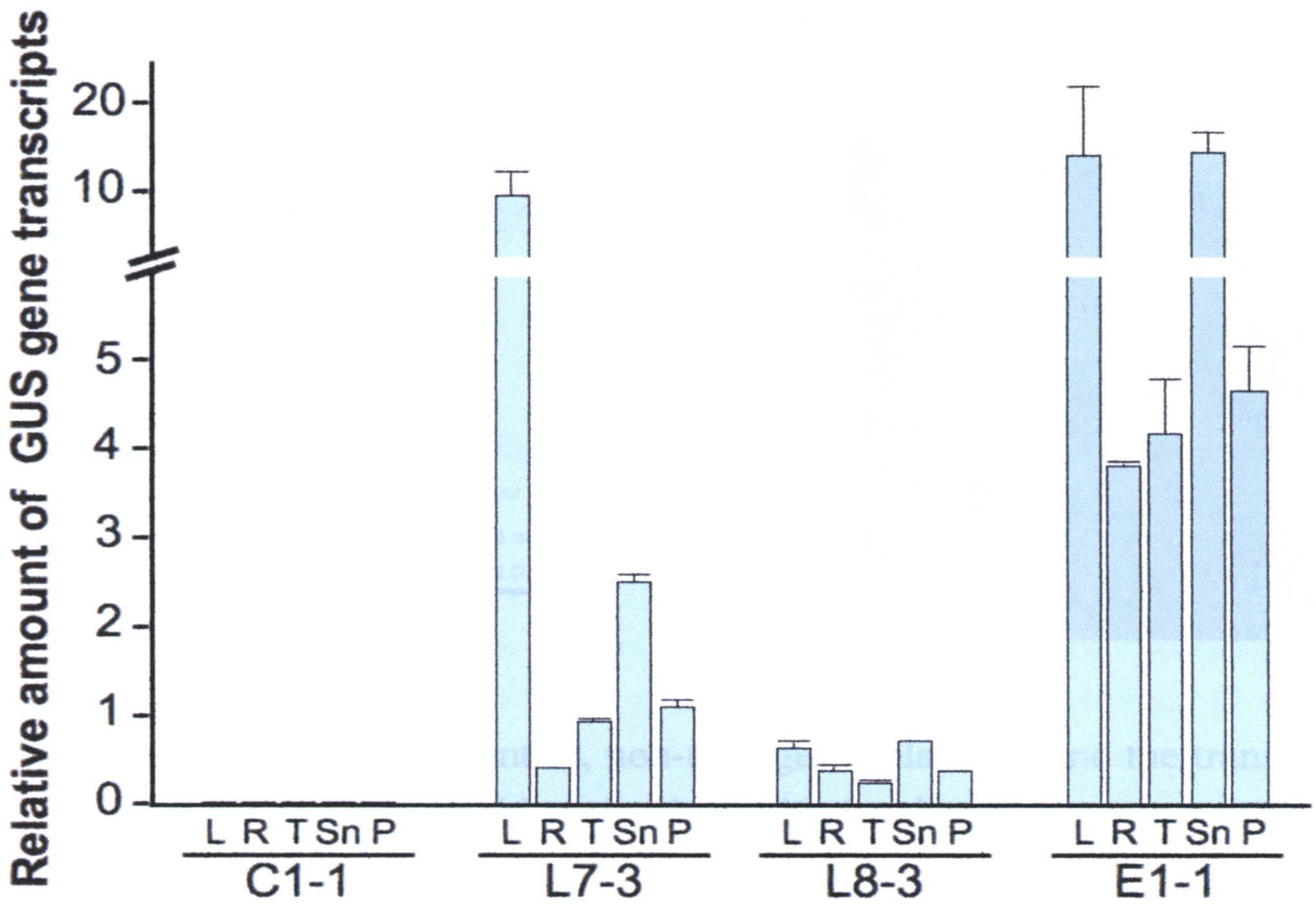


Figure 10.

Relative amount of GUS gene transcripts by real-time RT-PCR analysis in various organs of the control, non transgenic plant C1-1 and the transgenic plants L7-3, L8-3 and E1-1 of *Agapanthus praecox* ssp. *orientalis* 'ES-1'. The investigation was performed in 2005. Data were normalized to the actin gene of *A. praecox* ssp. *orientalis* (*ApAct1*). Values represent the mean ± SE of triplicates. L, leaves; R, roots; T, tepals; Sn, stamens; P, pistils.

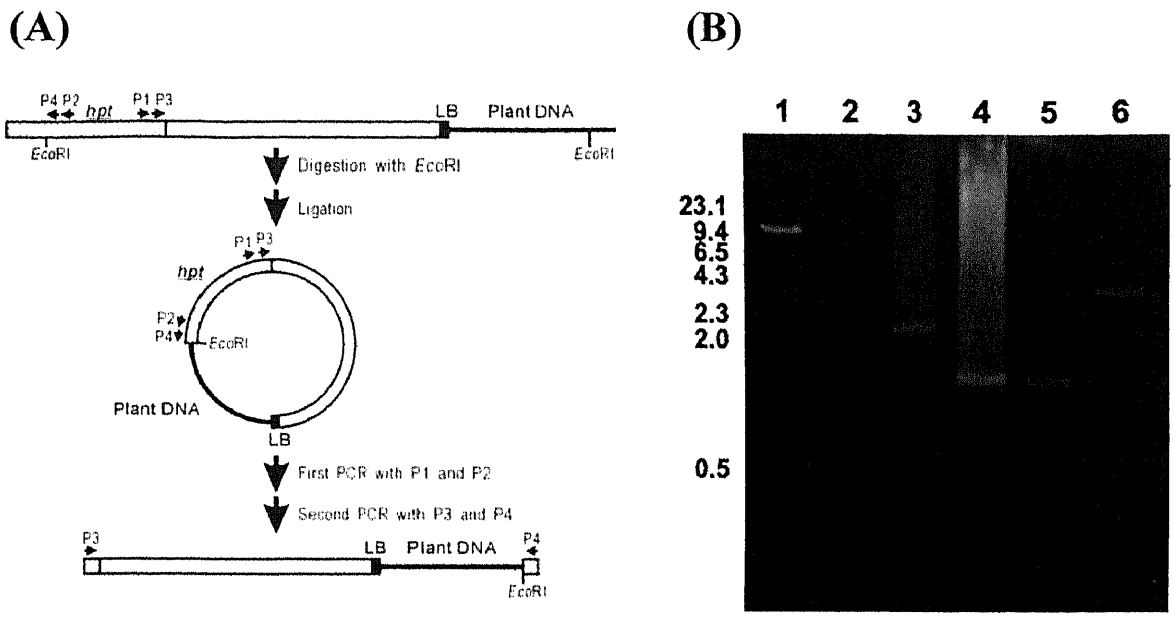


Figure 11.

Inverse PCR analysis of the control, non-transgenic plant C1 and the transgenic plants T1, T2, T3 and T4 of *Tricyrtis hirta*. (**A**) A schematic representation of the inverse PCR analysis (Hoshi et al., 2004). Genomic DNA was digested with *Eco*RI, ligated to generate circular molecules, and subjected to PCR to amplify the fragments of the junctions of inserted DNA and plant genomic DNA. LB, left border; open box, T-DNA region between the HPT gene and LB; black line, plant genomic DNA; P1, P2, P3 and P4, PCR primers. (**B**) Electrophoretic patterns of the amplified products. Lane 1, pIG121Hm plasmid as a positive control; lane 2, C1 as a negative control; lane 3, T1; lane 4, T2; lane 5, T3; lane 6, T4. Numerals indicate kbp.

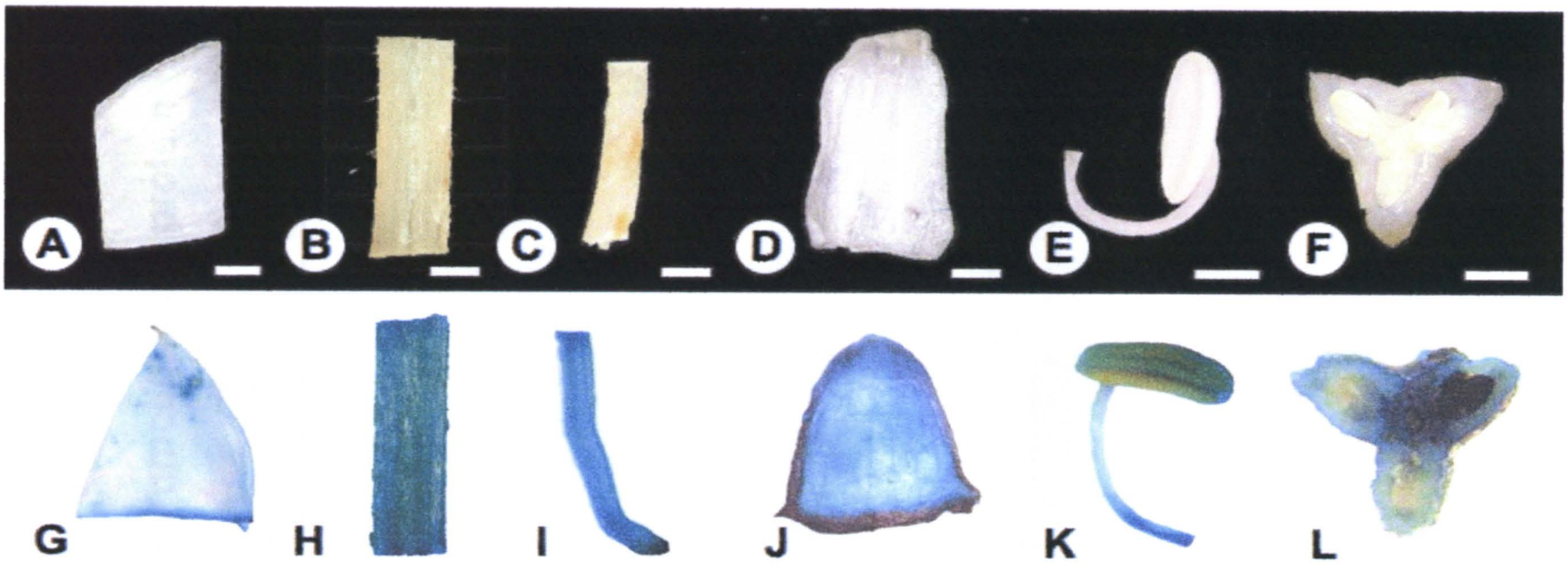


Figure 12.
GUS histochemical assay of leaves (**A**, **G**), stems (**B**, **H**) roots (**C**, **I**), tepals (**D**, **J**), stamens (**E**, **K**) and pistils (cross sections) (**F**, **L**) of the control, non transgenic plant C1 (upper; **A–F**) and the transgenic plant T1 (lower; **G–L**) of *Tricyrtis hirta*. The investigation was performed in 2005. Bars = 2 mm.

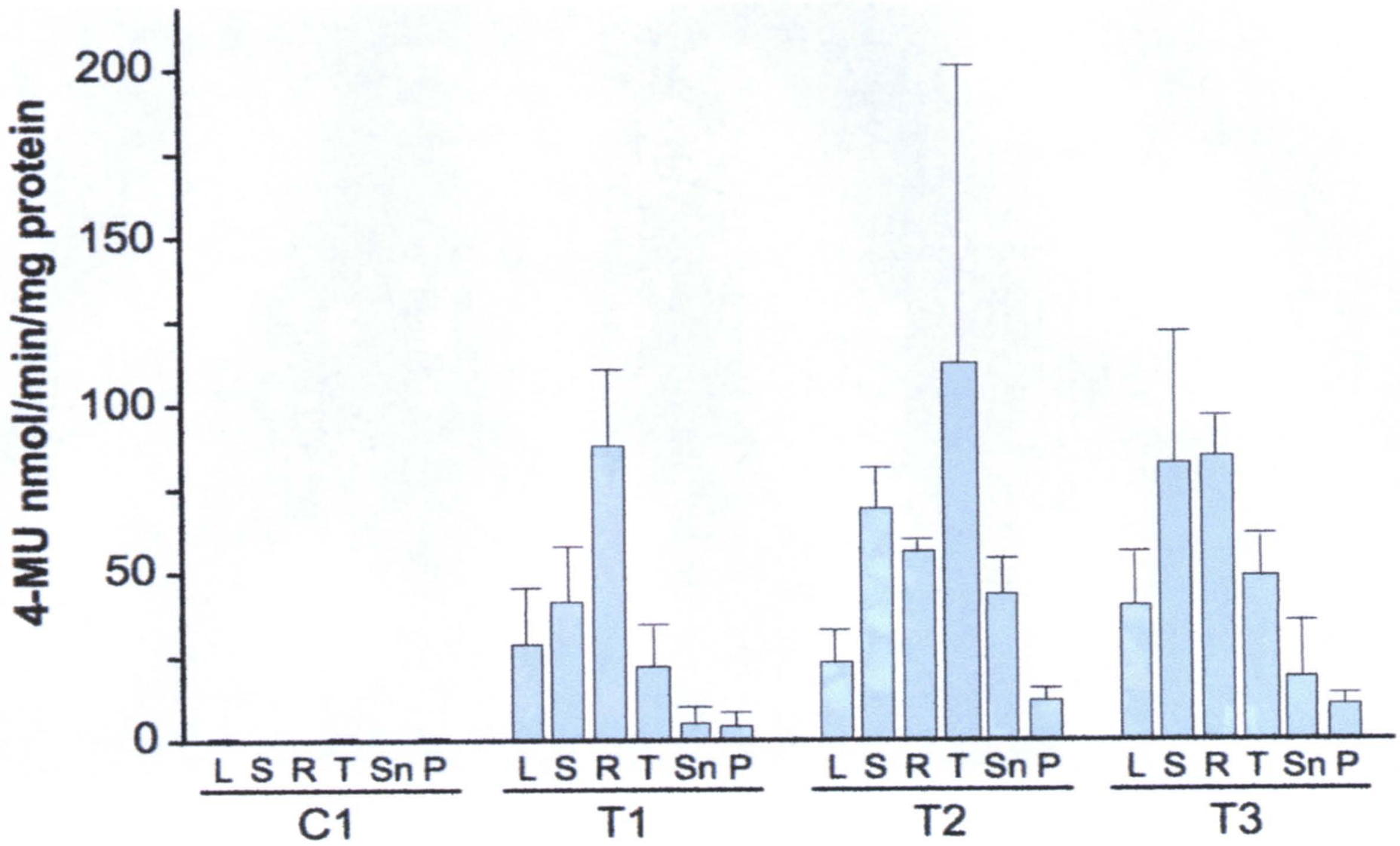


Figure 13.
Fluorometric quantification of GUS activity in various organs of the control, non-transgenic plant C1 and the transgenic lines T1, T2 and T3 of *Tricyrtis hirta*. The investigation was performed in 2005. Values represent the mean ± SE of triplicates. L, leaves; S, stems; R, roots; T, tepals; Sn, stamens; P, pistils.

