Plant Biotechnology 29, 323–337 (2012)
DOI: 10.5511/plantbiotechnology.12.0521a

Invited Review

Genetic engineering of chrysanthemum (*Chrysanthemum morifolium*): Current progress and perspectives

Harue Shinoyama[1,*], Ryutaro Aida[2], Hiroaki Ichikawa[3], Yukio Nomura[1], Atsushi Mochizuki[4]

[1]Fukui Agricultural Experiment Station, Fukui 918-8215, Japan; [2]National Institute of Floricultural Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8519, Japan; [3]Division of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan; [4]National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki 305-8604, Japan
*E-mail: harue_shinoyama@fklab.fukui.fukui.jp Tel: +81-776-54-5100 Fax: +81-776-54-5106

Received March 13, 2012; accepted May 21, 2012 (Edited by M. Nakano)

Abstract We give an overview of the advances of an *Agrobacterium*-mediated transformation system, clarifying its problems and their solutions, and then show the latest version of our transformation system and examples of the introduction of agronomically important traits into chrysanthemums. Typical problems with the *Agrobacterium*-mediated transformation in chrysanthemum include low transformation efficiency, high chimerism and cultivar specificity. Using a co-cultivation medium containing acetosyringone and casamino acids for high transformation efficiency and an antibiotic-selection step for transgenic calli before plant regeneration to eliminate the chimerism, we established an efficient and stable transformation system for chrysanthemum. In addition, this system was used to successfully introduce useful agronomical traits, such as insect resistance and new flower color, into chrysanthemums. These traits have been stably and highly expressed to confer the expected characteristics upon the transgenic chrysanthemums. Before applying a field trial of the genetically modified (GM) chrysanthemums, male and female sterility were introduced into the transformants to exclude the transgene flow from the GM plants to their wild relatives. So far, using RNAi technology, some of the transgenic chrysanthemums have displayed complete male sterility with very weak female fertility.

Key words: *Agrobacterium*, callus induction system, bi-directional promoters, agronomic traits, environmental safety

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most popular ornamental flowers cultivated all over the world, second only to the rose. Chrysanthemums originated from interspecific crossing between wild relatives native to China. Fukai et al. (1995) and Kitamura (1950) suggested that florist chrysanthemums ($2n=54$) originated by crossing and doubling between *C. zawadskii* var. *latilobum* (Maxim.) Kitamura ($2n=18$) and *C. indicum* var. *procumbense* (Lour.) Kitamura ($2n=36$). The contemporary cultivars are hexaploids with a loss or gain of several chromosomes (Dowrick 1958; Dowrick and El-Bayoumi 1966), and they display a self-incompatible trait (Drewlow et al. 1973).

Florist chrysanthemums are mostly cultivated by vegetative stem cuttings or suckers. In addition, the plants can be regenerated from adventitious shoots from various chrysanthemum tissues or calli using *in vitro* culture methods (Teixeira da Silva 2003). Since the late 1970s, its extensive ranges of flower colors, shapes and form have been created by conventional crossbreeding techniques or artificial mutation breeding techniques using X-rays (Broertjes et al. 1976; Huitema et al. 1987; Preil et al. 1983), gamma rays (De Jong and Custers 1986), heavy-ion beams (Nagatomi et al. 1998) or chemical substances such as ethylmethane sulphonate (EMS) (Dalsou and Short 1987). In conventional crossbreeding, hereditary elements from the same or different species are combined by sexual reproduction to create completely new gene combinations. The artificial mutation breeding techniques can only change a few useful traits.

Abbreviations: *Bt*, *Bacillus thuringiensis*; *cab*, *chlorophyll-a/b-binding protein* gene; CaMV *35S*, Cauliflower mosaic virus *35S* RNA; *CmCCD4a*, *carotenoid cleavage dioxygenase 4a* gene from chrysanthemum; *CHS*, *chalcone synthase* gene; CRES-T, Chimeric REpressor gene-Silencing Technology; *EF1α*, *elongation factor 1α* gene; GM, genetically modified; *GUS*, *β-glucuronidase* gene; ICPs, insecticidal crystal proteins; *ipt*, *isopentenyl transferase* gene; *Lhca3.St.1*, *apoprotein 2 of the light-harvesting complex of photosystem I* gene from *Solanum tuberosum*; *nptII*, *neomycin phosphotransferase II* gene; *NtADH*, tobacco *alcohol dehydrogenase* gene; *PhyB1*, *phytochrome B1* gene; *rbcS1*, *ribulose-1,5-bisphosphate carboxylase small-subunit* gene; SAAT, sonication-assisted *Agrobacterium* transformation; TSWV, *Tomato spotted wilt virus*; *UEP1*, *ubiquitin extension protein 1* gene; 5′UTR, 5′-untranslated region.

This article can be found at http://www.jspcmb.jp/
Published online September 20, 2012

Many agronomically important and commercially attractive traits are impossible to be introduced by conventional breeding or artificial mutation breeding because utilizable gene resources and modified traits are limited. Recently, advances in biotechnology have made possible the addition of new traits that are unachievable via conventional or mutation breeding (Shinoyama et al. 2006). After the first report on the susceptibility of chrysanthemum plants to *Agrobacterium* (Miller 1975), many researchers have tried to introduce useful agronomical traits into chrysanthemums via *Agrobacterium*-mediated transformation.

Here, we briefly review advances in the *Agrobacterium*-mediated transformation of chrysanthemums by discussing their problems and solutions and then describing our improved transformation system with examples of the introduction of agronomically important traits into chrysanthemums.

1. Genetic transformation systems

1.1. Biolistic-mediated genetic transformation

Several methods for gene transfer are applicable to the chrysanthemum. Biolistic-mediated transformation, which uses particle bombardment to deliver exogenous genetic material (Hosokawa et al. 1998; Teixeira da Silva and Fukai 2002a, 2002b; Yepes et al. 1995, 1999), has been applied in many plant species to overcome the problem of strain specificity that limits the use of *Agrobacterium* transformation. However, in the chrysanthemum, it is often difficult to directly induce shoot or callus formation from the cells into which foreign genes have been introduced via particle bombardment (Hosokawa et al. 1998). There is also a high level of cultivar specificity on the efficiency of plant regeneration and transformation (Teixeira da Silva and Fukai 2002a, 2002b; Yepes et al. 1995, 1999). Accordingly, biolistic-mediated transformation is not frequently used in the chrysanthemum.

1.2. Agrobacterium-mediated genetic transformation

Once the susceptibility of chrysanthemums to *Agrobacterium tumefaciens* was reported (De Cleene and De Ley 1976; Hooykaas and Beijersbergen 1994; Miller 1975), the *Agrobacterium*-mediated transformation of chrysanthemum was studied worldwide. However, some barriers to the establishment of a chrysanthemum transformation system have been reported: low transformation efficiency (De Jong et al. 1994; Renou et al. 1993; Urban et al. 1994), regeneration of chimeric plants comprising both transgenic and non-transgenic tissues (Benetka and Pavingerová 1995; Pavingerová et al. 1994) and transgene inactivation, also known as gene silencing (Takatsu et al. 2000).

