농학석사학위논문

Development of genetically modified chrysanthemums resistant to *Chrysanthemum stunt viroid*

국화왜화바이로이드 저항성 유전자 조작 국화개발

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농생명공학부 식물미생물학전공

조경민

A THESIS FOR DEGREE OF MASTER SCIENCE

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BY

KYOUNG-MIN JO

Department of Agricultural Biotechnology

The Graduate School of Seoul National University

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A THESIS FOR DEGREE OF MASTER SCIENCE

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UNDER THE DIRECTION OF
DR. KOOK-HYUNG KIM

SUBMITTED TO FACULTY OF THE GRADUATE
SCHOOL OF SEOUL NATIONAL UNIVERSITY

BY KYOUNG-MIN JO

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICUTURAL BIOTECHNOLOGY

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BY THE COMMITTEE MEMBERS

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ABSTRACTS

Development of genetically modified chrysanthemums resistant to *Chrysanthemum stunt viroid*

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Major in Plant Microbiology

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Chrysanthemum (*Dendranthema X grandiflorum*) belonging to the family *Asteraceae* has been cultivated for more than 3,000 years in Asia and Europe. It is currently one of the most popular flowers and also important for the worldwide floriculture industry. Viroids are the smallest pathogens composed of circular RNAs and cause infectious diseases in the plant host. So far, two viroids including *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) infecting chrysanthemum has been reported. Especially,

CSVd infection leads to serious damage in chrysanthemum production with following symptoms like bleached leaves, stunting, and reduced level of anthocyanin. Therefore, it is desirable to make genetically modified (GM) chrysanthemums resistant to CSVd. To engineer viroid resistant chrysanthemums, RNAi mediated silencing system using partial RNA sequences of the viroid was applied. Four different vectors containing different sense/antisense RNA sequences of CSVd were constructed and used for *Agrobacterium* mediated transformation. Initially, a total of 39 transgenic lines carrying the individual CSVd RNA sequence were finally obtained. To test resistance against CSVd infection, 60 out of 145 GM chrysanthemum plants were inoculated with CSVd by the sap inoculation. Relative resistance level was determined by semi-quantitative RT-PCR using total RNAs extracted from both inoculated and upper systemic leaves and CSVd specific primers. Of 16 tested GM chrysanthemums, nine GM chrysanthemum lines showed very strong resistance against CSVd infection and/or replication. In general, GM chrysanthemums grow uniformly and are much healthier than non-GM chrysanthemums. This study is the first report which develops GM chrysanthemums resistant to CSVd using CSVd sense/antisense RNAs.

Keywords: *Chrysanthemum stunt viroid*, viroid resistance, *Agrobacterium*-mediated transformation, genetically modified chrysanthemums,

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Introduction

The chrysanthemum (*Dendranthema X grandiflorum*) is one of the popular flowers in the world belonging to the family *Asteraceae*. The international market for cut and potted chrysanthemums is increasing, and chrysanthemums in many European and Asian countries are commercially very important for the floral industry (Tomassoli et al., 2004). Several pathogens, including viruses, viroids, and phytoplasma cause serious diseases in chrysanthemums. To date, nine viruses such as (*Tomato aspermy virus*, *Cucumber mosaic virus*, *Chrysanthemum virus B*, *Tomato spotted wilt virus*, *Turnip mosaic virus*, *Tobacco mosaic virus*, *Potato virus Y*, and *Potato virus X*) and two viroids (*Chrysanthemum stunt viroid* and *Chrysanthemum chlorotic mottle viroid*) are known to infect chrysanthemums (Verma et al., 2003). Viroids are the smallest known plant pathogens. They consist of a circular, single-stranded RNA, which does not encode a protein. Viroid RNAs range from 246 to 401 nucleotides (Navarro and Flores, 1997; Ding, 2010). Viroids traffic from cell-to-cell via plasmodesmata (Ding and Wang, 2009). To date, over 30 species of viroids have been reported; these can be divided into two families, the *Pospiviroidae* and the *Avsunviroidae* (Góra-Sochacka, 2004). The family *Pospiviroidae* includes five genera, such as *Apscaviroid*, *Cocadviroid*, *Coleviroid*, *Hostuviroid*, and *Pospiviroid*. So far, ten species including *Chrysanthemum stunt viroid* (CSVd) and *Potato spindle tuber viroid*