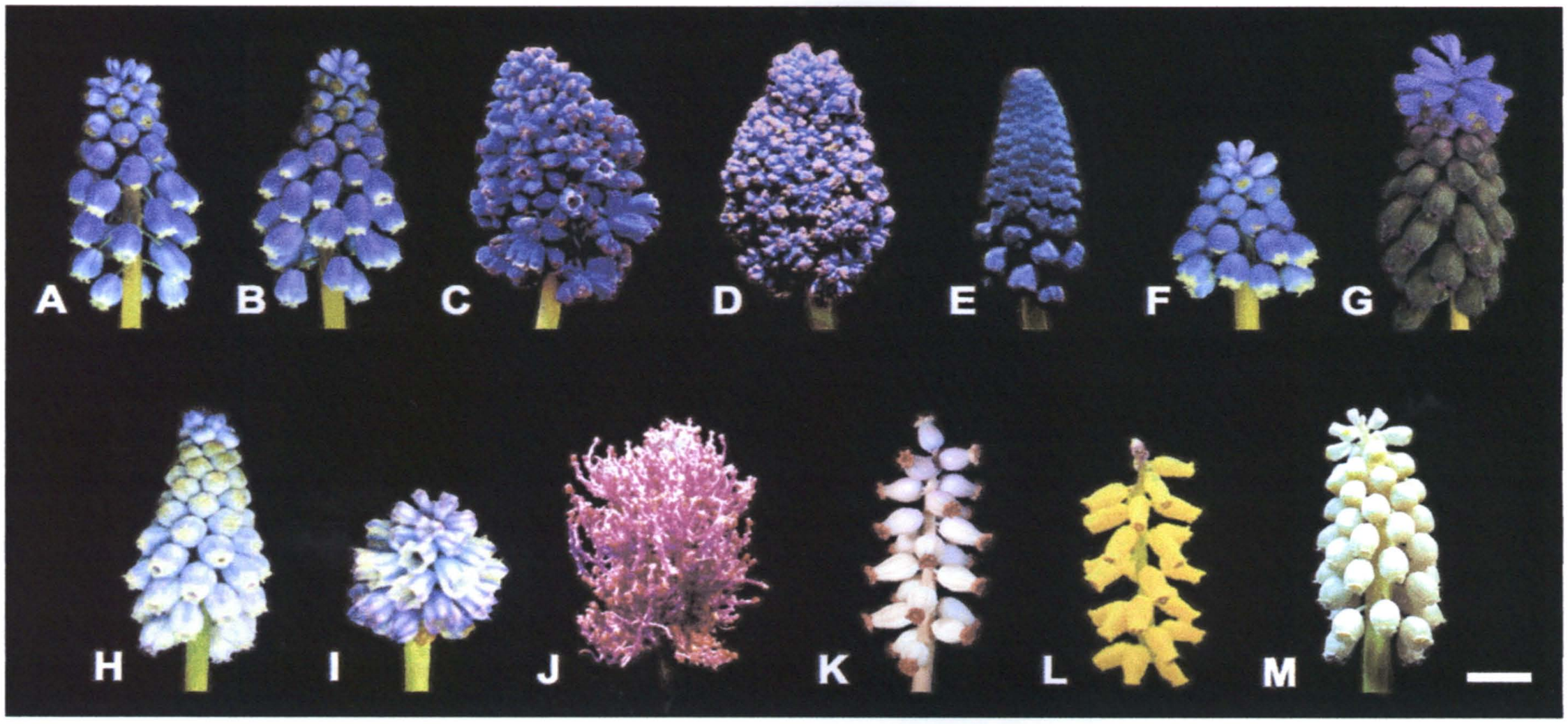


Figure 14.
Flowers of *Muscari* species and cultivars used in Chapter 3, Experiment 1. (**A**) *M. armeniacum*, (**B**) *M. armeniacum* 'Blue pearl', (**C**) *M. armeniacum* 'Blue Spike', (**D**) *M. armeniacum* 'Fantasy Creation', (**E**) *M. paradoxum*, (**F**) *M. tubergenianum*, (**G**) *M. latifolium*, (**H**) *M. armeniacum* 'Valerie Finnis', (**I**) *M. azureum*, (**J**) *M. comosum* var. *plumosum*, (**K**) *M. moschathum*, (**L**) *M. moschathum* var. *flavum*, (**M**) *M. botryoides* 'Album'. Bar = 2 cm.

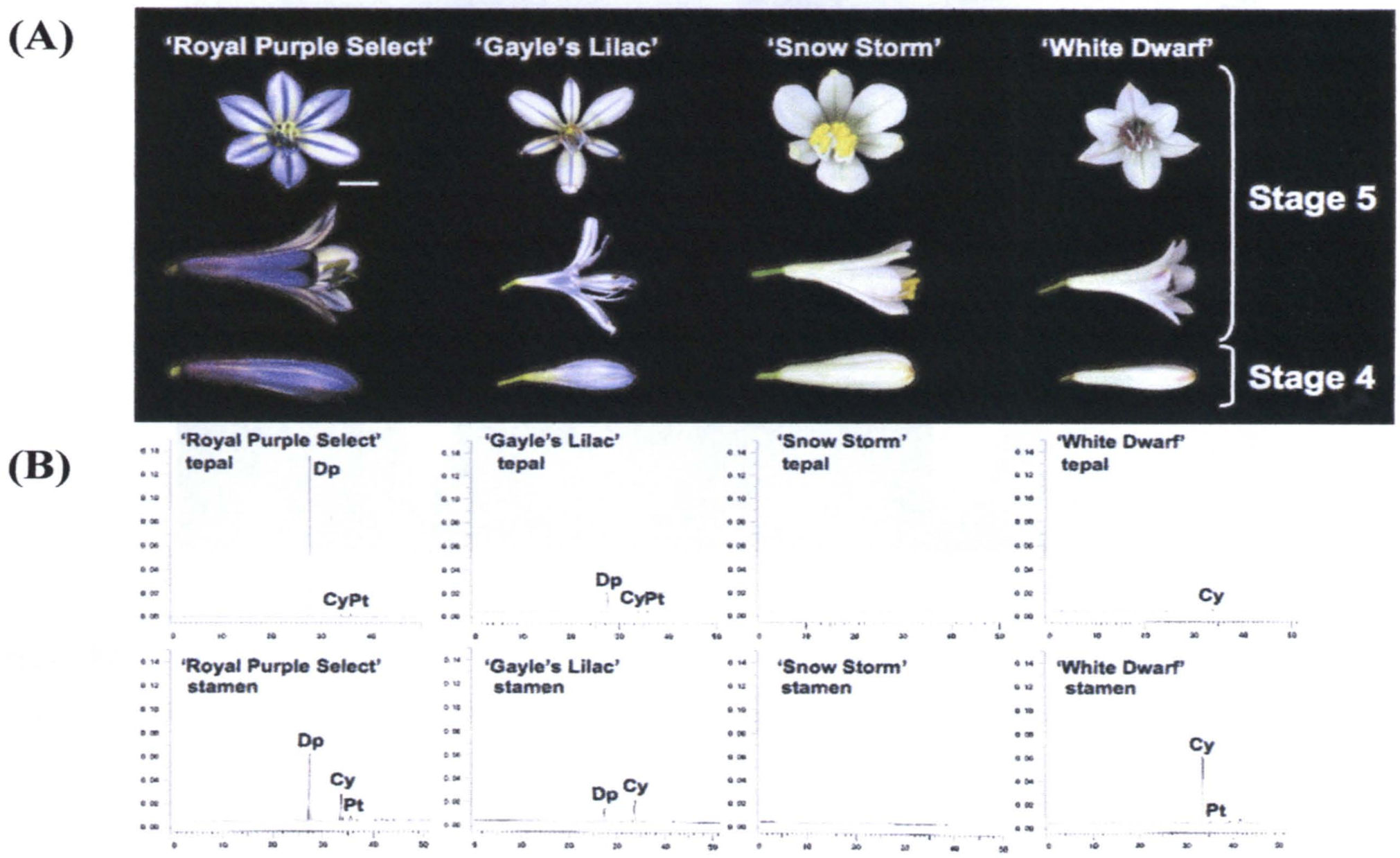


Figure 15.

Flowers and anthocyanidin accumulation in tepals and stamens of *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select', *Agapanthus* 'Gayle's Lilac', *Agapanthus* 'Snow Storm', and *Agapanthus* 'White Dwarf'. (**A**) The stage 5 (fully opened flower) flowers and the stage 4 (flower bud just before anthesis) flower buds. Bar = 1 cm. (**B**) HPLC profiles for the accumulation of anthocyanidins in tepals and stamens of the stage 4 flower buds. Dp, delphinidin; Cy, cyanidin; Pt, petunidin.

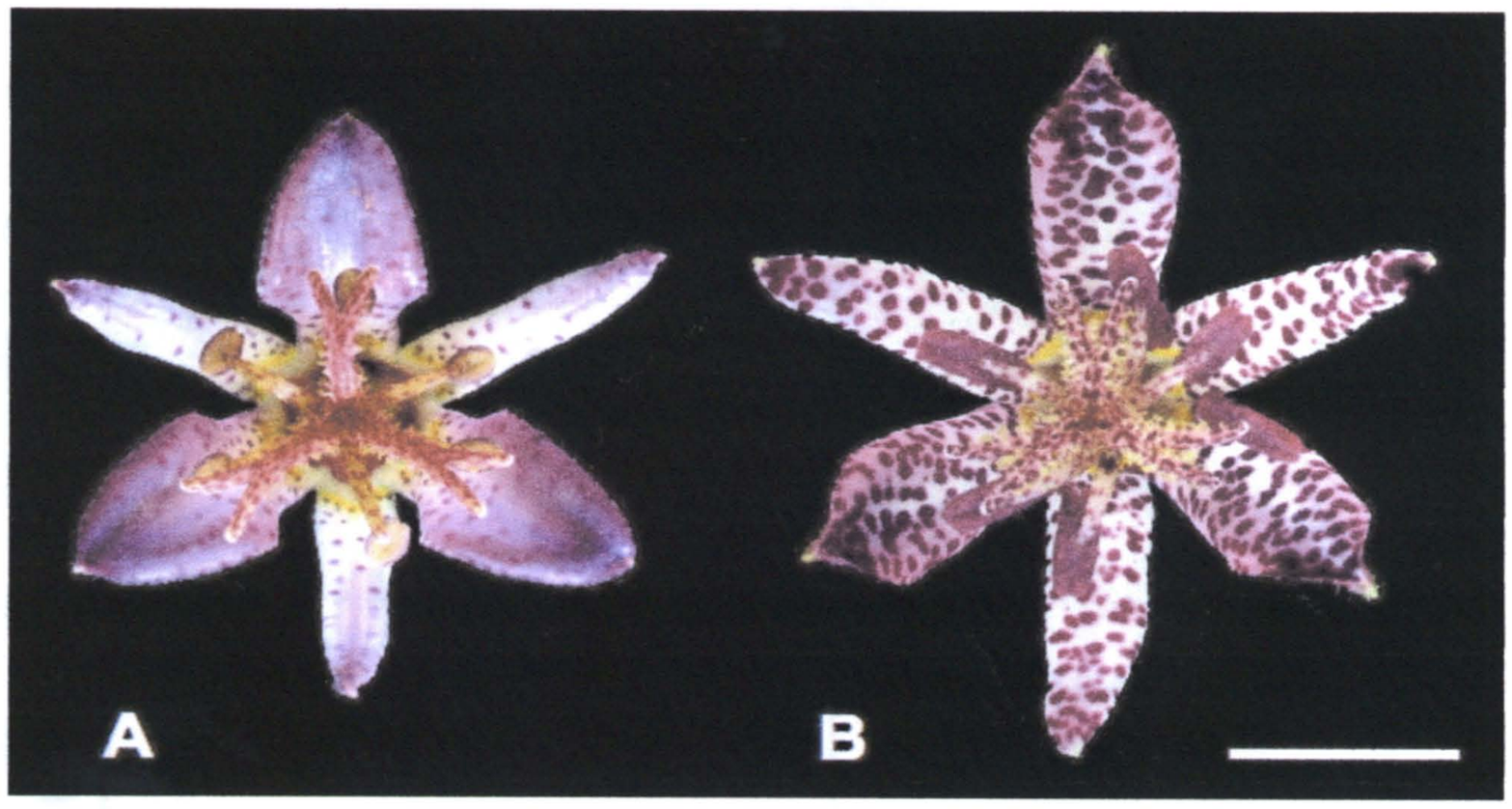


Figure 16.
Flowers of *Tricyrtis hirta* (**A**) and *T. formosana* (**B**). Bar = 1 cm.

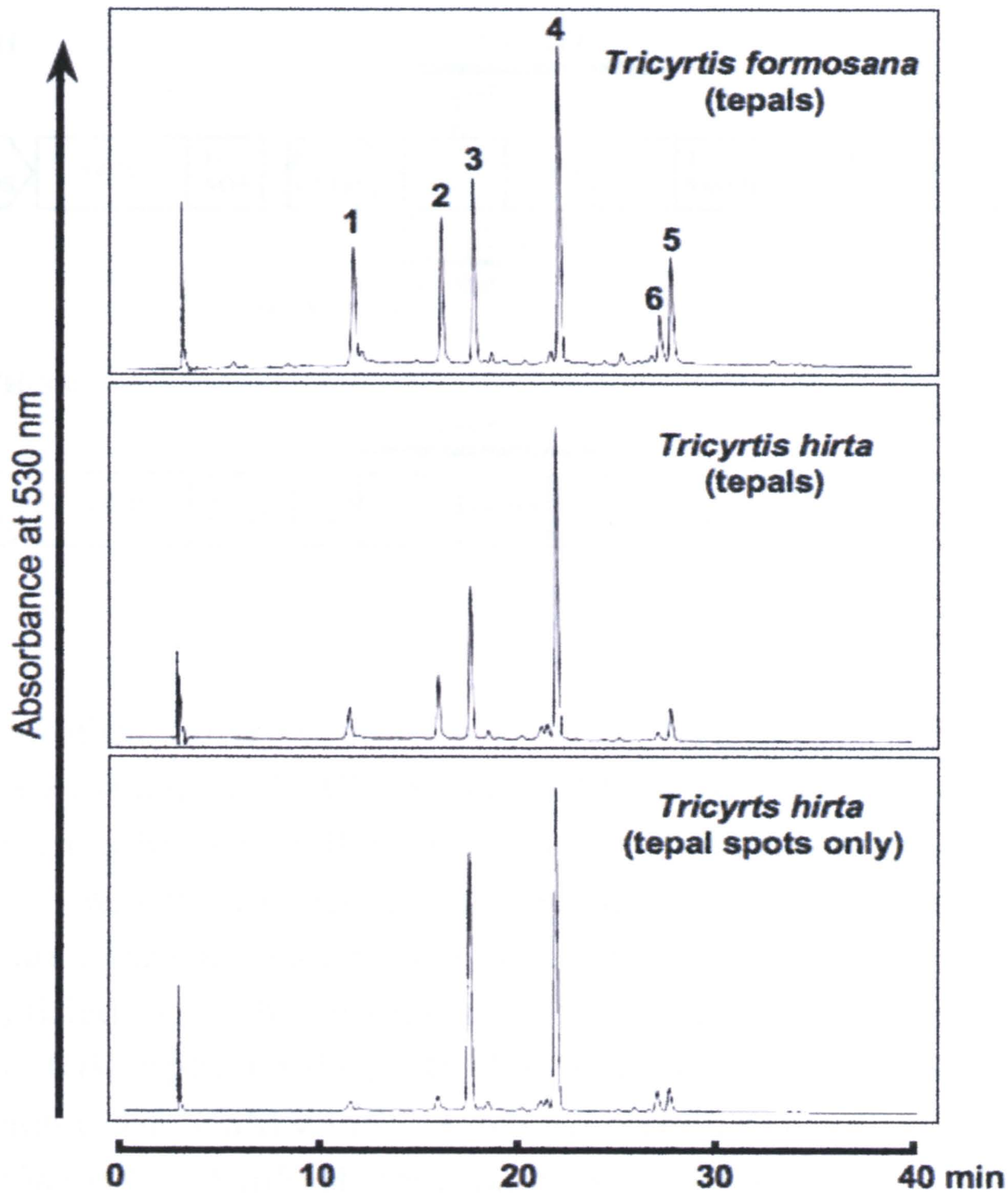
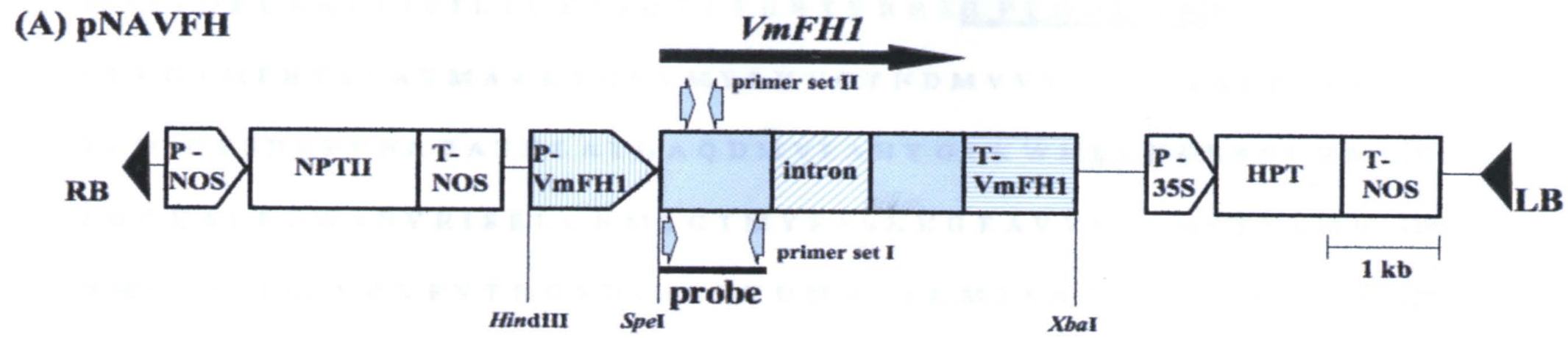


Figure 17.