1.2.1. Agrobacterium strains and their specificity to chrysanthemum cultivars

Ledger et al. (1991) first tried to generate transgenic chrysanthemum (*Dendranthema indicum* 'Korean') using *A. tumefaciens* strain LBA4404, but the transformation frequency was extremely low (1.7%). Renou et al. (1993) achieved a higher transformation efficiency (5 to 40%) using *A. tumefaciens* EHA101. Shinoyama and colleagues used two *Agrobacterium* strains, LBA4404 and EHA101, to transform the cultivar 'Shuho no chikara' and showed relatively high transformation efficiencies: 5.2% for LBA4404 (Shinoyama et al. 2002b) and 4.4% (Shinoyama et al. 2002a) and 8.8% (Shinoyama et al. 2003) for EHA101. They also used two *Agrobacterium* strains, EHA101 and EHA105, to transform the cultivar 'Yamate shiro' and showed relatively high transformation efficiencies: 21.7% for EHA101 (Shinoyama et al. 2002a) and 22.0% (Shinoyama et al. 2008) and 23.9% (Shinoyama et al. 2012b) for EHA105. Other *Agrobacterium* strains, such as Ach5, AGL0, and those isolated from the crown gall of chrysanthemums, have successfully been used to obtain high transformation frequencies (Bush and Pueppke 1991; Ogawa et al. 2000; Vaudequin-Dransart et al. 1995) (Table 1).

The transformation efficiency has been reported to be dependent not only on *Agrobacterium* strains but also on the nature of chrysanthemum cultivars, including their susceptibility to *Agrobacterium* infection and their ability to regenerate plants *in vitro* (Aida et al. 2004; Deroles et al. 2002; Horsch et al. 1985; Shinoyama et al. 2002a; Teixeira da Silva 2004; Van Wordragen et al. 1991). Aida et al. (2004), Shinoyama et al. (2002a) and Shinoyama and Mochizuki (2006) compared transformation frequencies among several cultivars and found high transformation frequency for some cultivars (e.g., 'Hiroshima beni' [Aida et al. 2004; Shinoyama et al. 2002a; Shinoyama and Mochizuki 2006] and '94-787' [Aida et al. 2004]).

De Jong et al. (1994) and Urban et al. (1994) described the difference in transformation frequencies of chrysanthemum using different *Agrobacterium* strains and indicated that the AGL0 and EHA105 strains showed higher transformation frequencies than LBA4404. In contrast, no significant difference in the transformation frequency was recognized for four cultivars, 'Shuho no chikara' (Shinoyama et al. 2002a, 2002b, 2003), 'Yamate shiro' (Shinoyama et al. 2002a, 2008, 2012a, 2012b), 'Yamabiko' and 'New Summer Yellow' (Takatsu et al. 2000), even if different *Agrobacterium* strains were used. The discrepancy between the cultivar dependence and independence of the chrysanthemum transformation might be caused by the use of different transformation methods and binary vectors. These results at least suggest the importance of choosing the *Agrobacterium* strain(s) that confer the highest transformation frequency on

some chrysanthemum cultivars.

Agrobacterium rhizogenes causes hairy root syndrome in infected plant tissues by transferring T-DNA from the Ri plasmid into the plant genome (De Cleene and De Ley 1981). Genetic transformation mediated by *A. rhizogenes* is used in many plant species because *A. rhizogenes* strains are often more virulent than *A. tumefaciens* (Van Wordragen et al. 1992a). In chrysanthemum, a moderately higher transformation efficacy was observed for *A. rhizogenes* (6.0%) than for *A. tumefaciens* (3.3%). However, the *rol* genes of *A. rhizogenes* Ri T-DNA were detected in only four of 38 transgenic chrysanthemum plants (i.e., 10.5%), and none of them exhibited hairy root syndrome (Tsuro et al. 2005). This result implies that unidentified barriers to the transfer of Ri T-DNA to chrysanthemum may exist. Because the difference in the transformation frequencies of chrysanthemum by *A. rhizogenes* and *A. tumefaciens* is not very large, the advantages of disarmed *A. tumefaciens* strains have overshadowed the usefulness of oncogenic *A. rhizogenes* strains as reagents for chrysanthemum transformation.

1.2.2. Explants for infection and its treatment

Successful transformation also depends on the source and physiological condition of the explants to be infected with *Agrobacterium*. Explants from leaves and stems have been frequently used for *Agrobacterium tumefaciens*-mediated transformation (Table 1). Most of those explants are harvested from plants aseptically cultured *in vitro*. In many cases, juvenile explants are used, such as newly formed expanding leaves (Ledger et al. 1991). Remarkably, De Jong et al. (1994, 1995) succeeded in the transformation using pedicels that were removed from the flowers of chrysanthemum plants that were non-aseptically grown in greenhouses.

The timing of *Agrobacterium* infection is considered an important factor for raising the infection efficiency, resulting in increased plant transformation efficiency (De Jong et al. 1993; Shinoyama et al. 2002a; Teixeira da Silva and Fukai 2002a). Shinoyama et al. (1998) demonstrated that the *Agrobacterium* culture in the logarithmic growth phase confers high infectability on plants and that the number of transformed cells increases when using the *Agrobacterium* in this phase.

The addition of chemical compounds such as acetosyringone, detergent and casamino acids to co-cultivation medium can increase the *Agrobacterium* infection frequency. De Jong et al. (1994) first succeeded in increasing the *Agrobacterium* infectability of chrysanthemum by adding 100 μM acetosyringone to the co-cultivation medium. Fukai et al. (1995) and Takatsu et al. (1998, 2000) confirmed the effect of acetosyringone. Shinoyama et al. (1998) reported that 50 μM acetosyringone is enough to increase the infectability. When added to the infection solution, detergent, such as 5% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20®), increased the adhesion of *Agrobacterium* to explants (Shinoyama et al. 1998). The infection frequency of *Agrobacterium* in chrysanthemums was further increased when 1% (w/v) casamino acids was added to the co-cultivation medium (Shinoyama et al. 1998).

An alternative method to increase chrysanthemum transformation frequency is sonication-assisted *Agrobacterium* transformation (SAAT), which helps *Agrobacterium* to seep deep inside the tissue and thus infect more tissues and cells (Teixeira da Silva and Fukai 2002b). Sonication could destroy bristles (trichomes) on the surface of the chrysanthemum plants, which inhibit the adhesion of *Agrobacterium* (Teixeira da Silva and Fukai 2002b).

Increasing the regenerative ability of plantlets from explants is also an effective way to increase transformation efficiency. Several combinations of plant growth regulators, such as indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) (Aida et al. 1992; De Jong et al. 1995; Fukai et al. 1995; Ledger et al. 1991; Urban et al. 1994), 1-naphthaleneacetic acid (NAA) and BAP (Renou et al. 1993; Takatsu et al. 1998), and NAA, BAP and Gibberellin A_3 (Shinoyama et al. 2002b) are reported to be effective.