(PSTVd) are members of the genus *Pospiviroid* (Owens et al., 2012). The genomic RNA of members of the family *Pospiviroidae*, which replicate in the nucleus, assumes rod-like or quasi-rod-lke conformation in which, based on local sequence similarity, five domains have been proposed: the left-terminal, pathogenicity, central, variable, and right-terminal domains(Keese and Symons, 1985). Moreover, several conserved regions have been identified in the rod-like conformation, including the central conserved region (CCR), likely involved in replication (McInnes and Symons, 1991; Baumstark et al., 1997; Gas et al., 2007; Gas et al., 2008) and the terminal conserved region (TCR) or the terminal conserved hairpin (TCH), which appear mutually exclusive (Koltunow and Rezaian, 1988; Puchta et al., 1988; Flores et al., 1997). Interestingly, chrysanthemums are a common host for two different viroids, the CSVd and the CChMVd which are quite different from each other in structure and function (Table 1 and Fig. 1) (Prins et al., 2008). CSVd and CChMVd have caused serious damage in the production of chrysanthemums (Matoušek et al., 1994). Thus, it is necessary to generate genetically modified (GM) chrysanthemums resistant to viroids. Several methods have been applied to develop GM plants resistant to viruses or viroids. For example, many GM plants expressing a viral protein such as capsid protein, movement protein, and replicase protein have been developed (Atkins et al., 1995). In addition, viral sense or antisense RNAs have been frequently used to confer RNA interference mediated resistance to GM plants

Table 1. Characteristics of CSVd and CChMVd viroids (Cho, W.K et al., 2013).

Characteristics	CSVd	CChMVd
Disease	Chrysanthemum stunt	Chrysanthemum chlorotic mottle
Symptoms	Light green young leaves, chlorotic spots, stunting, small leaves and flowers, and decreased rooting ability	Yellow-green mottling, chlorosis, and dwarfed size
Family and genus	*Pospiviroidae*, *Pospiviroid*	*Avsunviroidae*, *Pelamoviroid*
Genome size	354-356 nt	398-401nt
Replication method	Asymmetric rolling circle mechanism	Symmetric rolling circle mechanism with the hammerhead ribozymes
Replication localization	Nucleus	Chloroplast
Structure	Rod-like structure including central conserved region (CCR)	Branched conformation including hammerhead ribozymes
Transmission	Sap, grafting, and seed	Sap, grafting
Host	Chrysanthemums, *Petunia hybrida*, tomato, *Gynura aurantiaca*, *Ageratum*, dahlia, *Senecio*, *Vinca major*, *Argyranthemum frutescens* and many plants belonging to the families *Solanaceae* and *Asteraceae*	Restricted to chrysanthemums