HPLC profiles for the accumulation of anthocyanidins in tepals of *Tricyrtis formosana* and *T. hirta*. Peak No. 1, 8-*C*-glucosylcyanidin 3-*O*-malonylglucoside; peak No. 2, cyanidin 3-glucoside; peak No. 3, cyanidin 3-rutinoside; peak No. 4, cyanidin 3-*O*-malonylglucoside ; peak No. 5, 8-*C*-sinapylglucosylcyanidin 3-*O*-malonylglucoside; peak No. 6, unidentified anthocyanin.



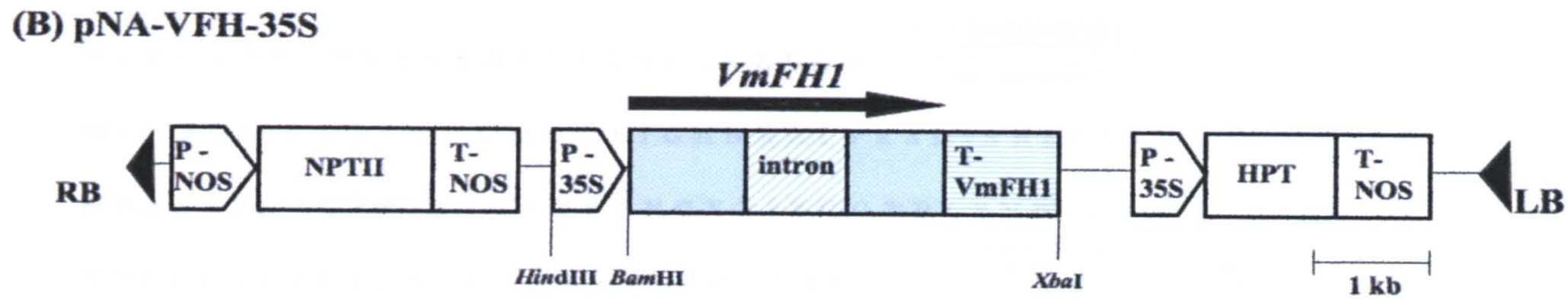


Figure 18.

T-DNA region of the binary vectors, pNAVFH (**A**) and pNA-VFH-35S (**B**). The positions of the primer set I (VFH8 and cVFH8), which was used for detecting *VmFH1* transcripts by RT-PCR in *Vinca major*, and the primer set II (VFH2L and cVFH1L), which was used for detecting foreign gene(s) by PCR in transgenic plants, are indicated over and under the map of pNAVFH. PCR products amplified with the primer set I were used as a probe for Southern hybridization. RB, right border; LB, left border; P-NOS, promoter of NOS; P-35S, promoter of CaMV35S; T-NOS, terminator of NOS; P-VmFH1, promoter of *VmFH1*; T-VmFH1, terminator of *VmFH1*.

Proline-rich region

M A I V D F L A A F L I F I L T Q K L I Q T L F R R T Y R R K L P P G P K G W P V I G A L (45)

P Y L G T M P H T S L A Y M A K K Y G P V M Y L K V G T N D M V V V T T P D A A R A F L K (90)

T L D I N F S N R P P N A G A T H L A Y G A Q D M V F A H Y G P K W K L L R K L S N L H M (135)

L G G K A L E D W S N V R I S E L G H M L G T M Y E S S K K G E A V V V A E M L T Y A M A (180)

N M I G Q V I L S R R V F V T K G S D S N E F K D M V V E L M T S A G L F N I G D F I P S (225)

I A W M D L Q G I E G G M K R L H K K F D V L L T K L L E E H K E S S S K R K G K P D L L (270)

Oxygen-binding pocket

D Y V L A N R D N S E G E R L T T T N I K A L L L N L F S A G T D T S S S I I E W A L S E (315)

M L R N P S I L K R A Q H E M D Q V I G R N R R L V E S D I S R L P Y L Q A I C K E T F R (360)

K H P S T P L N L P R I A T E A C E V N G Y Y I P K G T R L S V N I W A I G R D P D V W E (405)

Heme-binding region

N P L E F N P D R F L S G K N A K I D P R G N D F E L I P F G A G R R I C A G T R M G I L (450)

L V E Y I L G T L V H S F D W N L P S S V T K L N M D E S F G L A L Q K V V P L A A L V T (495)

P R L P I N A Y S P (540)

Figure 19.

Deduced amino-acid sequence of *VmFH1* isolated from *Vinca major*. Gray-boxed residues indicate those conserved in the F3'5'H genes previously reported for *Petunia hybrida Hf1* and *Hf2*, *Solanum melongena*, *Gentiana triflora*, *Eustoma grandiflorum*, *Eustoma russellianum*, *Catharanthus roseus*, and *Campanula medium*. White boxes indicate a putative proline-rich region, oxygen-binding pocket, and heme-binding region.

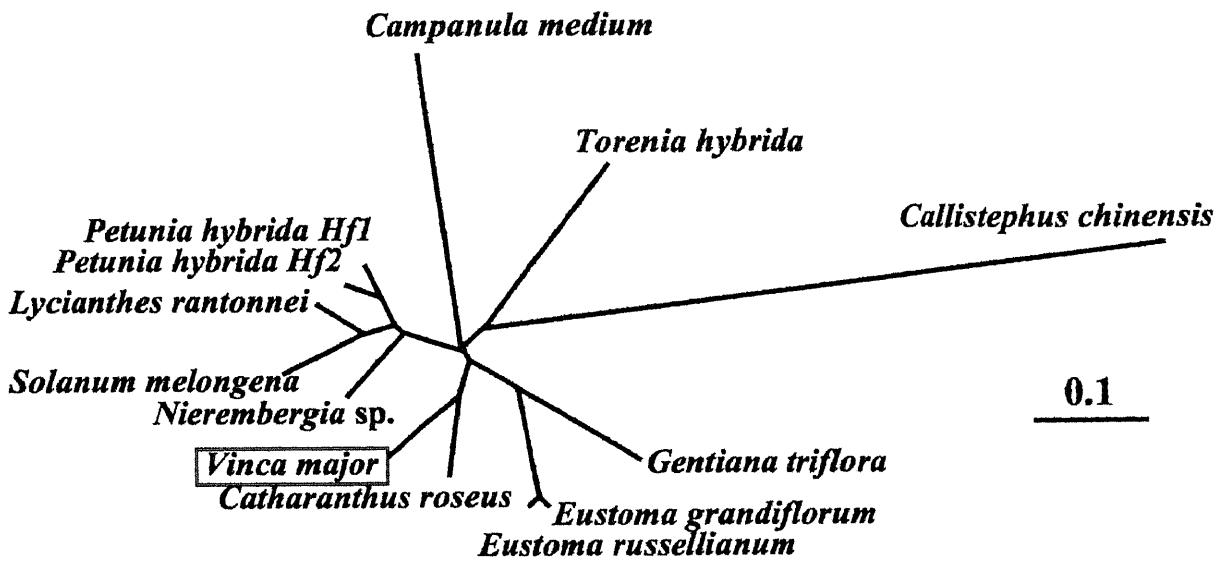
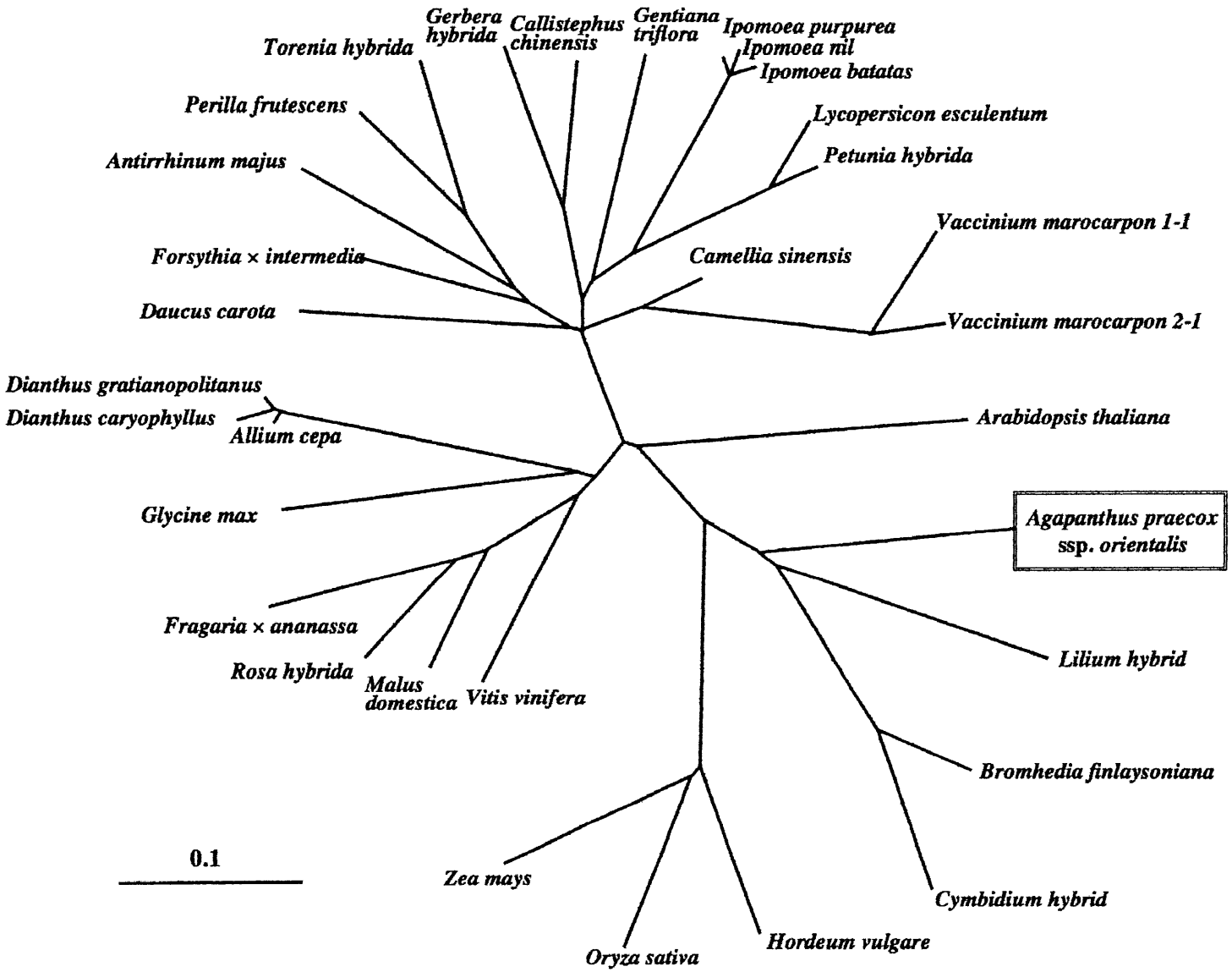


Figure 20.

Molecular phylogenic tree of the deduced amino acid sequences of *VmFH1* from *Vinca major* and the *F3'5'H* genes from several other plant species. This phylogenic tree was generated by using CLUSTAL W (Thomopson et al., 1994) and TREEVIEW programs (Page, 1996). The scale bar indicates 0.1 amino acid substitutions per site. The accession number in the GenBank/EMBL/DDBJ databases is as follows: *V. major VmFH1* (this study; AB078781), *Petunia hybrida Hf1* (Z22545) and *Hf2* (Z22544), *Lycianthes rantonnei* (AF313490), *Solanum melongena* (E05111), *Nierembergia* sp. (AB078514), *Catharanthus roseus* (AJ11862), *Eustoma russellianum* (D14589), *Eustoma grandiflorum* (U72654), *Gentiana triflora* (D85184), *Callistephus chinensis* (AF313489), *Torenia hybrida* (AB12925), and *Campanula medium* (D14590).


Gerbera hybrida
Callistephus chinensis
Gentiana triflora
Ipomoea purpurea
Ipomoea nil
Ipomoea batatas
Torenia hybrida
Perilla frutescens
Lycopersicon esculentum
Petunia hybrida
Antirrhinum majus
Vaccinium marocarpon 1-1
Forsythia × intermedia
Camellia sinensis
Daucus carota
Vaccinium marocarpon 2-1
Dianthus gratianopolitanus
Dianthus caryophyllus
Allium cepa
Arabidopsis thaliana
Glycine max
Agapanthus praecox ssp. orientalis
Fragaria × ananassa
Lilium hybrid
Rosa hybrida
Malus domestica
Vitis vinifera
Bromhedia finlaysoniana
0.1
Zea mays
Cymbidium hybrid
Hordeum vulgare
Oryza sativa

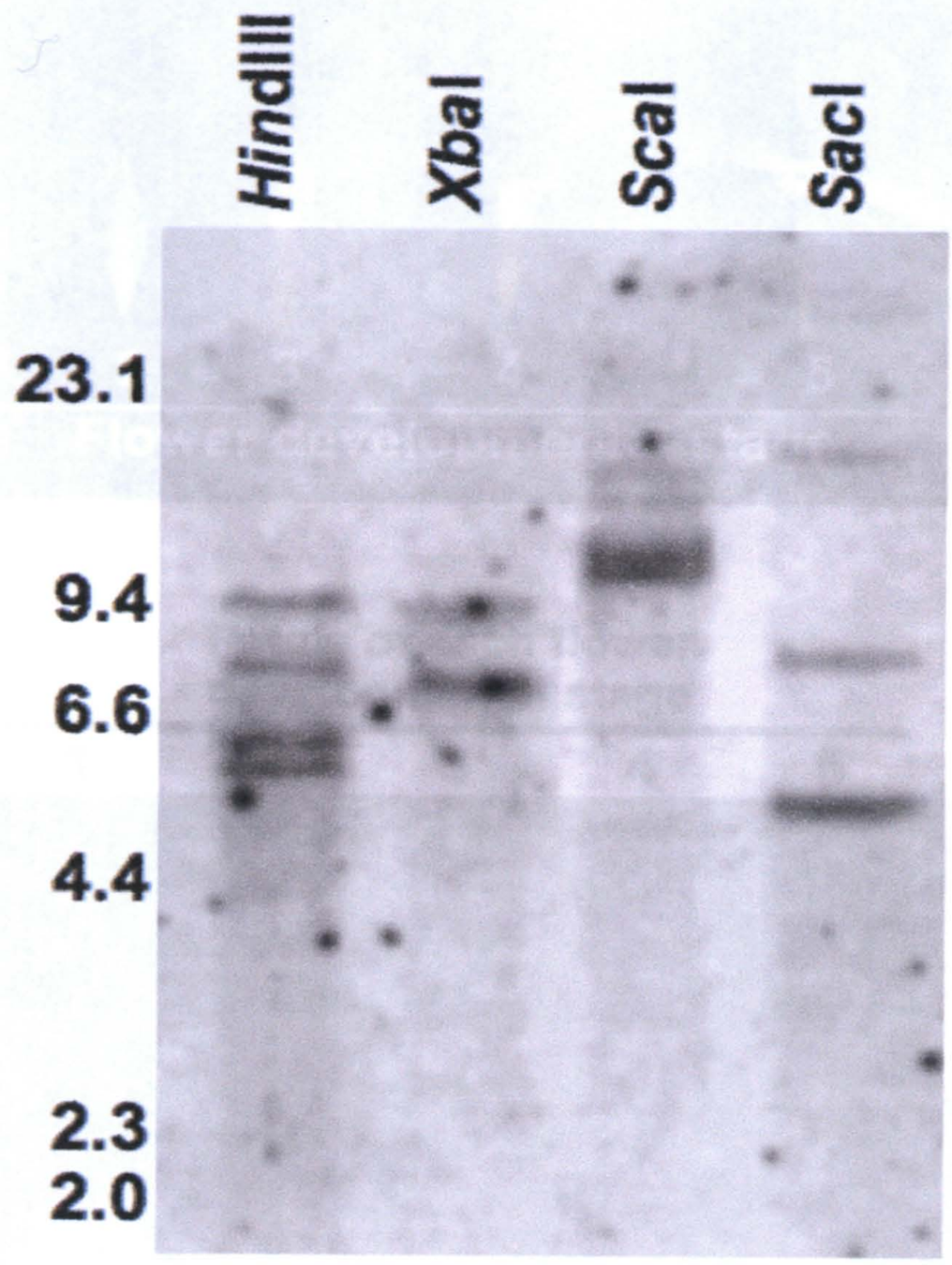


Figure 21.