Because both *Agrobacterium*-mediated transformation and shoot regeneration are promoted by wounding the explants (De Jong et al. 1993), Shinoyama et al. (1998) compared the difference in transformation frequencies with the two leaf-cutting methods using scalpels or cork-borers. More *Agrobacterium*-infected cells were obtained from the segments cut with cork-borers than from those cut with scalpels. The cut surfaces made by the cork-borers are likely more convenient for *Agrobacterium* infection.

1.2.3. Selection markers

Because the *neomycin phosphotransferase II* gene (*nptII*) was first applied as a selectable marker gene in the transformation of florist chrysanthemum (Lemieux et al. 1990), kanamycin has been the main selection agent of transformed cells and tissues (Table 1). Florist chrysanthemums are sensitive to kanamycin, and the application of a high concentration of kanamycin in the selection medium inhibits shoot formation (De Jong et al. 1994). Other antibiotics, such as hygromycin, paromomycin and geneticin, have also been successfully used for the selection of transgenic chrysanthemums (Aida et al. 2004; Renou et al. 1993; Sherman et al. 1998b; Shinoyama et al. 1998). Renou et al. (1993) used hygromycin for selection and showed the potential to avoid chimeras. Paromomycin is considered less toxic to cells than kanamycin, and its constant selection pressure during plant regeneration and rooting could reduce the chance of non-transgenic escapes (Aida et al. 2004;

Table 1. Studies on *Agrobacterium tumefaciens*-mediated transformation systems of chrysanthemums using *GUS* gene.

Agrobacterium strain(s)	Segment(s)	Antibiotics (Ab)	Ab for selection	Promoter(s)	Transgene(s)	Transformation frequency (%)	Localization of *GUS* gene expression	GUS activity (pmol 4-MU mg^{-1} protein min^{-1})	Reference
LBA4404	Leaf	CF, VA	none	CaMV *35S*	*GUS*	n.s.	n.s.	n.s.	De Jong et al. 1990
LBA4404, A2002	Leaf, stem	TI	K	CaMV *35S*	*nptII*, *GUS*	0.8	n.s.	n.s.	Ledger et al. 1991
LBA4404, A281, Ach5, C58	Leaf	CF, VA	K	*nos*, *TR-2′*	*nptII*, *GUS*	n.s.	Callus	0.0–466,000 (Callus)	Van Wordragen et al. 1991
EHA101	Stem	CF	K	CaMV *35S*	*nptII*, *HPT*, *GUS*	n.s.	n.s.	n.s.	Aida et al. 1992
LBA4404, LBA9402	Leaf	CF, VA	K	CaMV *35S*	*nptII*, *GUS*, *opines*	n.s.	None	—	Van Wordragen et al. 1992a
LBA4404, A281, Ach5	Leaf	CF, VA	none	CaMV *35S*	*nptII*, *GUS*, *opines*	n.s.	None	—	Van Wordragen et al. 1992b
LBA4404	Leaf	CF, VA	none	CaMV *35S*	*nptII*, *GUS*	n.s.	None	—	De Jong et al. 1993
EHA101, Ach5, C58, Bo542	Leaf, stem	CF, VA	K, H	CaMV *35S*	*nptII*, *HPT*, *GUS*	1.04–12.14	Callus, shoots, plants	histochemical assay+	Renou et al. 1993
LBA4404, C58	Stem	CF	K, B	CaMV *35S*	*nptII*, *GUS*, *luc*	n.s.	None	—	Lowe et al. 1993
A281	Leaf	CF, VA	K	CaMV *35S*	*nptII*, *GUS*, *cry1Ab*	n.s.	Callus	110–3,660 (Callus)	Van Wordragen et al. 1993
LBA4404, AGL0	Flower	CF, VA	K	CaMV *35S*	*nptII*, *GUS*	0.0–12.6	Shoot	histochemical assay +	De Jong et al. 1994
B6S3	Leaf	CF, TI	K	CaMV *35S*	*nptII*, *GUS*, *ocs*	17.0	Plant	25.61–73.31 (Plants)	Pavingerová et al. 1994
EHA105, Ach5, A281, Chry5	Leaf	CA	K	CaMV *35S*	*nptII*, *GUS*, TSWV *N*	4–7	Leaf	10–160 (Leaves)	Urban et al. 1994
B6S3	Leaf, stem	CF, TI	K	CaMV *35S*	*GUS*, *ocs*	n.s.	Plant	11.32–66.89 (Plants)	Benetka and Pavingerová 1995
AGL0	Pedicel	CF, VA	K	CaMV *35S*	*nptII*, *GUS*	0.0–45.0	Shoot	histochemical assay +	De Jong et al. 1995
AGL0	Stem	CF, VA	K	CaMV *35S*	*nptII*, *GUS*	5.6–15.6	Plant	histochemical assay +	Fukai et al. 1995
EHA105	Leaf	CA	P	CaMV *35S*	*nptII*, *GUS*	0.5–4.1	Plant	30–240 (Plants)	Sherman et al. 1998b
EHA101	Leaf	CF	G	*nos*, CaMV *35S*	*nptII*, *HPT*, *intron-GUS*	3.4	Leaf	histochemical assay +	Shinoyama et al. 1998
LBA4404	Stem	CF	K, H, G	CaMV *35S*	*nptII*, *GUS*	0.0–2.46	n.s.	n.s.	Takatsu et al. 1998
EHA101	Stem	CA	H	CaMV *35S*	*nptII*, *GUS*	2.5	Leaf	histochemical assay + (treated 5′-azacitidine)	Shirasawa et al. 2000
LBA4404, C58C1, MP90	Stem	CF	K	CaMV *35S*	*nptII*, *GUS*	1.12–1.91	Plant	30–250 (Plants)	Takatsu et al. 2000
AGL0	Stem	CF, TI	K	*Lhca3.St1*, *Lhaca3.St1* with MARs	*nptII*, *GUS*	n.s.	Plant	140,000 (Lhca3.St1; leaves)	Annadana et al. 2001
AGL0	Stem	CF, VA	K	CaMV *35S*, CaMV *35S* with MARs, *UEP1*, *ChsA*, *EPF2*, *CER6*, *PMC*	*nptII*, *GUS*	n.s.	Plant	16,500 (UEP1; ray florets)	Annadana et al. 2002a