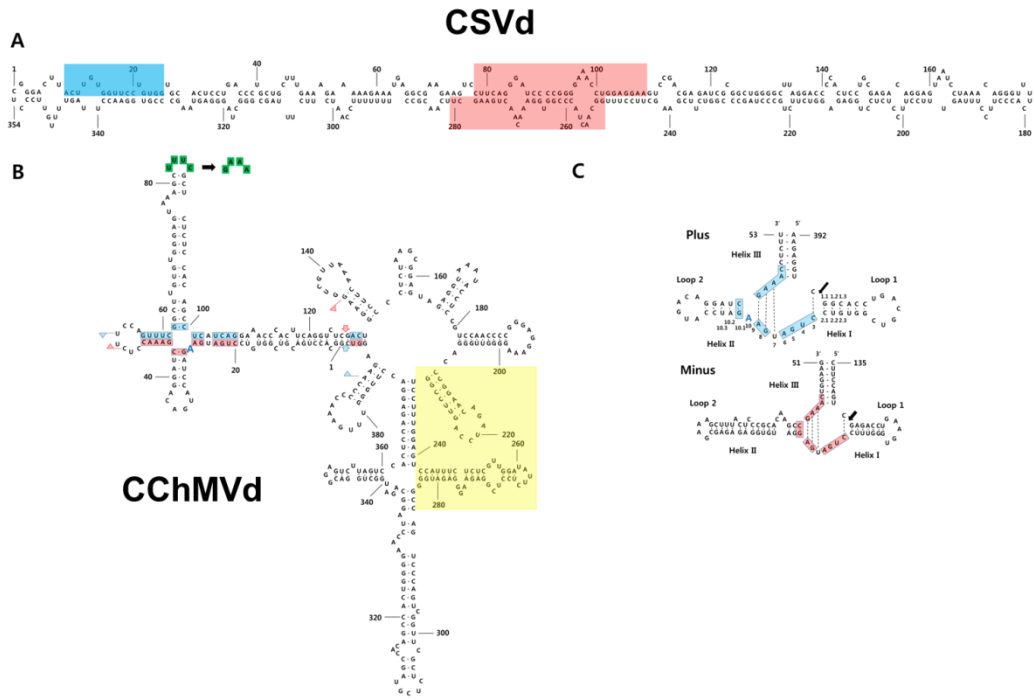

Fig. 1. Predicted secondary structures of CSVd and CChMVd (Cho, W.K et al., 2013).

(A) Predicted secondary structure of CSVd (adapted from Ref. (Yoon and Palukaitis, 2012) with minor modification). The central conserved region (CCR) is indicated by light-red shading and the terminal conserved region is indicated by light-blue shading. (B) Predicted secondary structure of CChMVd (adapted from Ref. (De la Peña and Flores, 2001) with minor modification). Plus and minus self-cleavage domains are delimited by flags, residues conserved in most natural hammerhead structures are boxed, and the self-cleavage sites are indicated by arrows. Light-blue shading and light-red shading in flags, boxes, and arrows refer to plus and minus polarities, respectively. The changes in the tetraloop delimited

by positions 82–85 (UUUC to GAAA) that convert a symptomatic variant into non-symptomatic are shown with green-colored boxes (De la Pena and Flores, 2002). The light-yellow square demarcates the domain that alternatively can form a kissing–loop interaction (Dufour et al., 2009). (C) Hammerhead structures of the plus and minus strands of CChMVd (adapted from Ref. (De la Peña and Flores, 2001) with minor modification). Residues conserved in most natural hammerhead structures are on light-blue shading and light-red shading in the plus and minus polarities, respectively, and the self-cleavage sites are indicated by arrows. Blue colored A indicates the position of the extra A in the CChMVd secondary structure and in its plus hammerhead structure. Numbering is done based on the Ref. (De la Peña and Flores, 2001).

(Shinoyama et al., 2012). Furthermore, host genes involving in pathogen resistance could be non-viral sources for the generation of GM plants (Seo et al., 2007). Among known approaches, previous studies reported that RNAi mediated silencing system using partial anti-sense RNA sequences of the viroid was very effective for viroid resistance (Saito et al., 1992; da Silva, 2005). There are two known methods for plant transformation; *Agrobacterium*-mediated transformation and biolistics method. *Agrobacterium*-mediated transformation is an ideal technique for producing transgenic plants which are valuable for agronomic and scientific purposes. In case of ornamental plants, it is an efficient technique in yielding desirable changes in cultivars without disturbing their important ornamental traits (Horst et al., 1977; Sherman et al., 1998). So far, many research groups have produced GM chrysanthemums by *Agrobacterium*-mediated transformation system (Table 2)(Sano et al., 1997; Goldbach et al., 2003; Seo et al., 2007; Sano et al., 2010).To successfully introduce useful and interesting foreign genes into plant genomes via *Agrobacterium*, a reproducible plant regeneration system is required. In case of garland chrysanthemums or other chrysanthemum species, it has been reported that the tissue-culture was quite difficult. In this study, I developed GM chrysanthemums resistant to CSVd using anti-sense RNAs of CSVd by *Agrobacterium*-mediated transformation. Furthermore, I developed a simple, reliable, and efficient genetic engineering system for regenerating chrysanthemum plants with a commercial cultivar from

leaf explants and improved the efficiency of chrysanthemum transformation. Using a sap inoculation and quantitative RT-PCR we identified several GM chrysanthemums showing strong resistance against CSVd infection.

Table 2. Studies on *Agrobacterium tumefaciens*-mediated transformation systems of chrysanthemums using GUS gene (Harue.S et al., 2012).