Southern hybridization of *Vinca major* using a 0.82-kbp DNA fragment from *VmFH1* as a probe. Genomic DNA isolated from young leaves were digested with *Hin*dIII, *Xba*I, *Sca*I, or *Sac*I, and hybridized with PCR products amplified using pNAVFH (Figure 18A) as a template and the primer set, VFH8 and cVFH8 (Figure 18A). Numerals indicate kbp.

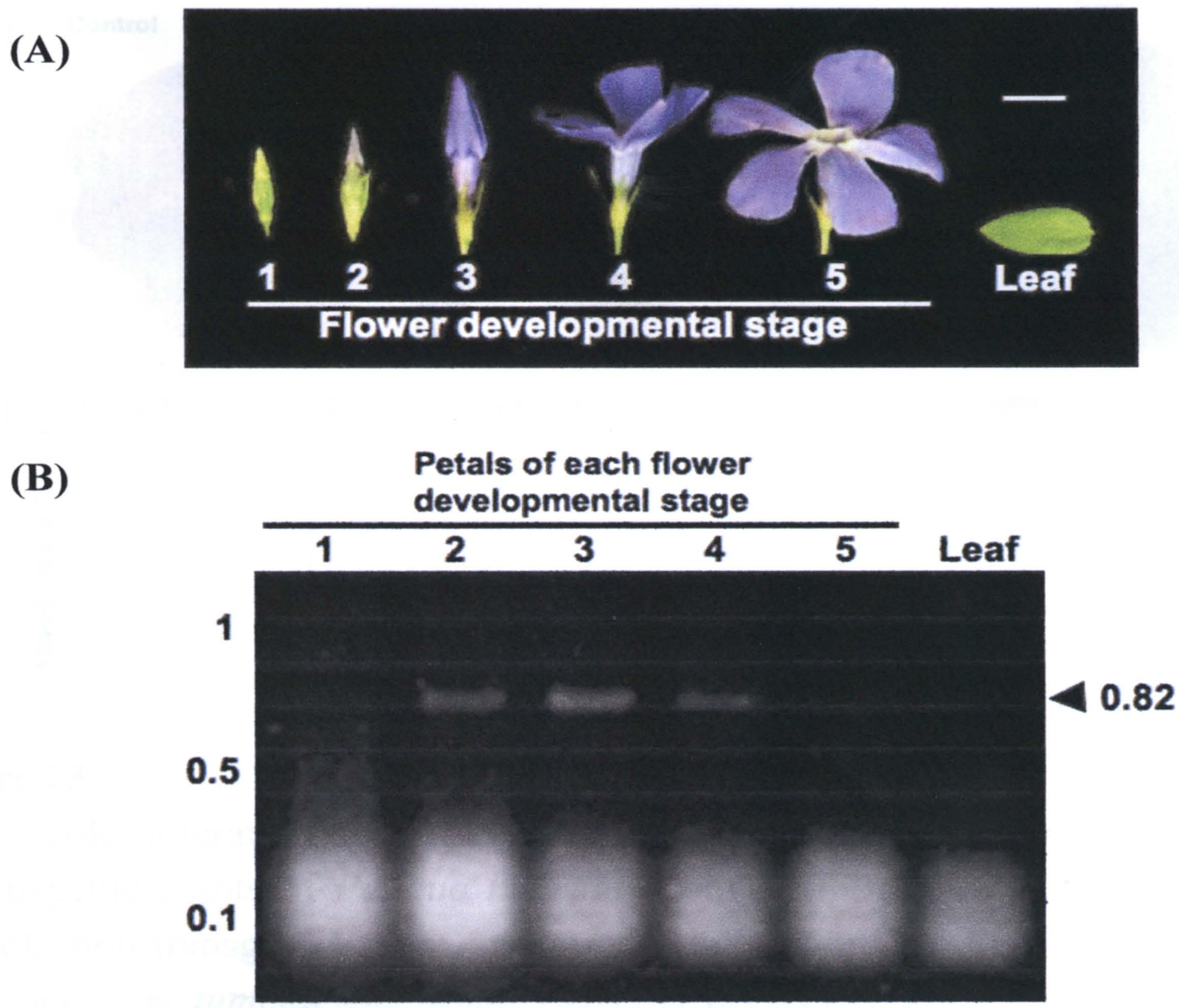


Figure 22.
Flower development and *VmFH1* expression in petals of *Vinca major*. (**A**) Developmental stages of the flower: stage 1, flower bud with non-pigmented (white) petals; stage 2, flower bud with pale blue petals; stage 3, flower bud with blue petals; stage 4, opening flower; and stage 5, fully opened flower. Bar = 1 cm. (**B**) RT-PCR analysis of *VmFH1* expression in petals of each developmental stage of the flower and in young leaves. RT-PCR was carried out using total RNA as a template and the primer set, VFH8 and cVFH8 (Figure 18A). Numerals indicate kbp.

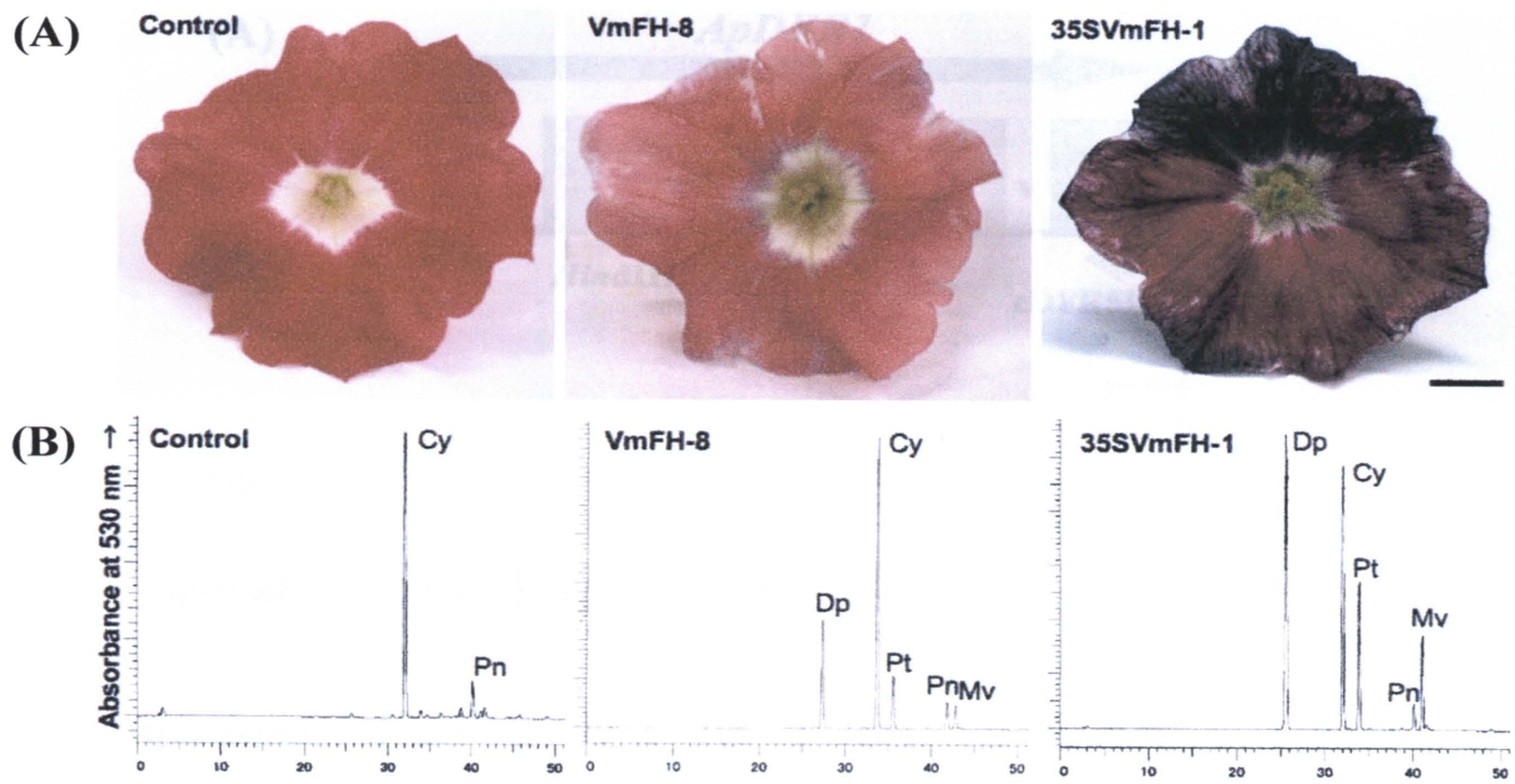


Figure 23.

Flower color alteration by heterologous expression of *VmFH1* from *Vinca major* in transgenic plants of *Petunia hybrida* 'Polo Red Target'. (**A**) Flowers of the control, non-transgenic plant, the transgenic plant VmFH-8 obtained with *Agrobacterium tumefaciens* strain EHA101/pNAVFH, and the transgenic plant 35SVmFH-1 obtained with *A. tumefaciens* strain EHA101/pNA-VFH-35S. Bar = 1 cm. (**B**) HPLC profiles for the accumulation of anthocyanidins in petals of the control, non-transgenic plant and the transgenic plants VmFH-8 and 35SVmFH-1. Dp, delphinidin; Cy, cyanidin; Pt, petunidin; Pn, peonidin; Mv, malvidin.

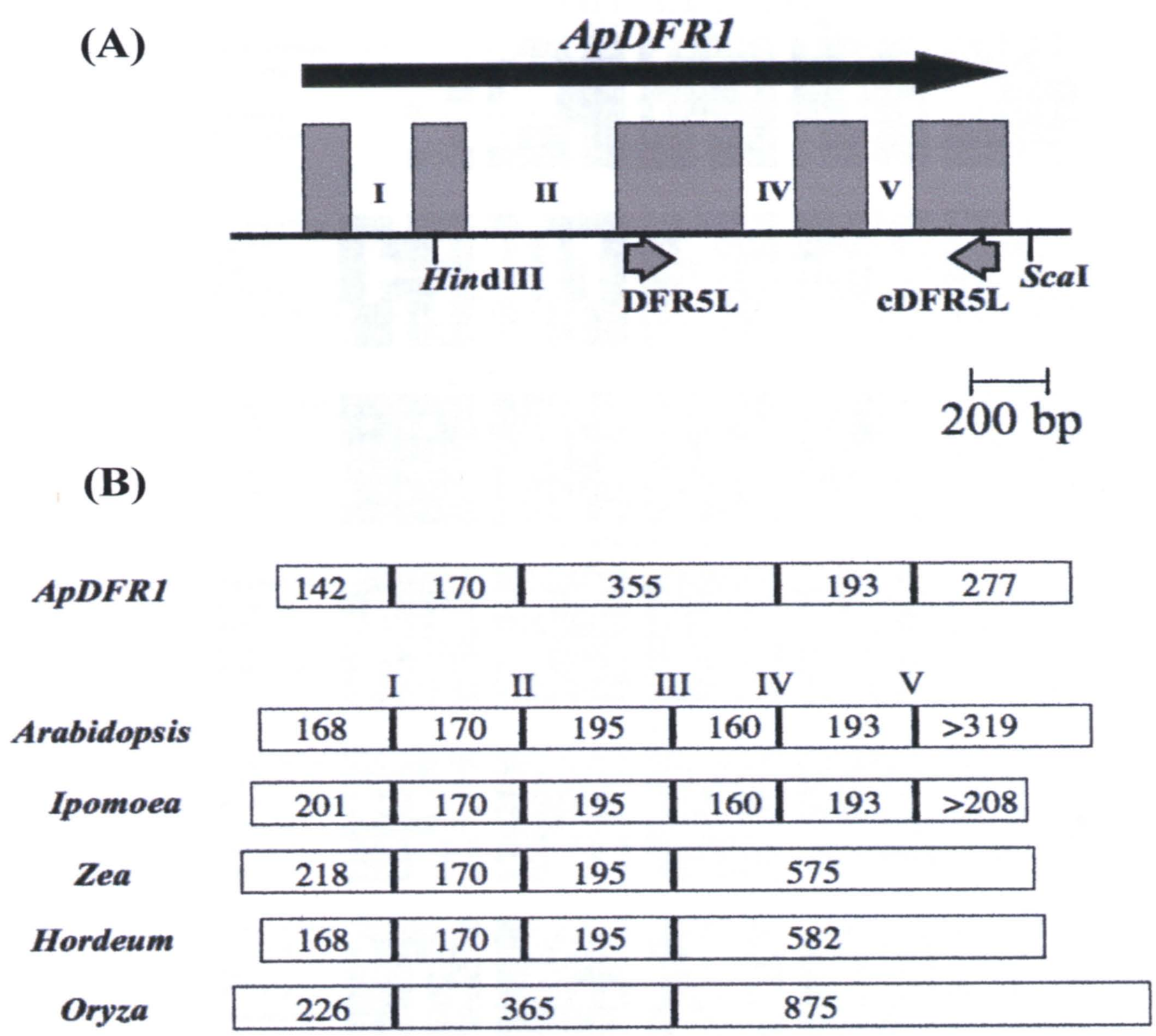


Figure 24.
Genomic structure of *ApDFR1* isolated from *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select'. (**A**) Physical maps of the genomic region harboring *ApDFR1*. Gray boxes represent exon. Roman numerals indicate the position of each intron. The positions of the primer set, DFR5L and cDFR5L, used for detecting *ApDFR1* transcripts by RT-PCR are indicated under the map. In addition, PCR products amplified with this primer set and p35S-ADFR (constracted in Chapter 4, Experiment 1) as a template were used as a probe for Southern hybridization. (**B**) Comparison of the genomic structure of *ApDFR1* and several DFR genes previously reported for *Arabidopsis thaliana* (Shirley et al., 1992), *Ipomoea nil* (Inagaki et al., 1999), *Zea mays* (Schwarz-Sommer et al., 1987), *Hordeum vulgare* (Kristiansen and Rohde, 1991), and *Oryza sativa* (Nakai et al., 1998). White boxes represent exon. Numerals in the white boxes and Roman numerals indicate the length of each exon in bp and the position of each intron, respectively.