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Agrobacterium strain(s)	Segment(s)	Antibiotics (Ab)	Ab for selection	Promoter(s)	Transgene(s)	Transformation frequency (%)	Localization of *GUS* gene expression	GUS activity (pmol 4-MU mg^{-1} protein min^{-1})	Reference
LBA4404, EHA101, AGL0, C58C1	Leaf	TI	K	*nos*, CaMV 35*S*	*nptII*, *HPT*, *GUS*	n.s.	Cell	blue spot +	Kudo et al. 2002
LBA4404	Leaf	CF	G	*nos*, CaMV 35*S*	*nptII*, *HPT*, *GUS*	0–23.9	Shoot, root, plant	histochemical assay +	Shinoyama et al. 2002a
LBA4404, AGL0	Stem	CF	K	CaMV 35*S* with enhancer	*nptII*, *GUS*	0.0–25.0	Plant	histochemical assay +	Teixeira da Silva and Fukai 2002a, b
AGL0	Stem	CF, VA	K	CaMV 35*S*, *rbc1*	*nptII*, *GUS*		Plant	82,000 (rbc1; leaves)	Outchkourov et al. 2003
EHA105, AGL0	Leaf	CA	P	CaMV 35*S*, *cab*	*nptII*, *GUS*	0.5–6.5	Plant	58,766 (cab; leaves)	Aida et al. 2004
EHA105	Leaf	CA	P	*EF1α*	*nptII*, *GUS*	0.5–6.8	Plant	14,000 (Leaves)	Aida et al. 2005
EHA105	Leaf	CA	P	CaMV 35*S* with enhancer	*nptII*, *GUS*	n.s.	n.s.	98,500	Aida et al. 2008b

CA: carbenicillin, CF: cefotaxime (sodium salt), VA: vancomycin, TI: ticarcillin, K: kanamycin, H: hygromycin, B: Basta, P: paromomycin, G: geneticin (G418), MARs: matrix-associated regions. n.s.: not specified.

Sherman et al. 1998b). Takatsu et al. (1998) described that the susceptibility against antibiotics depends on cultivars by comparing three antibiotics, kanamycin, hygromycin and geneticin (G418). Shinoyama and Mochizuki (2006) showed a high selection ability of the non-mutated type *nptII* gene, which was artificially synthesized by PCR, against 20 to 30 $mg\,l^{-1}$ of G418. Yenofsky et al. (1990) warned that the chimeric *nptII* gene, inserted in some of the commonly used binary vectors such as pBIN19 and pBI121, has an undesired point mutation, reducing the resistance of the transformants against kanamycin.

Recently, marker-free transgenic chrysanthemums have been generated using twin T-DNA binary vectors (Sun et al. 2009). Because these transgenic plants cannot be re-transformed using the same selectable marker gene, technology that can eliminate the selectable marker gene needs to be developed. This will be a very useful approach toward producing marker-free transgenic chrysanthemums and to relieve public or scientific concerns regarding the dispersal of antibiotic- and herbicide-resistant genes into the environment.

1.2.4. Eliminating chimerism

Chimerism, the regeneration of chimeric plants comprising both transgenic and non-transgenic cells, is one of the most serious problems in establishing a stable transformation system in chrysanthemum (Benetka and Pavingerová 1995; Pavingerová et al. 1994). To eliminate the chimerism, a method that allows for the regeneration of plantlets only from transformed cells must be used. Shinoyama et al. (2002a) compared the transformation efficiencies and chimerism of two transformation procedures: a regeneration system through callus-induction (CI) and a regeneration system with direct shoot-induction (SI). In the CI regeneration system, *Agrobacterium* infection and callus induction are simultaneously performed on CI medium (MS medium + 1.0 $mg\,l^{-1}$ NAA, 0.5 $mg\,l^{-1}$ BAP) containing 250 or 100 $mg\,l^{-1}$ cefotaxime sodium salt and 20 $mg\,l^{-1}$ G418, and plantlets are regenerated from the calli on regeneration medium (MS medium + 0.5 $mg\,l^{-1}$ BAP, 0.2 $mg\,l^{-1}$ GA_3) containing 100 $mg\,l^{-1}$ cefotaxime sodium salt. In the SI regeneration system, shoots are directly regenerated from leaf discs infected with *Agrobacterium* on SI medium (MS medium + 2.0 $mg\,l^{-1}$ NAA, 0.5 $mg\,l^{-1}$ BAP) containing 250 or 100 $mg\,l^{-1}$ cefotaxime sodium salt and 20 $mg\,l^{-1}$ G418. More plantlets were regenerated from the SI regeneration system than from the CI regeneration system. Fewer transformed plantlets were obtained using the SI regeneration system than using the CI regeneration system. All transformed plantlets regenerated from the CI regeneration system were non-chimeric, whereas 80% of the transformed plantlets regenerated from the SI regeneration system were

chimeric. These results indicate that transformation using the CI regeneration system can eliminate non-transformed cells and prevent chimerism.

1.2.5. Promoters and translational enhancer

When the *β-glucuronidase* (*GUS*) reporter gene was driven by the cauliflower mosaic virus *35S* RNA (CaMV *35S*) promoter, relatively low GUS activities in CaMV *35S*::*GUS*-transgenic chrysanthemums have been reported (Urban et al. 1994). When the *GUS* gene was under the control of a CaMV *35S*::*Intron*, which often confers higher GUS activity in some plants (Ohta et al., 1990), the highest GUS activity in the transgenic chrysanthemum leaves was 18,100 pmol 4-MU mg^{-1} protein min^{-1} (Aida et al. 2004).

Alternatively, several efficient promoters have been developed for high transgene expression in chrysanthemums using GUS as a reporter (see Table 1). Annadana et al. described two such high-level expression promoters: the promoter from the potato *Lhca3.St.1* gene encoding apoprotein 2 of the light-harvesting complex of photosystem I from *Solanum tuberosum* (Annadana et al. 2001) and the promoter from the chrysanthemum *UEP1* gene encoding ubiquitin extension protein 1 (Annadana et al. 2002a). A promoter-terminator cassette of the chrysanthemum *rbcS1* gene (encoding the ribulose-1,5-bisphosphate carboxylase small subunit) conferred high GUS activity to the transgenic leaves (Outchkourov et al. 2003). Aida et al. (2004, 2005) reported other promoters that induce high GUS expression: the promoter from the chrysanthemum *cab* gene encoding chlorophyll-*a*/*b*-binding protein and the promoter from the tobacco *EF1α* gene encoding elongation factor 1*α*. The *EF1α* gene promoter was used to delay leaf senescence with a mutated ethylene receptor gene (Narumi et al. 2005) and to modify flower shape with a MADS-box gene (Aida et al. 2008b).

We cloned a novel bi-directional promoter fragment (465 bp) for the *mannopine synthase-1′* and *-2′* (*mas1′-2′*) genes from an *Agrobacterium tumefaciens* strain isolated from crown galls formed on chrysanthemum plants (Shinoyama et al. unpublished). The bi-directional promoters could induce the expression of genes fused at both ends of the promoters, which was first demonstrated in transgenic chrysanthemums with insect resistance by Shinoyama and Mochizuki (2006). We have also been successful in producing transgenic chrysanthemums in which the *mas* bi-directional promoters drive both insect resistance and sterility (Shinoyama et al. 2012b) and in which the small promoter cassette drives both antibiotic resistance and sterility (Shinoyama et al. 2012a).

Inducible promoters would be necessary to efficiently cope with the environmental stresses. Recently, such promoters, including that from the tomato *ACC oxidase* (*LEACO1*) gene (Khodakovskaya et al. 2009) and the stress-inducible *rd29A* promoter (Ma et al. 2010), were used for transgene expression in chrysanthemum (Table 1).