***Agrobacterium* strain(s)**	**Segment(s)**	**Antibiotics (Ab)**	**Ab for selection**	**Promoter(s)**	**Transgene(s)**	**Localization of *GUS*gene expression**	**Reference**
LBA4404, EHA101, AGL0, C58C1	Leaf	TI	K	*nos*, CaMV*35S*	*nptII*, *HPT*, *GUS*	Cell	Kudo et al. 2002
LBA4404	Leaf	CF	G	*nos*, CaMV*35S*	*nptII*, *HPT*, *GUS*	Shoot,root, plant	Shinoyama et al. 2002a
LBA4404, AGL0	Stem	CF	K	CaMV*35S* with enhancer	*nptII*, *GUS*	Plant	Teixeira da Silva and Fukai 2002a, b
AGL0	Stem	CF, VA	K	CaMV*35S*, *rbc1*	*nptII*, *GUS*	Plant	Outchkourov et al. 2003
EHA105, AGL0	Leaf	CA	P	CaMV*35S*, *cab*	*nptII*, *GUS*	Plant	Aida et al. 2004
EHA105	Leaf	CA	P	*EF1_*	*nptII*, *GUS*	Plant	Aida et al. 2005
EHA105	Leaf	CA	P	CaMV*35S* with enhancer	*nptII*, *GUS*	n.s.	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.: notspecified.

Materials and methods

Generation of constructs for chrysanthemum transformation

Four different regions of CSVd were selected as shown in Fig. 2 and primers were designed. Using region specific primers, four different PCR products were obtained. After PCR purification using TOPO® Cloning Kit (Invitrogen, Carlsbad, CA, USA), the PCR products were cloned into TOPO-D entry vector. For chrysanthemum transformation, a gateway compatible destination vector named as pBH7-att vector was used. The pBH7-att vector is a binary vector carrying 35S promotor, basta resistance gene, spectinomycin resistance gene, left border (LB), and right border (RB) (Fig. 3). In final, four different constructs referred as CSVd-Sense-1, CSVd-Anti-Sense-2, CSVd-Sense-3, and CSVd-Anti-Sense-3 were generated (Table 3).

Preparation of medium for chrysanthemum tissue culture and propagation

Basic medium forchrysanthemum tissue culture is composed of Murashige and Skoog (MS) medium (Duchefa Biochemie BV, Haarlem, Netherlands) 4.4 g/L, sucrose (Junsei Chemical, Tokyo, Japan) 30 g/L, Indole-3-acetic acid (IAA) (Sigma-Aldrich, St. Louis, USA) 0.5 mg/L and N6-benzyladenine (BA) (Duchefa

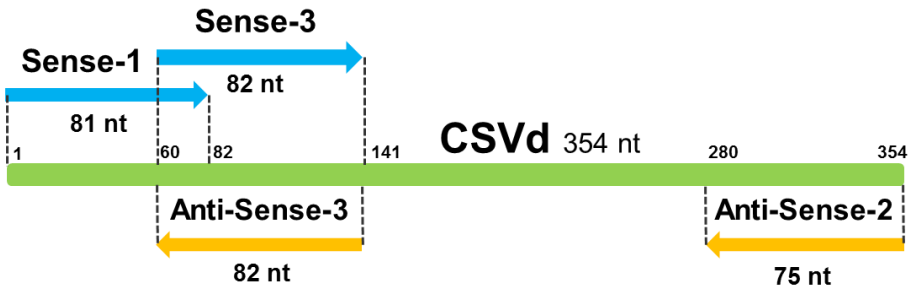

Fig. 2. Position of CSVd partial RNAs used for generation of vectors.

Green and orange colors indicate sense and anti-sense strands, respectively. The two regions including sense 3 and anti-sense 3 represent the conserved central regions of CSVd which might be highly conserved in different CSVd variants. Blue and yellow colored arrows indicate sense and antisense RNA, respectively. The numbers indicates positions of CSVd RNA sequences.

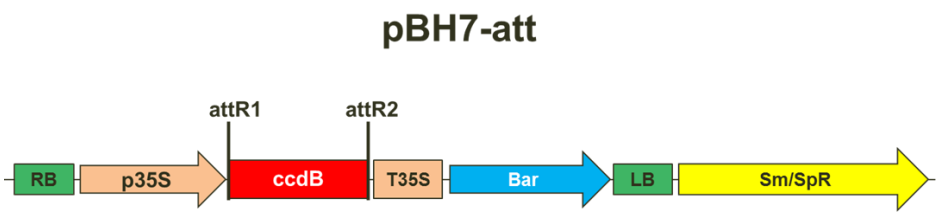

Fig. 3. The vector used for chrysanthemum transformation.