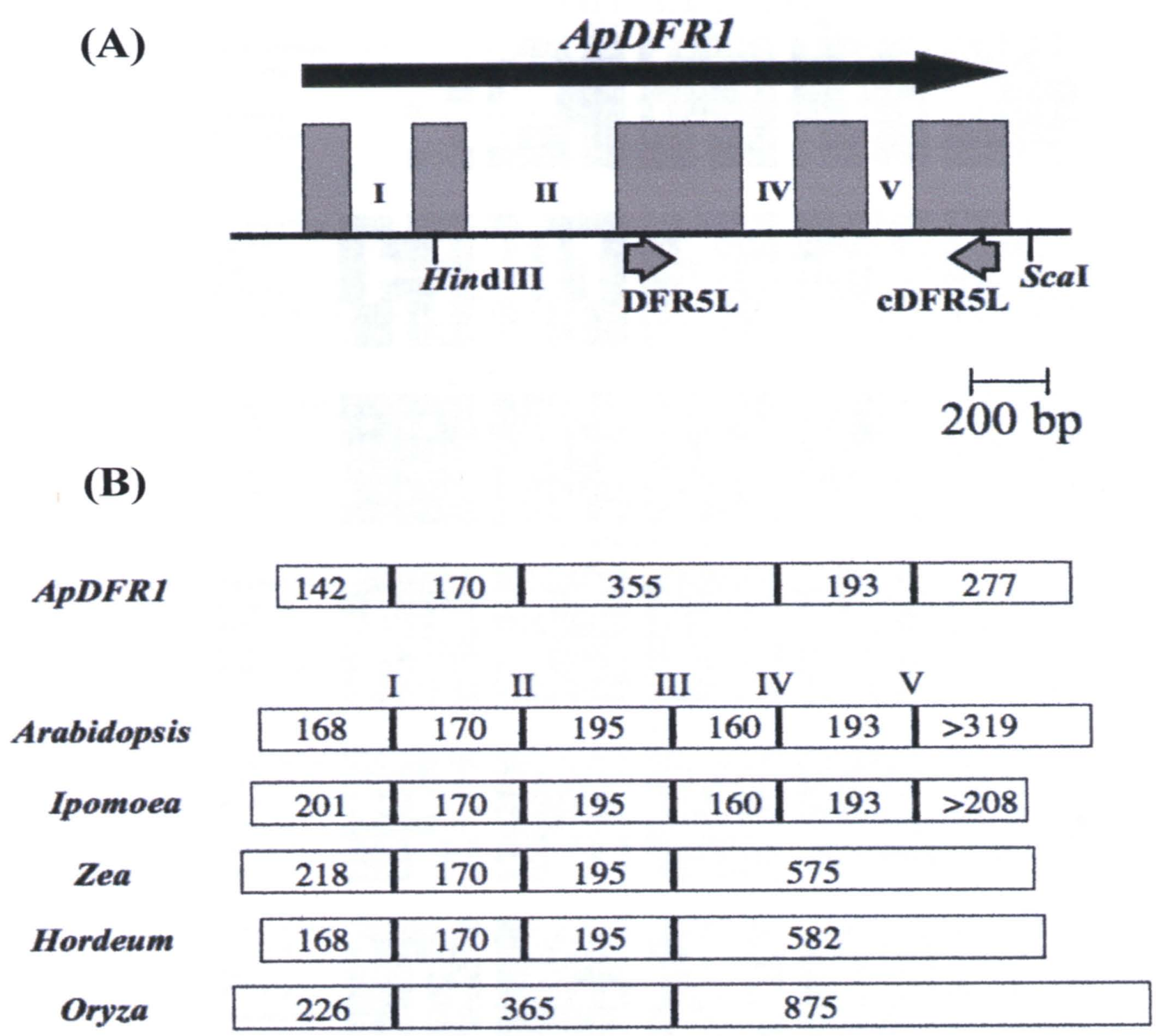


Figure 24.
Genomic structure of *ApDFR1* isolated from *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select'. (**A**) Physical maps of the genomic region harboring *ApDFR1*. Gray boxes represent exon. Roman numerals indicate the position of each intron. The positions of the primer set, DFR5L and cDFR5L, used for detecting *ApDFR1* transcripts by RT-PCR are indicated under the map. In addition, PCR products amplified with this primer set and p35S-ADFR (constracted in Chapter 4, Experiment 1) as a template were used as a probe for Southern hybridization. (**B**) Comparison of the genomic structure of *ApDFR1* and several DFR genes previously reported for *Arabidopsis thaliana* (Shirley et al., 1992), *Ipomoea nil* (Inagaki et al., 1999), *Zea mays* (Schwarz-Sommer et al., 1987), *Hordeum vulgare* (Kristiansen and Rohde, 1991), and *Oryza sativa* (Nakai et al., 1998). White boxes represent exon. Numerals in the white boxes and Roman numerals indicate the length of each exon in bp and the position of each intron, respectively.

Figure 25.

Alignment of the deduced amino acid sequences of *ApDFR1* from *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select' and the DFR genes previously reported for *Gerbera hybrida*, *Rosa hybrida*, *Petunia hybrida*, *Hordeum vulgare* and *Zea mays*. Identical amino acid residues are shown with reverse contrast characters. Arrows indicate a putative NADP-binding domain and substrate-binding site. A single amino acid residue directly influencing the substrate specificity, asparagine 134, in the DFR gene of *Gerbera hybrida* (Johnson et al., 2001) is represented by an asterisk.

Figure 26.

Molecular phylogenic tree of the deduced amino acid sequences of *ApDFR1* from *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select' and the DFR genes from several other plant species. This phylogenic tree was generated by using the CLUSTAL W (Thomopson et al., 1994) and TREEVIEW programs (Page, 1996). The scale bar indicates 0.1 amino acid substitutions per site. The accession number in the GenBank/EMBL/DDBJ databases are as follows: *A. praecox* ssp. *orientalis* (this study; AB099529), *Allium cepa* (AF268383), *Antirrhinum majus* (X15536), *Arabidopsis thaliana* (AB033294), *Bromhedia finlaysoniana* (AF007096), *Callistephus chinensis* (Z67981), *Camellia sinensis* (AB018685), *Cymbidium hybrid* (AF017451), *Daucus carota* (AF184271), *Dianthus caryophyllus* (Z67983), *Dianthus gratianopolitanus* (AF291097), *Forsythia* × *intermedia* (Y09127), *Fragaria* × *ananassa* (AF029685), *Gentiana triflora* (D85185), *Gerbera hybrida* (Z17221), *Glycine max* (AF167556), *Hordeum vulgare* (S69616), *Ipomoea batatas* (AB019243), *Ipomoea nil* (AB006792), *Ipomoea purpurea* (AB018438), *Lilium* hybrid (AB058641), *Lycopersicon esculentum* (Z18277), *Malus domestica* (AF117268), *Oryza sativa* (AB003495), *Perilla frutescens* (AB002817), *Petunia hybrida* (AF233639), *Rosa hybrida* (D85102), *Torenia hybrida* (AB012924), *Vaccinium marocarpon 1-1* (AF483835), *Vaccinium marocarpon 2-1* (AF483836), *Vitis vinifera* (X75964) and *Zea mays* (Y16040).

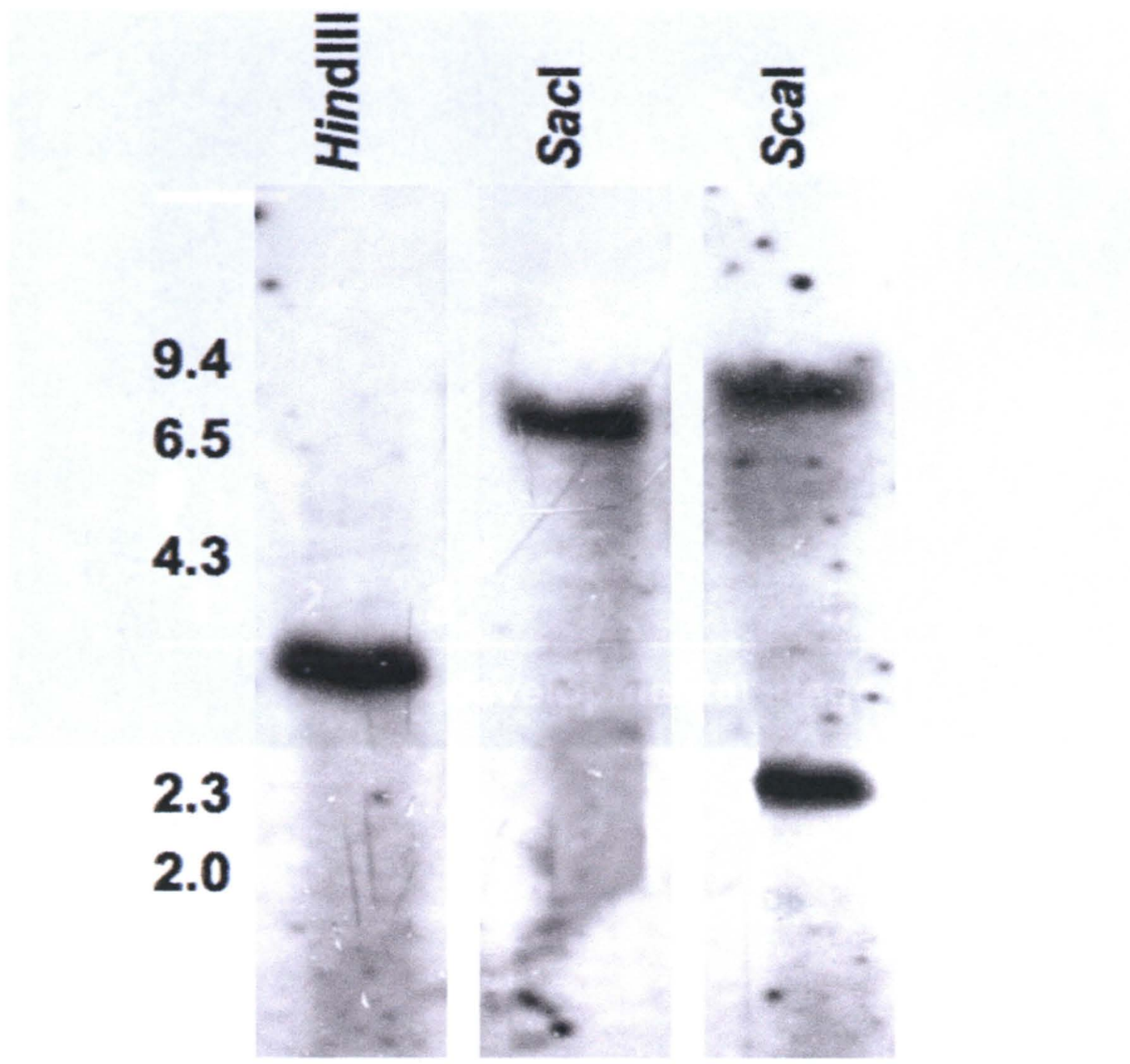


Figure 27.
Southern hybridization of *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select' using a 0.81-kbp DNA fragment from *ApDFR1* as a probe. Genomic DNA isolated from young leaves were digested with *Hin*dIII, *Sac*I or *Sca*I, and hybridized with PCR products amprifhed using p35S-ADFR (constracted in Chapter 4, Experiment 1) as a template and the primer set, DFR5L and cDFR5L (Figure 24A). Numerals indicate kbp.

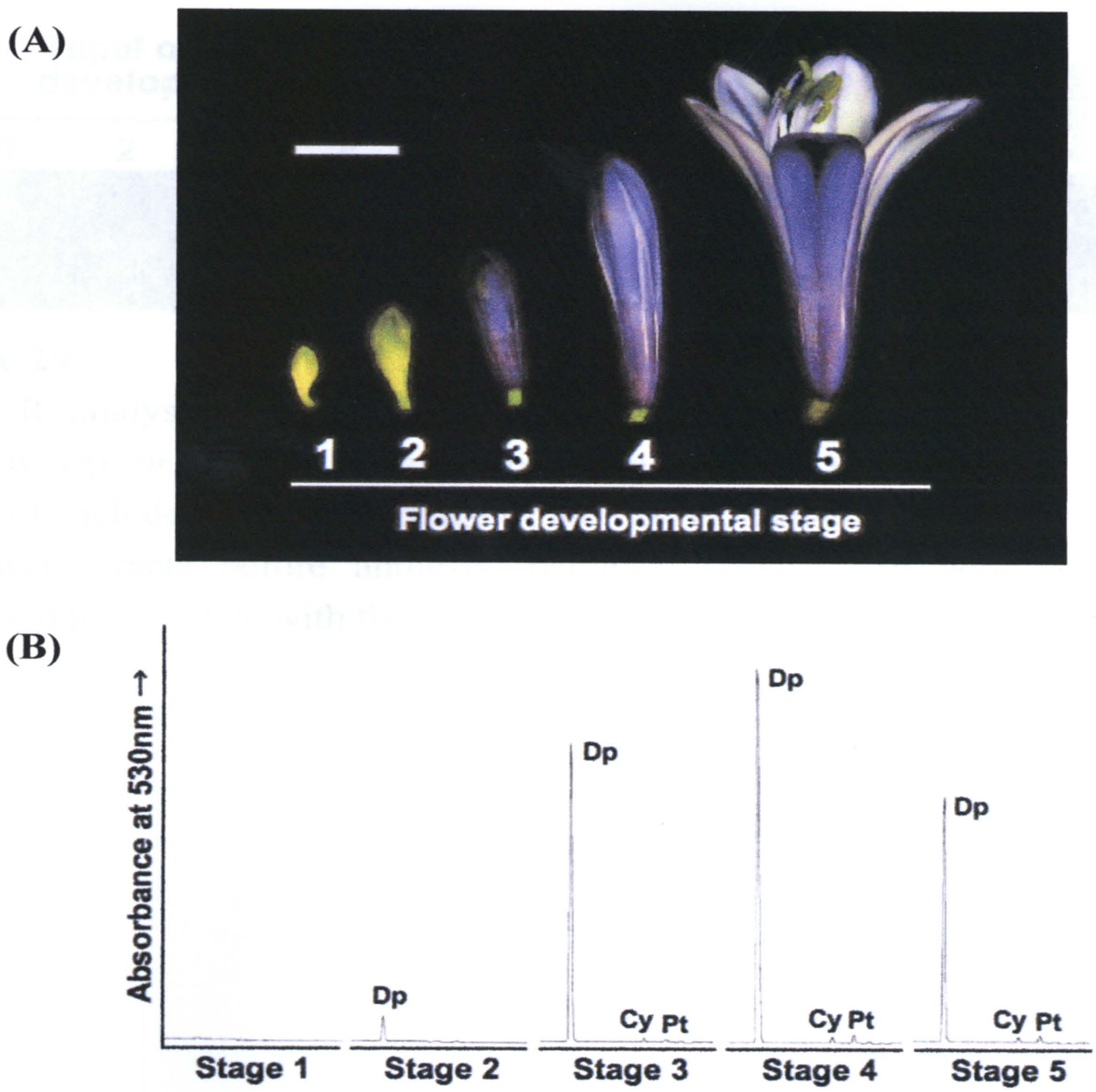


Figure 28.

Flower development and anthocyanidin accumulation in tepals of *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select'. (**A**) Developmental stages of the flower: stage 1, flower bud with non-pigmented (light green) tepals; stage 2, flower bud with pale blue tepals; stage 3, flower bud with blue tepals; stage 4, flower bud just before anthesis; and stage 5, fully opened flower. Bar = 1 cm. (**B**) HPLC profiles for the accumulation of anthocyanidins per 300 mg of tepal tissues of each developmental stage of the flower. Dp, delphinidin; Cy, cyanidin; Pt, petunidin.

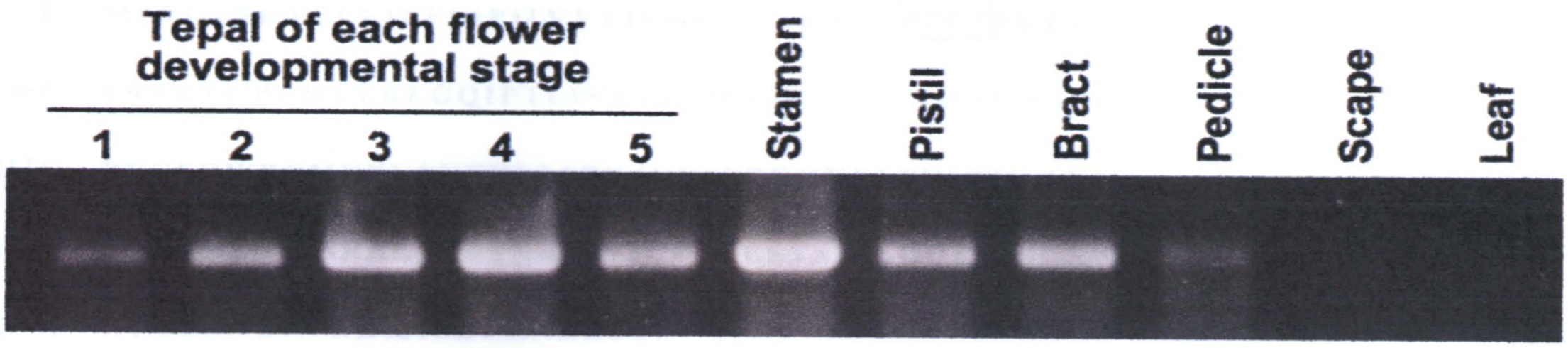


Figure 29.

RT-PCR analysis of *ApDFR1* expression in various organs of *Agapanthus praecox* ssp. *orientalis* 'Royal Purple Select'. Total RNA was extracted from tepals of each developmental stage of the flower, stamens and pistils of the stage 4 flower, bracts before anthesis, pedicles, scapes, and young leaves, and subjected to RT-PCR with the primer set, DFR5L and cDFR5L (Figure 24A).

Proline-rich region

(1) MSFTDHHYLLLILFLIPILVYTIRRKISSTKSKLPPCPPKLPLIGNLHQMGTLPHQSLHA

(61) LSVKYGPLMLLKLGQIPTLIVSSADMAREIMKTHDHIFASRPSLMTAGIILYGSMDVVFA

(121) PYGEHWRQMRKLCVNHLLSPKAVQSFRRMHEEEVATMVAKISEVSSSSGVVNMSETLNLF

(181) ASNAMLKAISRKLFRDERRSRVICELNEETAAILGQFSVSDFMPLLAWFDMVFGVGARAK

(241) KTARLWDRVLHEIIEDCRNRRDSEVNTDFVNVLLALLEDNDMDFSLNKDIIKAVLQDMIA

Oxygen-binding pocket K-region

(301) AGTETSSTAMDWCMAELVRNPEAMKKLQDEVRGIANTKPMITDDDLSKMGYLKAVIKELL

Aromatic region

(361) RLHPPVPLLIPRESMDHCEVQGFDIPKQTRVIVNAWSIGRDPNVWEAPEEFRPERHLDCA

Heme-binding region

(421) INFRGHDFELIPFGAGRRICPGMQFAVSTLELALANLVRSFDWELPDGMNNEDLGMGDGP

(481) GLSARRRQSLLLVAKPFLGLKCM

Figure 30.