For the efficient transgene expression, appropriate levels of protein production are required. Although a promoter with high transcriptional activity results in a high-level accumulation of transgene mRNA, such accumulation tends to induce post-transcriptional gene silencing (Vaucheret et al. 1998). The 5′-untranslated region (5′UTR) of the tobacco *alcohol dehydrogenase* gene (*NtADH*-5′UTR) (Satoh et al. 2004) has been reported to be an efficient translational enhancer in chrysanthemums (Aida et al. 2008a). Transgene silencing has occasionally occurred in chrysanthemum transformants (Takatsu et al. 2000). The *NtADH*-5′UTR translational enhancer may have the potential to solve transgene silencing in transgenic chrysanthemum through its high transgene-expression mechanism.

1.2.6. Agrobacterium-mediated transformation protocol

We established an efficient *Agrobacterium*-mediated transformation protocol using the chrysanthemum cultivar 'Shuho no chikara' (Shinoyama et al. 1998, Table 3). Initially, aseptic plant materials were produced by meristem culture. Their shoot tips were surface-sterilized briefly by dipping in 70% ethanol and then 1% sodium hypochlorite for 15 min. They were rinsed with sterilized distilled water three times. Then, they were cultivated *in vitro* on basal MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.3% Gellan Gum (Wako Pure Chemical Industries, Osaka, Japan) adjusted to pH 5.8 prior to autoclaving at 120°C for 15 min. They were incubated at 25°C under a 16-h photoperiod using cool-white fluorescent lamps [photosynthetic photon flux (PPF, 400–700 nm) of 60 $\mu mol\ m^{-2}\ s^{-1}$] or at 25°C in darkness.

We routinely apply the CI system because it efficiently inhibits chimerism in the transformants (Shinoyama et al. 2002a). We use *A. tumefaciens* strains LBA4404 (Ooms et al. 1982), EHA101 (Hood et al. 1986) or EHA105 (Hood et al. 1993) for conformity with cultivars or vectors. Individual frozen stocks of *Agrobacterium* strains are pre-cultured on AB minimal medium plates (Clark and Maaloe 1967) containing 50 $mg\ l^{-1}$ kanamycin and 50 $mg\ l^{-1}$ rifampicin at 30°C for three days in an incubator. The antibiotics should be changed depending on the antibiotic resistance genes in the binary vectors and *Agrobacterium* strains. They are then cultured in 10 ml of liquid YEP medium (5 $g\ l^{-1}$ NaCl, 10 $g\ l^{-1}$, yeast extract, 10 $g\ l^{-1}$ peptone, pH 7.2) in an incubator shaker at 200 rpm for 5 h at 28°C.

Leaf discs are cut from newly formed expanding leaves (1 cm^2) by a cork-borer ($\varphi = 6$ mm) and immersed for 15 min at room temperature in MS liquid

medium containing 5% (v/v) Tween 20® and 50 μM acetosyringone with *Agrobacterium* (final OD_{600}=0.1). After immersion, the leaf discs are placed onto co-cultivation CI medium (MS medium+1.0 mg l^{-1} NAA, 0.5 mg l^{-1} BAP) (containing 1.0 g l^{-1} casamino acids) and co-cultivated for three days at 25°C in darkness. The leaf discs are transferred to bacteria elimination CI medium (containing 250 mg l^{-1} cefotaxime sodium salt) for the elimination of *Agrobacterium* and, after 10 days, are transferred to the selection CI medium I (containing 250 mg l^{-1} cefotaxime sodium salt and 20 mg l^{-1} G418) for the selection of putatively transformed calli. After three subcultures on the selection CI medium I, the leaf discs are transferred to selection CI medium II (containing 100 mg l^{-1} cefotaxime sodium salt and 20 mg l^{-1} G418) in which the concentration of cefotaxime sodium salt is reduced to promote callus proliferation. In the CI system, the leaf discs forming green calli are transferred to plantlet regeneration medium (MS medium+0.5 mg l^{-1} BAP, 0.2 mg l^{-1} gibberellin A_3 (GA_3) and 100 mg l^{-1} cefotaxime sodium salt) to obtain putatively transformed plantlets. The regenerated plantlets are transferred to rooting medium (MS medium without plant growth regulators), acclimatized under the same culture conditions in vermiculite and then transferred to a closed greenhouse at 25°C.

This CI regeneration system is applicable to other cultivars of chrysanthemum with appropriate changes in the concentration of G418 (between 20 and 30 mg l^{-1}), depending on the cultivar (Shinoyama et al. 2002a).

2. Recent examples of agronomically practical transgenic chrysanthemums

At present, several agronomic traits have been introduced to chrysanthemums using GM technology (Table 2). Several reports, however, seem to describe unclear results because only a small number of transgenic lines were tested or because the transgenes were expressed at low levels. Therefore, whether the new transgene-based agronomic traits have practical potential is not obvious. To obtain agronomically useful transgenic plants, generating a great number of independent transgenic lines and selecting lines that stably express the transgenes at appropriate levels would be necessary. Here, we show recent examples of agronomically practical transgenic chrysanthemums with high and stable expression of transgenes.

One example is insect-resistant chrysanthemums. Insect damage brings yield loss, and thus, expensive pesticides are required in chrysanthemum cultivation to control insects. In particular, lepidopteran insects cause heavy damage and are responsible for a substantial proportion of insect loss. To protect chrysanthemums from the damage, Shinoyama et al. (2003) introduced the *mcbt* gene, which was a modified *cry1Ab* sequence (encoding *Bacillus thuringiensis* insecticidal protein Cry1Ab) that maintained the amino acid sequences; eliminated AT-rich sequences such as ATTTA, which destabilize mRNA in eukaryotes (Murray et al. 1991; Perlak et al. 1991; Van Aarssen et al. 1995); and increased the preferred codon of the *Compositae* family. A total of 317 transgenic chrysanthemum lines were obtained, and of 20 randomly sampled transgenic lines, 11 highly expressed the Cry1Ab protein. In addition, all tobacco budworm larvae (*Helicoverpa armigera*) that fed on the leaves were dead during the first instar, resulting in a high insecticidal effect against lepidopteran insect larvae. Shinoyama and Mochizuki (2006) obtained 1,586 transgenic chrysanthemum lines in another 5 chrysanthemum cultivars and successfully induced 10 lines of 20 randomly sampled transgenic lines to confer a high lepidopteran insect resistance using the same protocol.

The second example of practical chrysanthemum transformation is flower color modification. Chrysanthemums contain anthocyanins and carotenoids as pigments in their ray florets. The pink to violet flower color is controlled by the existence of anthocyanins alone, the cream to yellow color is controlled by carotenoids alone, and the blond to orange color is directed by both anthocyanins and carotenoids. White flowers lack both anthocyanins and carotenoids. Ohmiya et al. (2006) demonstrated white petals could be converted into yellow petals by suppressing the expression of *carotenoid cleavage dioxygenase* (*CmCCD4a*) using RNAi technology. Although carotenoids are initially synthesized in both yellow and white flowers, carotenoids are degraded by the carotenoid cleavage dioxygenase (CCD) protein in white flowers. This strategy was used to produce "Yellow Jimba" from 'Jimba', which is the most popular white-flower chrysanthemum cultivar in Japan (Ohmiya et al. 2009; Fig. 1). They first obtained 61 transformed plants carrying a *CmCCD4a* RNAi construct, but the petals of the transformed plants were very pale yellow. They then chose the most yellow transformed plant to perform the second transformation with another *CmCCD4a* RNAi construct bearing different DNA sequences. They finally obtained 50 double-transformed plants, and more than half showed yellow-colored petals. Recently, expression of the *flavonoid 3′,5′-hydroxylase* gene in chrysanthemums generated delphinidin-based anthocyanins, which resulted in a flower color shift toward blue (Noda et al., in preparation).