The vector named as pBH7-att is a gateway compatible binary vector carrying 35S promoter, *ccdB gene*, 35S terminator, and *bar gene*.

Table 3. Information of CSVd RNA sequences to generate sense and antisense RNA constructs.

Construct name	Size of CSVdRNA fragment	Position of RNA sequences in CSVd	Sequences
CSVd-Sense-1	81 nt	1 -> 82	AAAGAAATGAGGCGAAGAAGTCCTTCAGGGATCCCCGGGGAAACCTGGAGGAAGTCCGACGAGATCGCGGCTGGGGCTTAG
CSVd-Anti-Sense-2	75 nt	354 <-280	TTCTTTCAAAGCAGCAGGGTCAGGAGTGCACCACAGGAACCACAAGTAAGTCCCGAGGGAACAAAACTAAGGTTC
CSVd-Sense3	82 nt	60 -> 141	CGAGATCGCGGCTGGGGCTTAGGACCCCACTCCTGCGAGACAGGAGTAATCCTAAACAGGGTTTTCACCCTTCCTTTAGTTT
CSVd-Anti-Sense-3	82 nt	141 <- 60	AAACTAAAGGAAGGGTGAAAACCCTGTTTAGGATTACTCCTGTCTCGCAGGAGTGGGGTCCTAAGCCCCAGCCGCGATCTCG

Biochemie BV, Haarlem, Netherlands) 1.0 mg/L. Cefotaxime (Duchefa Biochemie BV, Haarlem, Netherlands) 125 mg/L was used to eliminate *Agrobacterium*. To select transgenic chrysanthemums, bastar (Sigma-Aldrich, St. Louis, USA) 0.75 mg/L was used. Medium for chrysanthemum propagation is consist of MS medium 4.4 g/L, sucrose 30 g/L and activated charcoal (Junsei Chemical, Tokyo, Japan) 1 g/L. For co-culture with chrysanthemum leaf disk and *Agrobacterium*, only 0.5X Liquid MS medium was used.

Preparation of plant hormones and antibiotics

To prepare stocks for IAA and NA, IAA (20 mg) and BA (20 mg), respectively, were dissolved in 1N NaOH (200 μL) and then 100 mL of distilled water were added. To prepare stocks for antibiotics, cefotaxime (6.25 g) and Bastar (5 g), respectively, were dissolved in 100 mL of distilled water. Finally, all prepared hormones and antibiotics were sterilized using 0.22 μm filter (Millipore Corporation, Billerica, MA, USA). Stock concentrations for hormones and antibiotics are 200 mg/L (IAA), 200 mg/L (BA), 62.5 g/L (cefotaxime), 50 g/L (bastar). The final concentrations for hormones and antibiotics are 0.5 mg/L (IAA), 1.0 mg/L (BA), 125 mg/L (cefotaxime), 0.75 mg/L (bastar).

Methods for *Agrobacterium*-mediated transformation and tissue culture

For chrysanthemum transformation, chrysanthemum cultivar vivid scarlet was used. Prepared leaf discs were incubated on the MS medium (IAA 0.5 mg/L and BA 1 mg/L) for 24 hour. After that, the leaf discs were co-inoculated with cultured *Agrobacterium* (O.D. 600 rate 0.7) in 0.5X MS medium for 15 minutes. Inoculated chrysanthemum leaf discs were dried on the sterilized filter paper and incubated on the MS medium (IAA 0.5 mg/L and BA 1 mg/L) in dark condition for three days. The leaf discs were transferred to selection medium (IAA 0.5 mg/L, BA 1 mg/L, and cefotaxime 125 mg/L) to eliminate the Agrobacteria by cefotaxime. After that, the leaf discs were transferred to fresh selection medium (IAA 0.5 mg/L, BA 1 mg/L, and cefotaxime 125 mg/L) every 10 days until shoots were formed. When shoots were formed, the linking point between leaf disc and shoot was cut. The shoots were transferred to selection medium containing bastar (0.75 mg/ L) until roots were appeared. In general, non-transgenic chrysanthemums could not develop roots in the medium containing bastar and GM chrysanthemums could be selected in this step. In final, the selected GM chrysanthemums were transferred the root inducing medium (cefotaxime 125 mg/L and Bastar 0.75 mg/L).