Deduced amino acid sequence of *MaP450* isolated from *Muscari armeniacum* 'Blue Pearl'. White boxes indicate a putative proline-rich region, oxygen-binding pocket, K-region, aromatic region and heme-binding region.

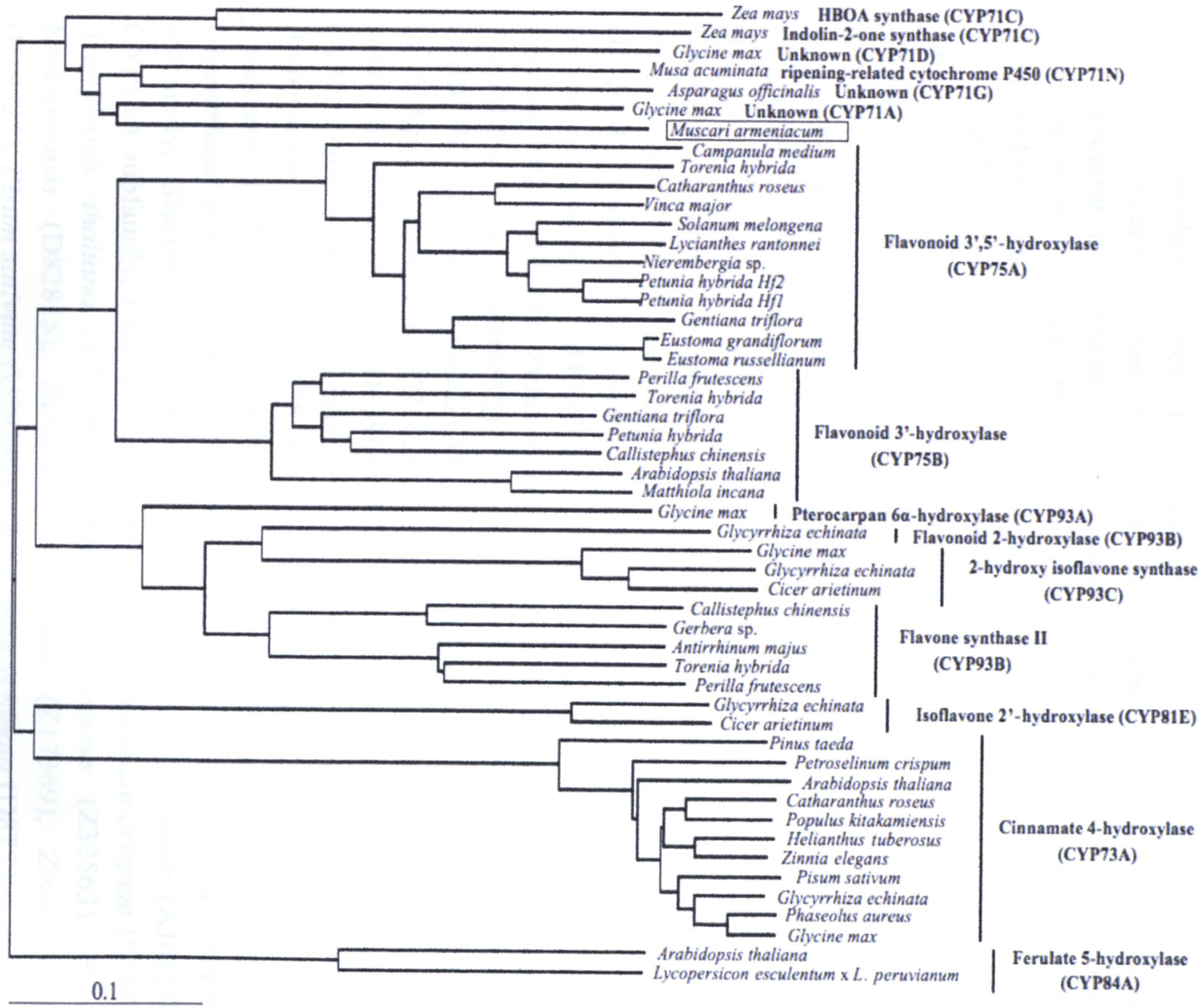

Zea mays HBOA synthase (CYP71C)
Zea mays Indolin-2-one synthase (CYP71C)
Glycine max Unknown (CYP71D)
Musa acuminata ripening-related cytochrome P450 (CYP71N)
Asparagus officinalis Unknown (CYP71G)
Glycine max Unknown (CYP71A)
Muscari armeniacum
Campanula medium
Torenia hybrida
Catharanthus roseus
Vinca major
Solanum melongena
Lycianthes rantonnei
Nierembergia sp.
Petunia hybrida Hf2
Petunia hybrida Hf1
Gentiana triflora
Eustoma grandiflorum
Eustoma russellianum
Flavonoid 3',5'-hydroxylase
(CYP75A)
Perilla frutescens
Torenia hybrida
Gentiana triflora
Petunia hybrida
Callistephus chinensis
Arabidopsis thaliana
Matthiola incana
Flavonoid 3'-hydroxylase
(CYP75B)
Glycine max
Pterocarpan 6α-hydroxylase (CYP93A)
Glycyrrhiza echinata
Flavonoid 2-hydroxylase (CYP93B)
Glycine max
Glycyrrhiza echinata
Cicer arietinum
2-hydroxy isoflavone synthase
(CYP93C)
Callistephus chinensis
Gerbera sp.
Antirrhinum majus
Torenia hybrida
Perilla frutescens
Flavone synthase II
(CYP93B)
Glycyrrhiza echinata
Cicer arietinum
Isoflavone 2'-hydroxylase (CYP81E)
Pinus taeda
Petroselinum crispum
Arabidopsis thaliana
Catharanthus roseus
Populus kitakamiensis
Helianthus tuberosus
Zinnia elegans
Pisum sativum
Glycyrrhiza echinata
Phaseolus aureus
Glycine max
Cinnamate 4-hydroxylase
(CYP73A)
Arabidopsis thaliana
Lycopersicon esculentum x L. peruvianum
Ferulate 5-hydroxylase
(CYP84A)
0.1

Figure 31.

Molecular phylogenic tree of the deduced amino acid sequences of *MaP450* from *Muscari armeniacum* 'Blue Pearl', the cytochrome P450 genes involved in the flavonoid biosynthetic pathway, and the cytochrome P450 genes not involved in this pathway but showing relatively high homology to *MaP450*. This phylogenic tree was generated by using CLUSTAL W (Thomopson et al., 1994) and TREEVIEW programs (Page, 1996). The scale bar indicates 0.1 amino acid substitutions per site. The accession number in the GenBank/ EMBL/DDBJ databases is as follows: CYP71C subfamily, *Zea mays* (HBOA synthase, X81827), *Zea mays* (indolin-2-one synthase, X81831); CYP71N subfamily, *Musa acuminata* (AY062168); CYP71A subfamily, *Glycine max* (Y10489); CYP71D subfamily, *Glycine max* (AF022459); CYP71G subfamily, *Asparagus officinalis* (AB037245); CYP75A subfamily, *Campanula medium* (D14590), *Torenia hybrida* (AB12925), *Catharanthus roseus* (AJ11862), *Vinca major* (AB078781), *Gentiana triflora* (D85184), *Eustoma grandiflorum* (U72654), *Eustoma russellianum* (D14589), *Solanum melongena* (E05111), *Lycianthes rantonnei* (AF313490), *Nierembergia* sp. (AB078514), *Petunia hybrida Hf2* (Z22544), *Petunia hybrida Hf1* (Z22545); CYP75B subfamily, *Perilla frutescens* (AB045593), *Torenia hybrida* (AB057672), *Gentiana triflora* (AB057673), *Petunia hybrida* (AF155332), *Callistephus chinensis* (AF313488), *Arabidopsis thaliana* (AF271651), *Matthiola incana* (AF313491); CYP93A subfamily, *Glycine max* (D83968); CYP93B subfamily, *Glycyrrhiza echinata* (AB001380), *Callistephus chinensis* (AF188612), *Gerbera* sp. (AF156976), *Antirrhinum majus* (AB028151), *Torenia hybrida* (AB028152), *Perilla frutescens* (AB045592); CYP93C subfamily, *Glycine max* (AF022462), *Glycyrrhiza echinata* (AB023636), *Cicer arietinum* (AJ243804); CYP81E subfamily, *Glycyrrhiza echinata* (AB001379), *Cicer arietinum* (AJ012581); CYP73A subfamily, *Pinus taeda* (AF096998), *Petroselinum crispum* (L38898), *Arabidopsis thaliana* (U71080), *Catharanthus roseus* (Z32563), *Populus kitakamiensis* (D82815), *Helianthus tuberosus* (Z17369), *Zinnia elegans* (U19922), *Pisum sativum* (U29243), *Glycyrrhiza echinata* (D87520), *Phaseolus aureus* (L07634), *Glycine max* (X92437); CYP84A subfamily, *Arabidopsis thaliana* (U38416), *Lycopersicon esculentum* × *L. peruvianum* (AF150881).

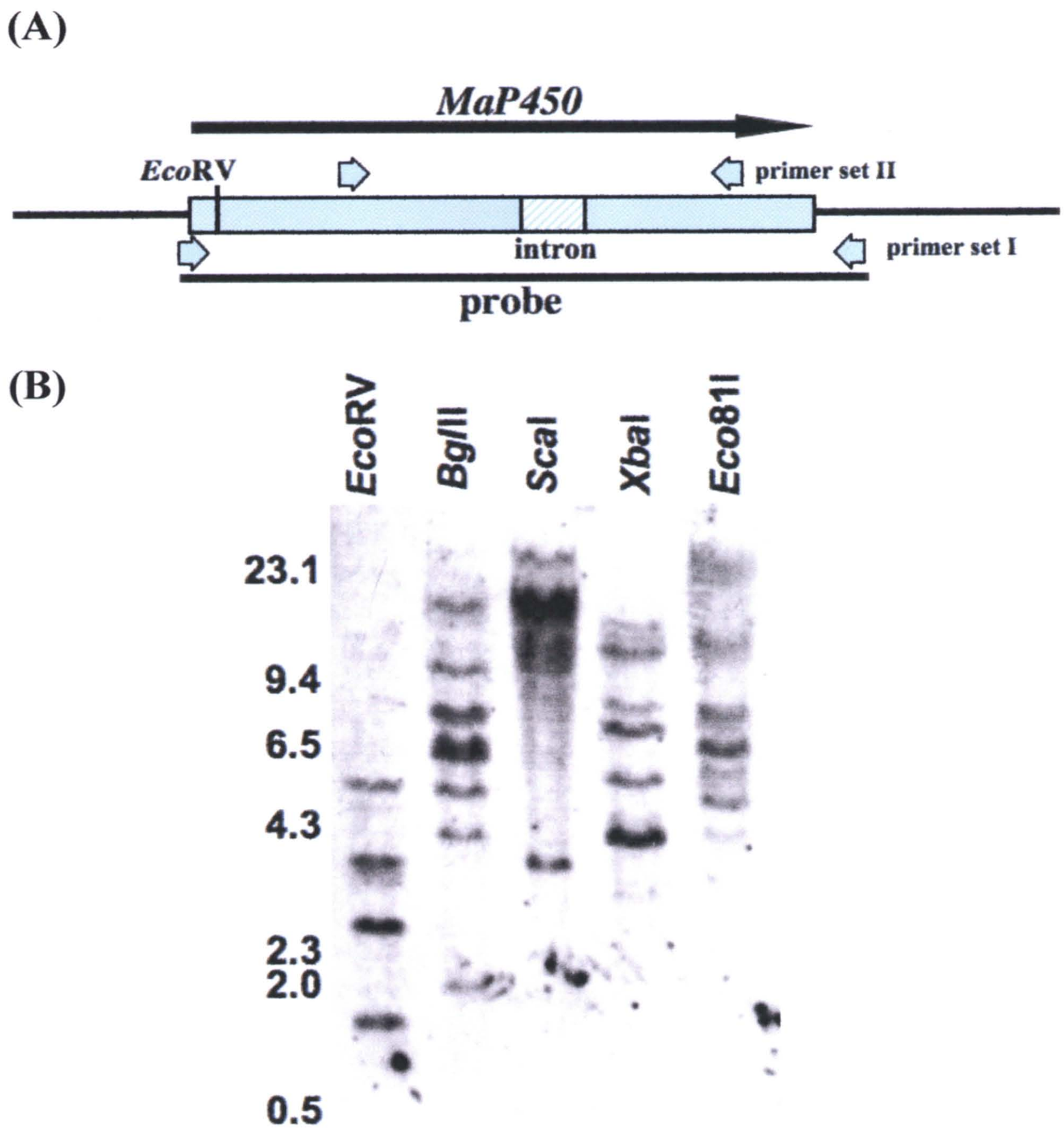


Figure 32.
Genomic structure and Southern hybridization of *MaP450* isolated from *Muscari armeniacum* 'Blue Pearl'. (**A**) Physical map of the genomic region harboring *MaP450*. The positions of the primer set I (MKFH1L and cMKFH1L) are indicated under the map. The positions of the primer set II (MFH2 and MFHR1), which was used for detecting *MaP450* transcripts by RT-PCR in *Muscari* spp., are indicated over the map. PCR products amplified with the primer set I were used as a probe for Southern hybridization. (**B**) Southern hybridization using a 1.67-kbp DNA fragment from *MaP450* as a probe. Genomic DNA was digested with *Eco*RV, *Bgl*II, *Sca*I, *Xba*I or *Eco*81I, and hybridized with PCR products amplified using pCR2.1-MaP450 (constructed in Chapter 3, Experiment 4) as a template and the primer set, MKFH1L and cMKFH1L. Numerals indicate kbp.

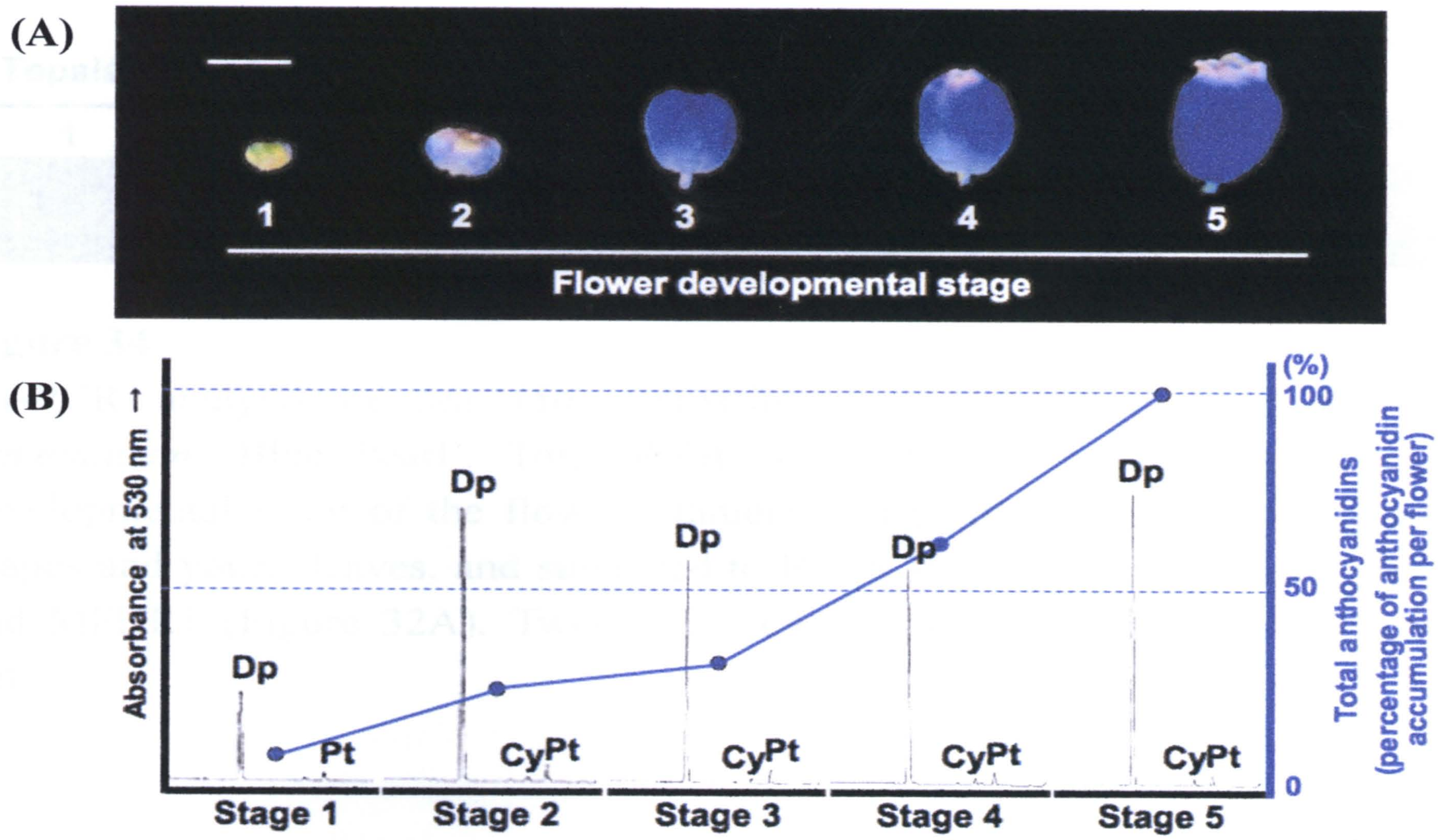


Figure 33.