3. Prevention of transgene flow for practical use of GM chrysanthemums

Transgenic crops were first cultured commercially in

Table 2. Studies on *Agrobacterium tumefaciens*-mediated transformation systems and introduction of agronomically important traits to chrysanthemums.

Agrobacterium strain(s)	Segment(s)	Antibiotics (Ab)	Ab for selection	Promoter(s)	Transgene(s)	Changed trait(s)	Reference
LBA4404	Peduncle	VA	K	*nos*, CaMV *35S*	*nptII*, *GUS*, *CHS*	Flower color	Lemieux et al. 1990
LBA4404	Leaf	CA	K	CaMV *35S*	*nptII*, *CHS*	Flower color	Courtney-Gutterson et al. 1993
A281	Leaf	CF, VA	K	CaMV *35S*	*nptII*, *GUS*, *cry1Ab*	Insect resistance	Van Wordragen et al. 1993
LBA4404	Leaf	CA	K	CaMV *35S*	*nptII*, *CHS*	Flower color	Courtney-Gutterson et al. 1994
B6S3	Leaf	CF, TI	K	CaMV *35S*	*nptII*, *GUS*, *ocs*	Plant shape	Pavingerová et al. 1994
EHA105, Ach5, A281, Chry5	Leaf	CA	K	CaMV *35S*	*nptII*, *GUS*, TSWV *N*	Virus and viroid resistance	Urban et al. 1994
B6S3	Leaf, stem	CF, TI	K	CaMV *35S*	*GUS*, *ocs*	Plant shape	Benetka and Pavingerová 1995
C58, A281	Leaf	CF	K	CaMV *35S*	*nptII*, *bt*	Insect resistance	Dolgov et al. 1995
A281, GV3101, C58, CBE21	Leaf	CF	K, H	*nos*, CaMV *35S*, *mas*	*nptII*, *HPT*, *AFP*, *bt*, *rolC*, *CHS*	Flower color, shape, insect resistance, stress resistance	Dolgov et al. 1997
LBA4404	Leaf	TI	K	*nos*, CaMV *35S*	*nptII*, *Lc*	Flower color	Boase et al. 1998
LBA4404	Leaf, stem	CF	K	CaMV *35S*	*nptII*, *NP-1*	n.s.	Fu et al. 1998
LBA4404	Stem	n.s.	n.s.	CaMV *35S*	*nptII*, *F3′,5′H*	Flower color	Kim et al. 1998
EHA105	Leaf	CA	P	CaMV *35S*	*nptII*, TSWV *N*	Virus and viroid resistance	Sherman et al. 1998a
LBA4404	Stem	n.s.	K	CaMV *35S*	*nptII*, *LFY*cDNA	Flowering time	Shao et al. 1999
C58, MP90	Stem	CF	K	CaMV *35S*	*nptII*, *RCC2*	Fungal resistance	Takatsu et al. 1999
GV3101	Leaf	CF	K	CaMV *35S*	*Agrobacterium rhizogenes rolC*	Plant shape	Mitiouchkina and Dolgov 2000
EHA101	Stem	CF, VA	K	CaMV *35S*	*nptII*, *Ac*	n.s.	Tosca et al. 2000
n.s.	Pedicel	CF, TI	K	CaMV *35S*	modified *cry1C*	Insect resistance	De Jong 2001
EHA105	Leaf, stem	CA	K	CaMV *35S*	Rice *phyA* and Arabidipsis *phyB*	Plant shape	Zheng et al. 2001
AGL0	Pedicel	CF, VA	K	*UEP1*	Potato *multicystatin*	Insect resistance	Annadana et al. 2002b
LBA4404, AGL0	Leaf	CF	K	CaMV *35S* with leader	*nptII*, *pacI*	Virus and viroid resistance	Ishida et al. 2002
LBA4404	Leaf	CF	K	*nos*, CaMV *35S*	*nptII*, *OsMADS1*		Jeong et al. 2002
LBA4404	Leaf	CF	G	CaMV *35S*	*nptII*, non-modified *cry1Ab*	Insect resistance	Shinoyama et al. 2002b
LBA4404, AGL0	Leaf	CF	K	CaMV *35S* with leader	*nptII*, *pacI*	Virus and viroid resistance	Toguri et al. 2003
AGL0	Pedicel	CF, VA	K	CaMV *35S*, *GAI*	*nptII*, *GAI*	Plant shape	Petty et al. 2003
EHA101	Leaf	CF	G	*nos*, CaMV *35S*	*nptII*, *HPT*, modified *cry1Ab*	Insect resistance	Shinoyama et al. 2003
n.s.	Leaf	CF	H	CaMV *35S*	*IbMADS4*	Plant shape	Aswath et al. 2004
LBA4404	stem	TI	K	*cor15a*	*ipt*	Leaf senescence	Khodakovskaya et al. 2005
EHA105	Leaf	CA	P	*EF1α*	*mDG-ERS1*	Leaf senescence	Narumi et al. 2005
LBA4404, AGL0	Leaf	CF	K	CaMV *35S*	*pac1*	Virus and viroid resistance	Ogawa et al. 2005
LBA4404	Leaf	CA	K, H	*rolC*	*rolC*	Plant shape	Kubo et al. 2006

Table 2. Studies on *Agrobacterium tumefaciens*-mediated transformation systems and introduction of agronomically important traits to chrysanthemums.