Propagation of chrysanthemums

The chrysanthemum stems were cut by a sterilized razor blade resulting in about 10 cm length of stems and they were planted in the Baroker-bed soil (Seoul-bio,

eumseoung-gun, Korea). The chrysanthemums were incubated for one week by covering with vinyl film (Fig. 4 A). When chrysanthemums are grown up to 70 cm tall, stems of the chrysanthemums were cut and propagated in new bed soil (Fig. 4 B).

Genomic DNA and total RNA extraction from chrysanthemums

Chrysanthemum leaves were harvested and frozen immediately in liquid nitrogen. To extract genomic DNA, chrysanthemum leaves were ground by mortar and pestle. The genomic DNA and the total RNA, respectively, were extracted by Qiagen DNeasy plant mini kit following and RNeasy plant mini kit following manufacturer's instruction (Qiagen, Hilden, Germany)..

Polymerase chain reaction (PCR) to check T-DNA insertion in transgenic chrysanthemums

PCR was performed with AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea) in 20 μL volumes, 10 pmol of forward primer, 10 pmol of reverse primer, 1 μL of DNA template and deionized water up to 20 μL. PCR conditions include *Taq* (*Thermus aquaticus*) DNA polymerase activation for 2 min, initial denaturation at 95°C for 3 minutes, 30 cycles of 20 seconds at 95°C for denaturation, 30

(A)

(B)

Fig. 4. Propagation of chrysanthemums.

Propagated chrysanthemum was grown in bed soil (A) and covered by vinyl film for incubation (B).

seconds at 55°C for annealing and 1 minute at 72°C for extension, and a final extension 5 minutes at 72°C. PCR was performed by c1000 thermal cycler (Bio-Rad, Hercules, USA).

Inoculation of CSVd in chrysanthemums

To identify GM chrysanthemum which are resistant to CSVd, CSVd infected chrysanthemum sap was inoculated on the leaves of wild type and GM chrysanthemums using carborundum (Fig. 5).

Reverse transcription (RT)-PCR

To examine CSVd replication, RT-PCR was performed using CSVd specific primers (354 nt of CSVd full length detection forward and reverse primers) with DiaStar™ OneStep RT-PCR Kit (Solgent, Daejeon, Korea) in 30 μL volumes, 10 pmol of forward primer, 10 pmol of reverse primer, 0.1 μg of RNA template, 6 μL of 5X one step RT-PCR buffer, 1 μL of 10 mM dNTP mix, 2 μL of one step RT-PCR enzyme mix and deionized water up to 30 μL. RT-PCR conditions include reverse transcriptase activation for 30 minutes at 50°C, initial denaturation at 95°C for 15 minutes, 35 cycles of 20 seconds at 95°C for denaturation, 40 seconds at 56°C for annealing and 30 seconds at 72°C for extension, and a final extension 5 minutes at 72°C. RT-PCR was performed by c1000 thermal cycler (Bio-Rad, Hercules, USA). *Actin* gene was used as positive control.