Flower development and anthocyanidin accumulation in tepals of *Muscari armeniacum* 'Blue Pearl'. (**A**) Developmental stages of the flower: stage 1, flower bud with non-pigmented (light green) tepals; stage 2, flower bud with pale blue tepals; stage 3, flower bud with blue tepals; stage 4, flower bud just before anthesis; and stage 5, fully opened flower. Bar = 5 mm. (**B**) HPLC profiles for the accumulation of anthocyanidins per 200 mg of tepal tissues of each developmental stage of the flower, and the percentage of anthocyanidin accumulation per flower as a percentage of the value of tepals of the stage 5 flower. Dp, delphinidin; Cy, cyanidin; Pt, petunidin.

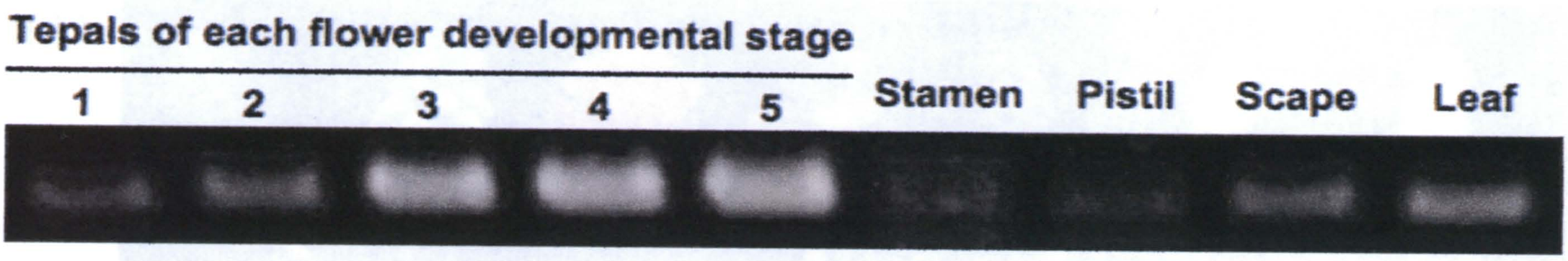


Figure 34.
RT-PCR analysis of *MaP450* expression in various organs of *Muscari armeniacum* 'Blue Pearl'. Total RNA was extracted from tepals of each developmental stage of the flower, stamens and pistils of the stage 4 flower, scapes and young leaves, and subjected to RT-PCR with the primer set, MFH2 and MFHR1 (Figure 32A). Twenty-five cycles of amplification were carried out.

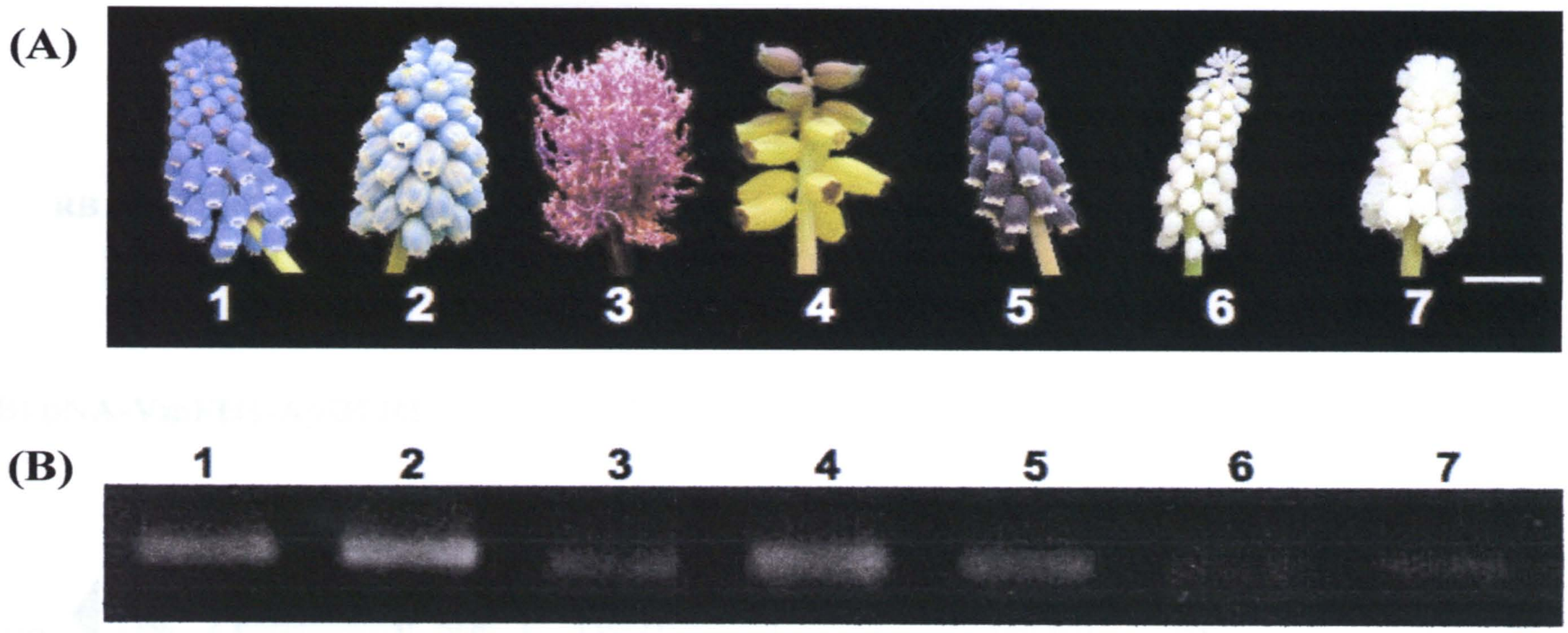
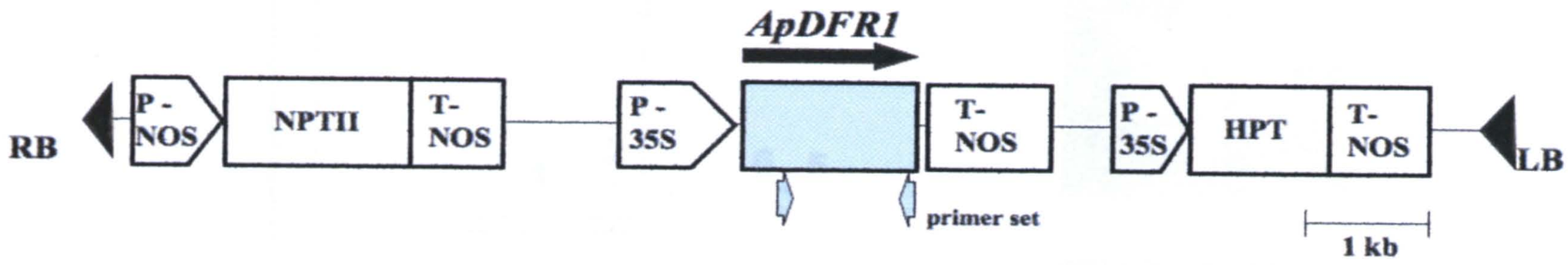


Figure 35.
RT-PCR analysis of *MaP450* expression in tepals of various *Muscari* genotypes with different flower colors. (**A**) Flowers of *Muscari* species and cultivars. 1, *M. armeniacum* 'Blue Pearl' (blue); 2, *M. armeniacum* 'Valerie Finnis' (pale blue); 3, *M. comosum* var. *plumosum* (reddish purple); 4, *M. macrocarpum* (yellow); 5, *Muscari* 'Sky Blue' (deep blue); 6, *M. botryoides* 'Album' (white); 7, *Muscari* 'White Beauty' (white). Bar = 2 cm. (**B**) RT-PCR analysis using total RNA isolated from tepals of the stage 4 flower as a template and the primer set, MFH2 and MFHR1 (Figure 32A). Twenty-five cycles of amplification were carried out. A number of each lane corresponds to a plant number in (**A**).

(A) pNA-ApDFR1-35S



(B) pNA-VmFH1-ApDFR1

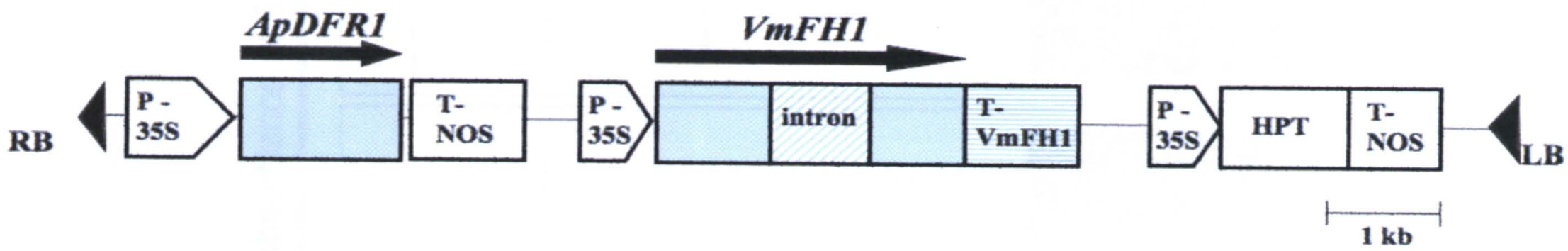


Figure 36.

T-DNA region of the binary vectors, pNA-ApDFR1-35S (**A**) and pNA-VmFH1-ApDFR1 (**B**). The positions of the primer set (DFR5L and cDFR5L), which was used for detecting foreign gene(s) by PCR in transgenic plants, are indicated under the map of pNA-ApDFR1-35S. RB, right border; LB, left border; P-NOS, promoter of NOS; P-35S, promoter of CaMV35S; T-NOS, terminator of NOS; and T-VmFH1, terminator of *VmFH1*.

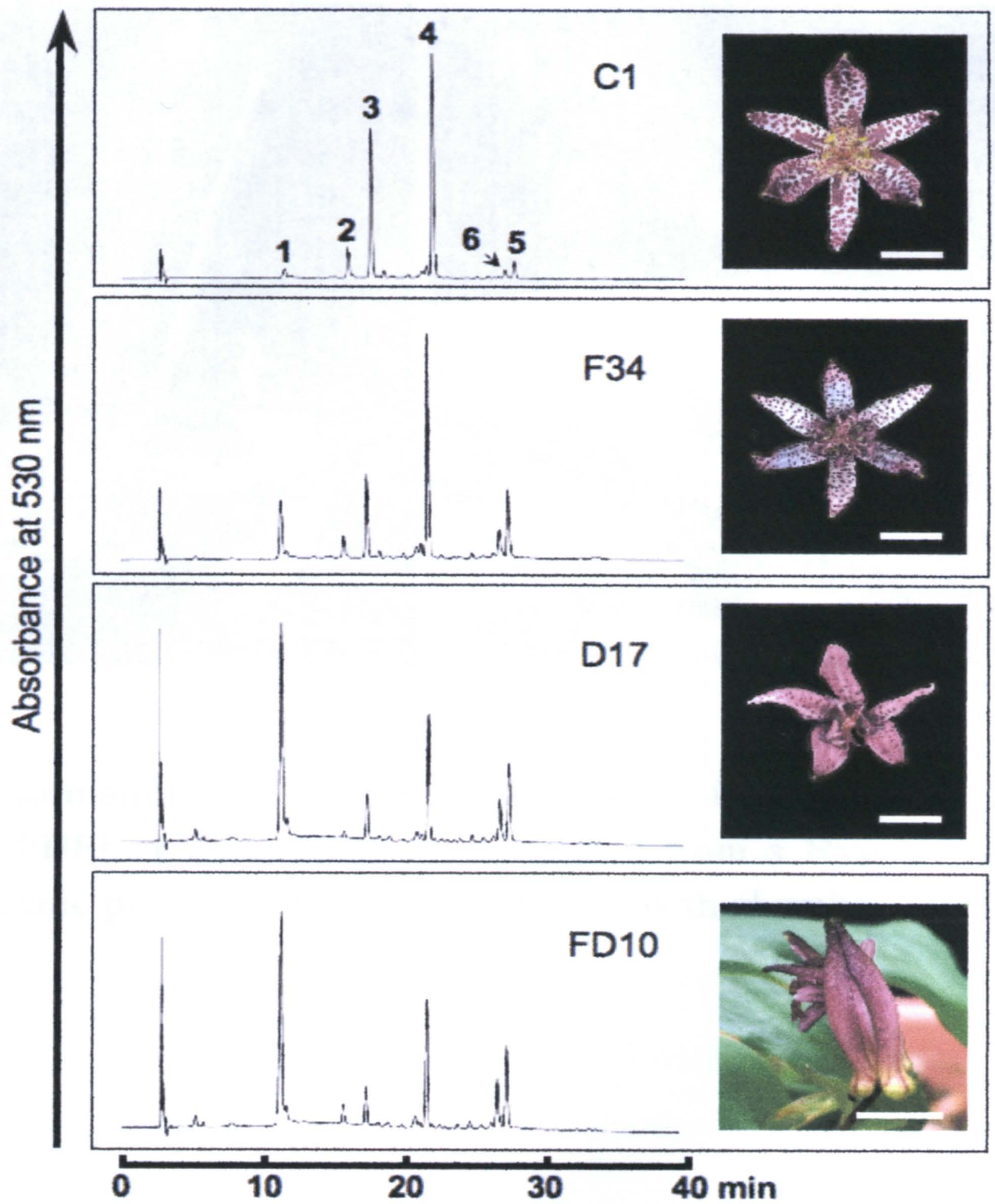


Figure 37.

Flower color phenotypes and HPLC profiles for the accumulation of anthocyanins in tepals of transgenic plants of *Tricyrtis hirta*. The control, non-transgenic plant C1, the transgenic plant F34 obtained with *Agrobacterium tumefaciens* EHA101/pNA-VFH-35S, the transgenic plant D17 obtained with *A. tumefaciens* EHA101/pNA-ApDFR1-35S, and the transgenic plant FD10 obtained with *A. tumefaciens* EHA101/pNA-VmFH1-ApDFR1 are shown. Bars = 1 cm. Peak No. 1, 8-*C*-glucosylcyanidin 3-*O*-malonylglucoside; peak No. 2, cyanidin 3-glucoside; peak No. 3, cyanidin 3-rutinoside; peak No. 4, cyanidin 3-*O*-malonylglucoside; peak No. 5, 8-*C*-sinapylglucosylcyanidin 3-*O*-malonylglucoside; peak No. 6, unidentified anthocyanin.