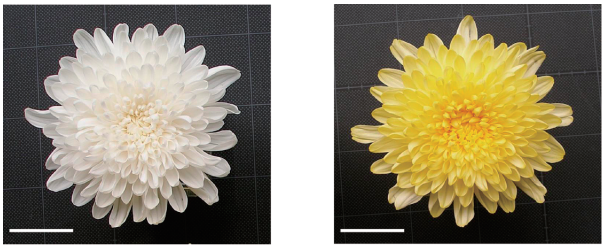
Agrobacterium strain(s)	Segment(s)	Antibiotics (Ab)	Ab for selection	Promoter(s)	Transgene(s)	Changed trait(s)	Reference
C58	Leaf	CF	K	*nos*, CaMV *35S* with enhancer, *rd29A*	*DREB1A*	Stress resistance	Hong et al. 2006
EHA105	Leaf	CA	P	*EF1α*	*CmCCD4a*-RNAi	Flower color	Ohmiya et al. 2006
EHA105	Leaf	CF	G	*mas*	*nptII,* modified *cry1Ab*	Insect resistance	Shinoyama and Mochizuki 2006
CBE21	Leaf, stem	CF	K	double CaMV *35S* with enhancer	*ORF5* encoding the CVB coat protein	Virus and viroid resistance	Skachkova et al. 2006
n.s.	n.s.	n.s.	n.s.	*rbcS1*	modified *cry1Ca*, engineered *seven-domain protein inhibitor* gene, strawberry *linalool synthase* gene	Insect resistance	Visser et al. 2007
LBA4404, C58C1	Leaf	CF	K	CaMV *35S*	*Ls*-like gene	Plantshape	Han et al. 2007
EHA105	Leaf	CA	P	*EF1α*	chrysanthemum *AGAMOUS*	Flower shape	Aida et al. 2008a
EHA105	Leaf	CF	G	*mas*	*nptII*, modified *cry1Ab*	Insect resistance	Shinoyama et al. 2008
LBA4404	Stem	TI	K	*LEACO1*	*ipt*	Plant shape	Khodakovskaya et al. 2009
EHA105	Leaf	CA	P	CaMV *35S*, *EF1α*	*CmCCD4a*-RNAi	Flower color	Ohmiya et al. 2009
n.s	Leaf	CF	K	*rd29A*, CaMV *35S*	*DREB1A*	Stress resistance	Ma et al. 2010
EHA105	Leaf	CA	K	CaMV *35S*	$hpaG_{Xoo}$	Fungal resistance	Xu et al. 2010
LBA4404	Leaf	CF	K	*nos*, CaMV *35S*	*nptII*, *HPT*, *CaXMT1*, *CaMXMT1*, *CaDXMT1*	Fungal resistance	Kim et al. 2011a
LBA4404	Leaf	CF	K	*nos*, CaMV *35S*	*nptII*, *HPT*, *CaXMT1*, *CaMXMT1*, *CaDXMT1*	Insect resistance	Kim et al. 2011b
EHA105	Leaf	CA	P	CaMV *35S*	*TCP3*-SRDX	Plant shape	Narumi et al. 2011
EHA105	Leaf	CF	G	*nos*, *mas*	*nptII*, *CmETR1/H69A*	Male/female sterility	Shinoyama et al. 2012a
EHA105	Leaf	CF	G	*nos*, *mas*	*nptII*, *CmDMC1*-RNAi, modified *cry1Ab*	Male/female sterility, insect resistance	Shinoyama et al. 2012b

CA: carbenicillin, CF: cefotaxime (sodium salt), VA: vancomycin, TI: ticarcillin, K: kanamycin, H: hygromycin, B: Basta, P: paromomycin, G: geneticin (G418). n.s.: not specified.

Table 3. Transformation time table using the callus induction (CI) system.

Day	Procedure	Key points
0	**[Inoculation with *Agrobacterium*]**	
	Culture of *Agrobacterium* in liquid YEP liquid medium for 5 h.	
	Prepare leaf discs with cork-borer from the aseptic plants.	Plant materials are produced by meristem culture. The newly formed expanding leaves are used for leaf discs.
	Immerse the leaf discs into MS liquid medium containing *Agrobacterium* for 15 min.	
	Coculture the leaf discs with *Agrobacterium* on cocultivation callus induction (CI) medium.	
3	**[Elimination of *Agrobacterium* after cocultivation]**	
	Transfer the discs to bacteria elimination CI medium.	
10	**[Selection of transformed cells]**	
	Transfer to selection CI medium.	
24	Transfer to fresh selection CI medium I.	
38	Transfer to fresh selection CI medium I.	Calli are induced on the edge of leaf discs.
52	Transfer to fresh selection CI medium II.	
66	Transfer to fresh selection CI medium II.	
80	**[Regeneration of plantlet from the transformed calli]**	
	Transfer to plant regeneration medium.	
101	Transfer to plant regeneration medium.	
122	Transfer to plant regeneration medium.	Shoots are formed on the calli.
	Collect elongated shoots (first collection) and trasfer to rooting medium.	
143	Transfer to plant regeneration medium.	The shoots are rooted.
	Collect elongated shoots (second collection) and trasfer to rooting medium.	
143–180	**[Acclimatizing the transgenic plants]**	
	Transfer rooted plants to a closed greenhouse.	In some cultivars, the low-temperature treatment (10°C, 40 days) must be performed.
200 onwards	**[Plants available for testing]**	

Medium constructions. CI medium: MS+1.0 mg l^{-1} NAA, 0.5 mg l^{-1} BA, 3% Sucrose (Suc.), 0.3% Gellan Gum (Gel.). Cocultivation CI medium: CI medium+1.0 g l^{-1} Casamino acids. Bacteria elimination CI medium: CI medium+250 mg l^{-1} Cefotaxine sodium salt (Cf.). Selection CI medium I: CI medium+250 mg l^{-1} Cf., 20 mg l^{-1} G418. Selection CI medium II: CI medium+100 mg l^{-1} Cf., 20 mg l^{-1} G418. Plant regeneration medium: MS+0.5 mg l^{-1} BA, 0.2 mg l^{-1} GA_3, 100 mg l^{-1} Cf., 3% Suc., 0.4% Gel. Rooting medium: MS+100 mg l^{-1} Cf., 3% Suc., 0.4% Gel.



Non-transgenic chrysanthemum 'Jimba'

Transgenic chrysanthemum "Yellow Jimba"

Figure 1. The suppression of the *carotenoid cleavage dioxygenase* gene converted white petals to yellow. Scale bars indicate 50 mm.

1996, and since then, the number of planted transgenic crops has been increasing worldwide. These increases are due to the many benefits of culturing transgenic crops, such as reduced production costs and farm labor. Recently, concerns have been raised about their potential harmful effects on biodiversity and the environment because transgenic crops are generated without ordinary crossings or intraspecies gene flow (Kamada 2001). Thus, risk assessment has been required for transgenic crops before their field cultivation.

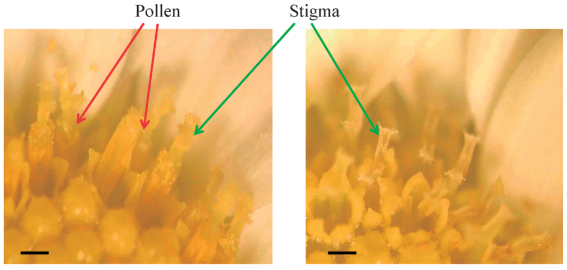
Items on the risk assessment of transgenic crops are well documented in the international templates of the Cartagena Protocol on Biosafety (Article 15 and Annex II). The actual assessment methods and procedures, however, vary depending on the types of genes introduced, the plant species, and the environment where the transgenic crops will be released.