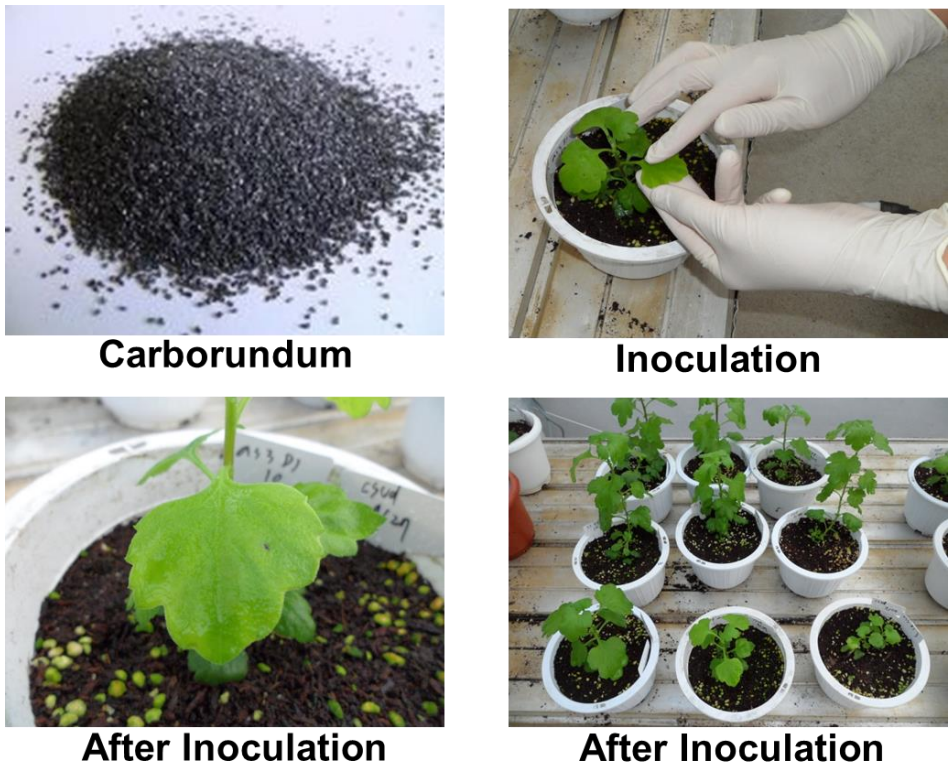

Fig. 5. Inoculation of CSVd on the leaves of chrysanthemum by sap using carborundum.

Examination of morphological characteristics

To examine morphological characteristics between WT and GM chrysanthemums, 10 plants from individual WT and GM line were propagated at the same time. After 2 weeks, CSVd was inoculated on the leaves of WT and GM. At 50 days after CSVd infection, stem length, number of leaves, length of leave, and stem girth were measured for WT and GM chrysanthemums.

Results

Generation of constructs for chrysanthemum transformation

To make GM chrysanthemum resistant to CSVd, sense/anti-sense RNAs of CSVd were used. Four different CSVd partial sequences ranged from 75 nt to 82 nt were amplified by RT-PCR. The amplified PCR products were cloned into entry clones and introduced into the pBH7-att vector by gateway system.

Agrobacterium-mediated transformationto generate GM Chrysanthemums

Agrobacterium-mediated transformation was used to generate GM chrysanthemums resistance to CSVd. As shown in Fig. 6 and Fig. 7, the transformed callus was induced on callus inducing medium at 14 dpi (days post inoculation). At 25 dpi, shoots could be appeared and mature shoots were developed at 30 dpi (Fig. 6). At 40 dpi, shoots displaying stems and leaves could be observed. At 45 dpi,the mature shoots could be transferred to the root inducing medium and roots were formed at 75 dpi. At 90 dpi, mature transgenic plants could be obtained (Fig. 7).