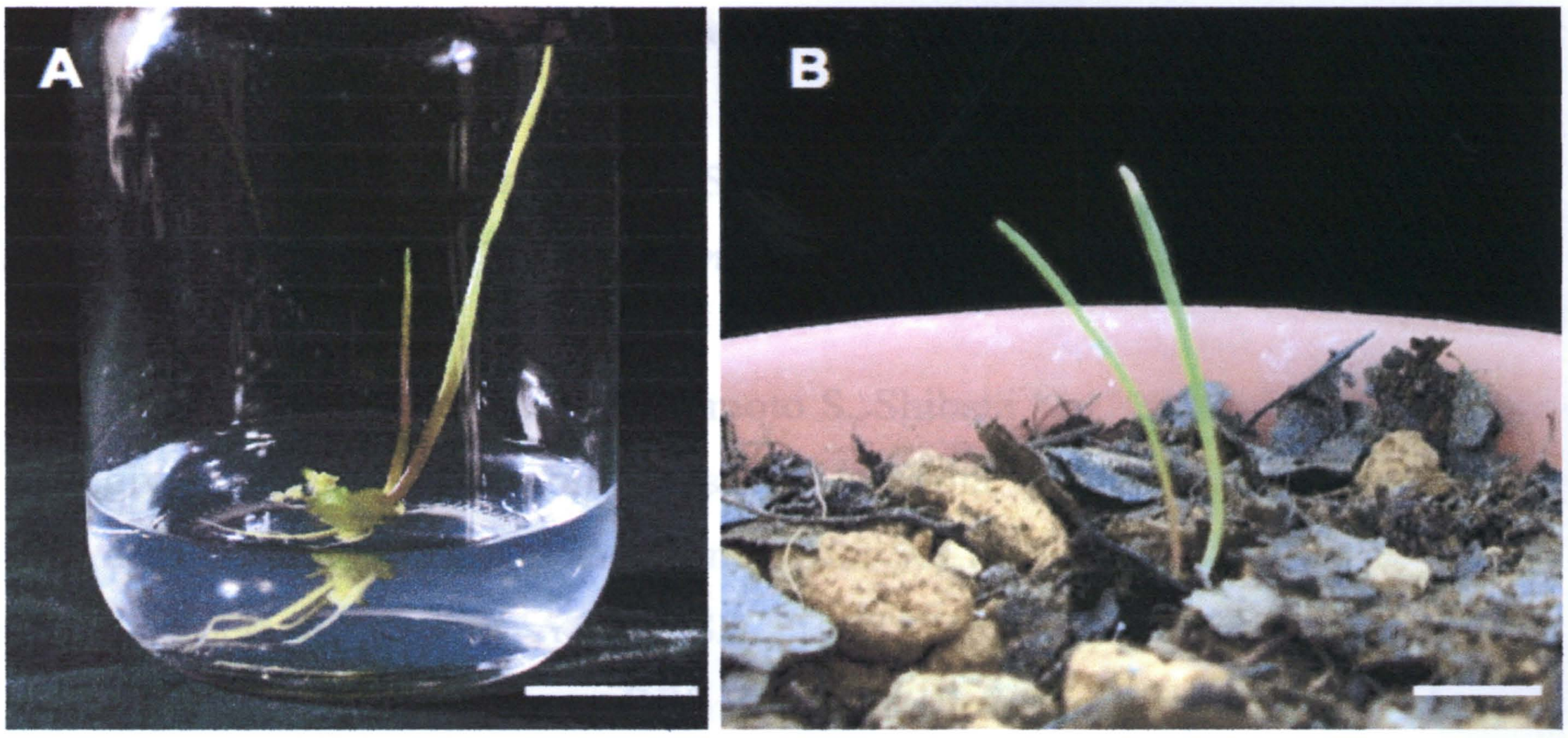


Figure 38.
Genetic transformation of *Tricyrtis hirta* with *Agrobacterium tumefaciens* EHA101/pNAFHFL. (**A**) A plantlet regenerated from a Hyg^r somatic embryo. (**B**) The transgenic plant FL 40 cultivated in a growth chamber. Bars = 1 cm.

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Summary

In the present study, the following experiments were carried out in order to apply molecular breeding for flower color modification to Liliaceous ornamentals.

1. Establishment of genetic transformation systems

As a first step toward the establishment of genetic transformation systems, development of fast growing and highly regenerable callus cultures was examined, since such cultures may be most suitable as a target material for transformation in Liliaceous ornamentals. Firstly, callus induction from cultured explants and plant regeneration from induced calluses were examined in 19 *Muscari* species and cultivars. Embryogenic callus cultures were induced on a medium containing 54 μM NAA from leaf and/or flower bud explants of six species and cultivars. Following transfer to PGR-free medium, the embryogenic calluses produced somatic embryos, which subsequently developed into plantlets. Secondly, callus induction and plant regeneration were examined in 33 *Lilium* species and cultivars. Organogenic callus cultures were induced on a medium containing 4.1 μM PIC from seed, bulb-scale, leaf and/or filament explants of 30 species and cultivars. Following transfer to media lacking PGRs or containing 22 μM BA, the organogenic calluses produced shoots, which subsequently developed into plantlets.

Development of an *Agrobacterium*-mediated transformation system of *Tricyrtis hirta* was examined by utilizing previously induced embryogenic callus cultures. The calluses were co-cultivated with *A. tumefaciens* strain EHA101/pIG121Hm for 7 days in the presence of AS, and then transferred onto a hygromycin-containing selection medium. After 8 weeks Hyg^r somatic

embryos were produced from the co-cultivated calluses, and these somatic embryos subsequently developed into plantlets. PCR analysis and GUS histochemical assay confirmed the transgenic nature of the regenerated plantlets. On average, about ten independent transgenic *T. hirta* plants could be obtained per 1 g FW of co-cultivated embryogenic calluses by using this transformation system.

For perennial and vegetatively propagated crops including almost all the Liliaceous ornamentals, long-term and stable expression of transgene(s) is one of the indispensable requisites for their improvement by genetic transformation. Therefore, firstly, morphological characterization and examination of the ploidy level and transgene (GUS gene) expression were carried out in transgenic plants of *Agapanthus praecox* ssp. *orientalis* following 5 years of cultivation in pots. Although polyploidization and morphological variations were observed in some transgenic plants, all the transgenic plants showed stable expression of the transgene. Secondly, transgene (GUS gene) expression was examined in transgenic *T. hirta* plants following 2 years of cultivation in pots. Stable expression of the transgene was observed in all the transgenic plants examined. No apparent transgene silencing was observed in these two Liliaceous ornamentals.

2. Biochemical and molecular biological information on the flavonoid biosynthetic pathway

In order to perform molecular breeding for flower color modification systematically, anthocyanin and/or anthocyanin analyses were carried out in flower organs of 13 *Muscari* species and cultivars, four *Agapanthus* cultivars, and two *Tricyrtis* species. For *Muscari* spp. and *Agapanthus* spp., blue or pale blue flower organs mainly contained delphinidin, whereas dark brown or reddish

purple flower organs mainly contained cyanidin. Pelargonidin was never detected in flower organs of *Agapanthus* spp. and *Muscari* spp. For two *Tricyrtis* spp., the same six anthocyanins, all cyanidin derivatives, were detected in tepals.

Isolation and characterization of genes for the flavonoid biosynthetic pathway were examined in several plant species. Firstly, the F3'5'H gene (*VmFH1*) was isolated from the Apocynaceous species *Vinca major*. *VmFH1* contained an ORF of 1,518 bp, encoding a polypeptide of 506 amino acid residues, and the percentage identities of the deduced amino acid sequence of *VmFH1* to the previously reported F3'5'H genes were 51–83%. Heterologous expression of *VmFH1* under the control of the CaMV35S promoter in transgenic *Petunia hybrida* resulted in drastic flower color alteration from red to deep red with deep purple sectors. These transgenic plants accumulated delphinidin, petunidin and malvidin in their petals, which were never detected in non-transgenic plants. Secondly, the DFR gene (*ApDFR1*) was isolated from *Agapanthus praecox* ssp. *orientalis*. *ApDFR1* contained an ORF of 1,137 bp, encoding a polypeptide of 379 amino acid residues, and the percentage identities of the deduced amino acid sequence of *ApDFR1* to the previously reported DFR genes were 59–75%. Thirdly, isolation of the F3'5'H from *Muscari armeniacum* was examined. The isolated cytochrome P450 gene (*MaP450*) contained an ORF of 1,512 bp, encoding a polypeptide of 504 amino acid residues. Although the deduced amino acid sequence of *MaP450* showed only low identities (34–37%) to that of the previously reported F3'5'H genes, a close correlation between *MaP450* expression and anthocyanidin accumulation as well as flower color indicates that this gene may be involved in the flavonoid biosynthetic pathway in *Muscari armeniacum*.

3. Attempts at flower color modification by genetic transformation

In order to modify flower color by genetic transformation, *Tricyrtis hirta* and *Muscari armeniacum* were transformed with gene(s) for flavonoid biosynthetic pathway. Firstly, the F3'5'H gene from *Vinca major* (*VmFH1*) and/or the DFR gene from *Agapanthus praecox* ssp. *orientalis* (*ApDFR1*), both under the control of the CaMV35S promoter, were introduced into *T. hirta*. However, no apparent alterations in flower color and anthocyanin composition in tepals were observed in eight independent transgenic plants containing *VmFH1*, three independent transgenic plants containing *ApDFR1* and one transgenic plant containing both *VmFH1* and *ApDFR1*. Secondly, *VmFH1* under the control of *VmFH1* original promoter was introduced into *M. armeniacum*. Two independent transgenic plants have so far been produced. Analyses of anthocyanidins and anthocyanins in flower organs should be carried out on these transgenic plants when they produced flowers.

摘　要

本研究では，花色改変分子育種をユリ科花き園芸植物に応用することを目的として，以下のような実験を行った．

1. 形質転換システムの確立

効率的な形質転換システムを確立するための前段階として，形質転換の材料となりうる，高い植物体再生能力を保持したカルス培養系の確立について検討した．まず，19 種・品種のムスカリ属植物 (*Muscari* spp.) を供試してカルス培養系の確立を試みたところ，54 μM NAA を含む培地上で，6 種・品種の葉または花蕾外植体からエンブリオジェニックカルスを誘導することができた．これらのエンブリオジェニックカルスを植物成長調節物質無添加の培地に移植したところ，不定胚を経由して小植物体が再生した．次に，33 種・品種のユリ属植物 (*Lilium* spp.) を供試してカルス培養系の確立を試みたところ，4.1 μM PIC を含む培地上で，30 種・品種の種子，鱗片，葉または花糸外植体から器官形成能力を保持したカルスを誘導することができた．これらのカルスを植物生長調節物質無添加または 22 μM BA を含む培地に移植したところ，小植物体が再生した．

すでに誘導されているホトトギス (*Tricyrtis hirta*) のエンブリオジェニックカルスを用いて，アグロバクテリウム法による形質転換システムの確立を検討した．エンブリオジェニックカルスを *Agrobacterium tumefaciens* EHA101/pIG121Hm 系統と AS 存在下で 7 日間共存培養した後，ハイグロマイシンを含む選抜培地に移植した．その結果，8 週間後にはハイグロマイシン耐性の不定胚が形成され，それらは小植物体に発達した．PCR 分析および組織化学的 GUS 分析により，再生植物体が形質転換体であることが確認された．この形質転換システムにより，1 g 新鮮重のエンブリオジェニックカルスから，平均で 10 系統の形質転換体を得ることができた．

多くのユリ科花き園芸植物のように多年生で栄養繁殖性の植物では，形質転換体における外来遺伝子の長期的・安定的な発現が求められる．そこで，まず，5 年間栽培したアガパンサス (*Agapanthus praecox* ssp. *orientalis*) 形質転換体において，倍数性，形態，および外来遺伝子である GUS 遺伝子の発現等を調査した．その結果，染色体

倍加や形態変異は観察されたものの，調査した全ての形質転換体において外来遺伝子の長期的かつ安定的な発現が示された．次に，2年間栽培したホトトギスの形質転換体において，外来遺伝子である GUS 遺伝子の発現を調査したところ，調査した全ての形質転換体において GUS 遺伝子の安定的な発現が示された．アガパンサスおよびホトトギスの形質転換体においては，外来遺伝子の明らかなジーンサイレンシングはみられなかった．

2. フラボノイド生合成経路に関する生化学的・分子生物学的調査

花色改変分子育種を効率的に行うことを目的として，13種・品種のムスカリ属植物 (*Muscari* spp.)，4品種のアガパンサス属植物 (*Agapanthus* spp.) および2種のホトトギス属植物 (*Tricyrtis* spp.) を供試し，花器官におけるアントシアニンおよびアントシアニジン分析を行った．その結果，ムスカリ属およびアガパンサス属植物では，青色または淡青色の器官における主要なアントシアニジンはデルフィニジンであり，褐色および赤紫色の花器官における主要なアントシアニジンはシアニジンであった．なお，ムスカリ属およびアガパンサス属植物の花器官においては，ペラルゴニジンは検出されなかった．一方，2種のホトトギス属植物の花被においては，ともにシアニジン系の6種類のアントシアニンが検出された．

花色改変分子育種を行う上で必要とされる，フラボノイド生合成経路に関連する遺伝子の単離および特徴づけを行った．まず，キョウチクトウ科のツルニチニチソウ (*Vinca major*) から F3'5'H 遺伝子 (*VmFH1*) を単離した．*VmFH1* の ORF 領域は 1,518 bp であり，506 アミノ酸をコードしていると推定された．*VmFH1* と既知の F3'5'H 遺伝子との推定アミノ酸配列の相同性は 83–51%であった．*VmFH1* をペチュニア (*Petunia hybrida*) の形質転換体で発現させたところ，花色は赤色から濃紫色の領域を伴った農赤色へ変化し，花弁において新たにデルフィニジン，ペチュニジンおよびマルビジンが検出された．次に，アガパンサス (*Agapanthus praecox* ssp. *orientalis*) から DFR 遺伝子 (*ApDFR1*) を単離した．*ApDFR1* の ORF 領域は 1,137 bp であり，379 アミノ酸をコードしていると推定された．*ApDFR1* と既知の DFR 遺伝子との推定アミノ酸配列の相同性は 75–59%であった．さらに，ムスカリ (*Muscari armeniacum*) から F3'5'H 遺伝子の単離を試みたところ，得られた遺伝子 (*MaP450*) の ORF 領域は 1,512

bp であり，504 アミノ酸をコードしていると推定された．*MaP450* と既知の F3'5'H 遺伝子との推定アミノ酸配列の相同性は低かった (37–34%)，しかし，*MaP450* の発現とアントシアニジンの蓄積に相関がみられたことから，*MaP450* はムスカリにおけるフラボノイド生合成経路に関与した遺伝子であると予想された．

3. 形質転換による花色改変の試み

形質転換による花色の改変を目的として，形質転換システムが確立しているホトトギス (*Tricyrtis hirta*) およびムスカリ (*Muscari armeniacum*) にフラボノイド生合成経路関連遺伝子を導入した．まず，CaMV35S プロモーター制御下のツルニチニチソウ (*Vinca major*) 由来 F3'5'H 遺伝子 (*VmFH1*) およびアガパンサス (*Agapanthus praecox* ssp. *orientalis*) 由来 DFR 遺伝子 (*ApDFR1*) をホトトギスに導入した．*VmFH1* が導入された 8 系統の形質転換体，*ApDFR1* が導入された 3 系統の形質転換体，および *VmFH1* および *ApDFR1* が導入された 1 系統の形質転換体について形質調査を行ったところ，いずれの形質転換体においても明らかな花色の変化はみられず，また，花被のアントシアニン分析においても質的な変化はみられなかった．次に，*VmFH1* オリジナルプロモーター制御下の *VmFH1* をムスカリに導入した．これまでに 2 系統の形質転換体が得られている．今後，これらの形質転換体の開花を待って，色素分析を含む形質調査を行う必要がある．

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid

ANS, anthocyanin synthase

AS, acetosyringone

BA, 6-benzyladenine

CaMV, cauliflower mosaic virus

CHI, chalcone isomerase

CHS, chalcone synthase

DFR, dihydroflavonol 4-reductase

DHK, dihydrokaempferol

DHM, dihydromyricetin

DHQ, dihydroquercetin

F3H, flavanone 3-hydroxylase

F3'H, flavonoid 3'-hydroxylase

F3'5'H, flavonoid 3',5'-hydroxylase

FLS, flavonol synthase

FNS, flavone synthase

FW, fresh weight

GUS, β-glucuronidase

HPLC, high-performance liquid chromatography

HPT, hygromycin phosphotransferase

MS, Murashige and Skoog (1962)

NAA, α-naphthaleneacetic acid

NOS, nopaline synthase

NPTII, neomycin phosphotransferase II

ORF, open reading frame

PCR, polymerase chain reaction

PGR, plant growth regulator

PIC, 4-amino 3,5,6-trichloropicolinic acid (picloram)

RACE, rapid amplification of cDNA ends

RT-PCR, reverse transcription-polymerase chain reaction