Florist chrysanthemums are predominantly self-incompatible plants and are easily cross-pollinated by certain insects, such as bees (Nakata and Takeuchi 1998). Many wild chrysanthemum relatives in the *Compositae* family are cross-compatible with chrysanthemum cultivars and are widely distributed throughout Japan. F_1 plants from natural crossbreeding between chrysanthemum cultivars and their wild relatives have been found in several relatively wild habitats (Taniguchi et al. 2009). As one of the risks of GM chrysanthemums, the pollen of transgenic chrysanthemums could be carried by insect pollinators and crossed with wild relatives, resulting in the production of F_1 plants with the transgene in relatively wild habitats. These F_1 plants

Table 4. Percentage of mature pollens in GM and non-GM chrysanthemums (modified from Shinoyama et al. 2012a and 2012b)

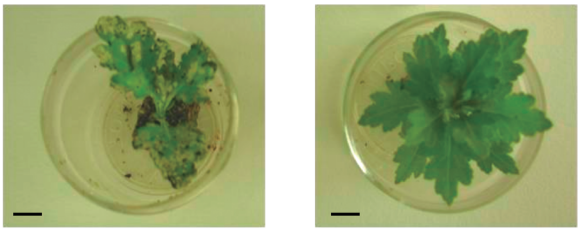
Line	non-GM	The *CmETR1/H69A* gene			The *CmDMC1*-RNAi segements and the modified *cry1Ab* gene						
Temp. (°C)		ETR91	ETR191	ETR324	DB194	DB260	DB315	DB395	DB569	DB576	DB613
35	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	62.0	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**
20	81.5	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**
15	50.4	10.3**	12.7**	10.9**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**
10	37.8	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**	0.0**

ETR: transgenic lins bearing the *CmETR1/H69A* gene, DB: transgenic lines bearing the *CmDMC1*-RNAi segments and the modified *cry1Ab* gene. Data are the percentages of no. of mature pollens/no. of total pollens per anther sac. Mature and immature pollens were differentially stained by Alexander staining (Alexander 1969). ** Significant at 1% level by Student *t* test.



Non-transgenic chrysanthemum 'Yamate-shiro' Transgenic chrysanthemum

Figure 2. The pollen induction of tubular flowers of non-transgenic and transgenic chrysanthemum plants carrying both a modified *cry1Ab* gene and *CmDMC1*-RNAi segments. Scale bars indicate 1 mm.



Non-transgenic chrysanthemum 'Shuho-no-chikara' Transgenic chrysanthemum

Figure 3. Bioassay of insects feeding on non-transgenic and transgenic chrysanthemum plants carrying both a modified *cry1Ab* gene and *CmDMC1*-RNAi segments using *Helicoverpa armigera* first instar larvae. Scale bars indicate 10 mm.

are thought to have various direct and indirect effects on biodiversity. For example, the insect-resistant plants may kill rare insect species that feed only on the wild plants. Alternatively, because the plants are not damaged by insects, they may grow thick and deprive the habitat of other wild plants.

To eliminate the risks of transgene flow, Shinoyama et al. (2012a) tried to create male-sterile transgenic chrysanthemums using the modified melon ethylene receptor gene, *CmETR1/H69A*. The overexpression of the *CmETR1/H69A* gene delayed the tapetum degradation of the anther sac, resulting in a reduction of mature pollen grains in some of the transgenic lines. In 15 of the 335 *CmETR1/H69A*-overexpression chrysanthemum lines, male and female fertility was significantly lower than in non-GM chrysanthemum. In particular, three of these lines produced no mature pollen grains in the temperature ranging 10 to 35°C, with the exception that 10% pollen grains in an anther sac were matured at 15°C (Table 4). Moreover, the female fertility of these three lines was decreased to half that of non-GM chrysanthemums. Overexpression of *CmETR1/H69A* gene most likely delays the maturation of the ovule.

Then, to produce transgenic chrysanthemums with both insect resistance and temperature-independent, complete male-sterility, Shinoyama et al. (2012b) transformed the *mcbt* gene and a 582-bp fragment of chrysanthemum meiosis-specific recombinase gene, *CmDMC1*, as an RNAi trigger segment (Fig. 2). We used the novel bi-directional *mas* promoter (Shinoyama et al. unpublished) to regulate the expression of the *mcbt* gene and the *CmDMC1*-RNAi segment located at both ends of the promoters. Transgenic lines showed high resistance for lepidopteran pest insects of chrysanthemums, such as the tobacco budworm (*H. armigera*), the cotton cutworm (*Spodoptera litura*) and the beet armyworm (*S. exigua*) (Fig. 3). Complete temperature-independent male sterility was achieved in seven of 682 transgenic lines (Table 4, Fig. 2). These seven lines produced no mature pollen grains from 10 to 35°C, which is the temperature range for chrysanthemum flowering. In the cross between GM lines and their wild relatives, a few F_1 seeds on the flowers of the GM lines, corresponding to 1/10 to 1/20 of non-GM chrysanthemum plants, were obtained (pollen parents were the wild relatives, and seed parents were the GM lines); no F_1 seeds were obtained on the flowers of the wild relatives (pollen parents were the GM lines, and seed parents were the wild relatives). Thus, these results suggested that these lines were completely male sterile and that their female fertility was partially retained. The F_1 plants obtained from the seeds of GM flowers showed male sterility, very weak

female fertility and strong insect resistance, indicating the stable inheritance of the transgenic phenotypes in the progeny. The *CmDMC1*-RNAi segment caused complete male sterility with incomplete female sterility. Because northern blot analysis showed the *mas* bi-directional promoters directed a lower transcription level of the *GUS* gene in ovules compared with leaves, stems, roots and pollens (Shinoyama et al. unpublished), the insufficient expression of the *CmDMC1*-RNAi segment in ovules might cause incomplete female sterility. Identifying more appropriate promoter(s) is desirable to realize high-level expression of the *CmDMC1*-RNAi segment in both pollen and ovules and to create completely male- and female-sterile transgenic chrysanthemums.

As a new method to suppress the function(s) of the transcription factor(s) of interest, Chimeric REpressor gene-Silencing Technology (CRES-T) has been successfully applied in chrysanthemums (Narumi et al. 2011). It would be very useful to silence the expression of a set of downstream genes under the control of the target transcription factor(s).

Advances in transgenic technology could reduce production costs and improve yield, cut-flower quality and commercial value. In the future, superior transgenic chrysanthemums would appear on the markets that possess agronomic traits with environmental safety.

Acknowledgments

We give special thanks to Drs. H. Kamada (University of Tsukuba), S. Ohki (Fukui Prefectural University), K. Tanaka (Kyoto Prefectural University), H. Ezura (University of Tsukuba), M. Mii (Chiba University), K. Wakasa (Tokyo University of Agriculture), K. Kondo (Tokyo University of Agriculture), M. Nakata (Toyama Botanical Garden), K. Taniguchi (Hiroshima University), Y. Yonezawa (Naruto University of Education), S. Fukai (Kagawa University) and Jaime A. Teixeira da Silva (Kagawa University) for their valuable suggestions for all of our experiments. In addition, we thank Ms. C. Amaya, F. Nogawa and R. Murai (Fukui Agricultural Experiment Station) for their technical support in all of our experiments. The authors are particularly indebted to Dr. Teixeira da Silva for improvements in the grammar, content and structure of this review.

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