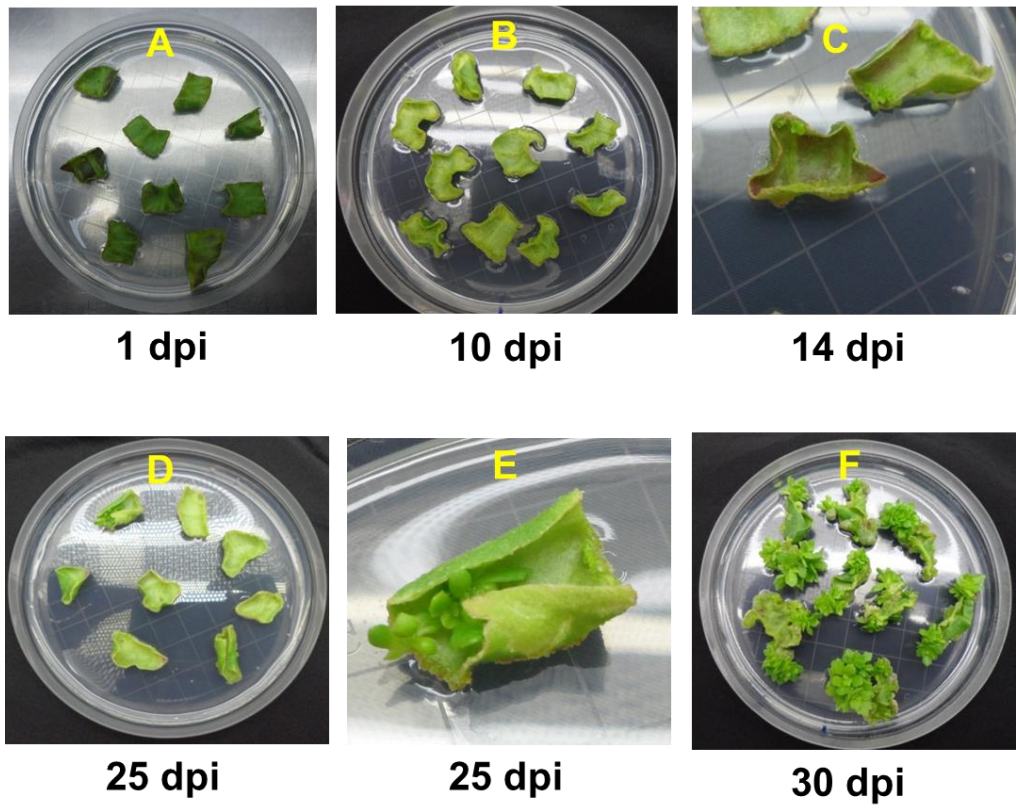

Fig. 6. Procedures for *Agrobacterium*-mediated transformation and induction of shoots.

(A) leaf discs which were co-inoculated with *Agrobacterium* at 1 dpi, (B) callus induced at 10 dpi, (C) shoots were induced at 14 dpi, (D) shoots were formed at 25 dpi, (E) magnified image for formed shoots at 25 dpi, (F) shoots were grown at 30 dpi.

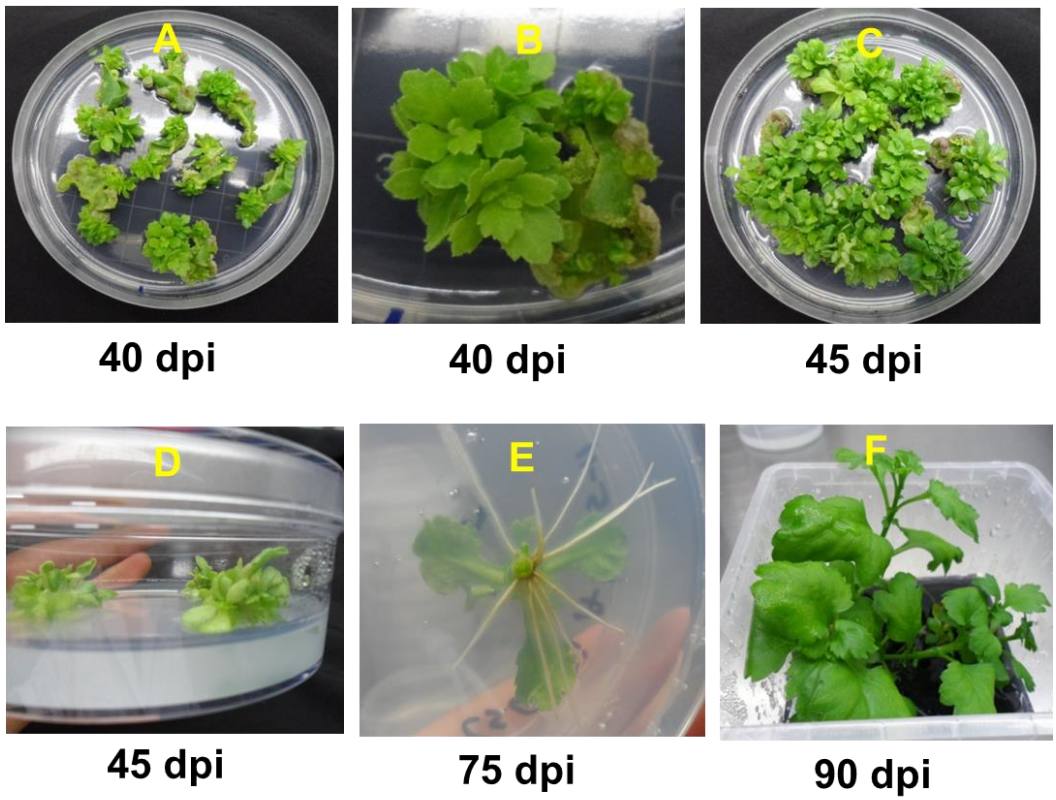

Fig. 7. Procedures to induce shoots and roots of transformed chrysanthemums. (A) mature shoots at 40 dpi, (B) magnified image for 40 days old shoots (C) 45 days old shoots were ready for propagation, (D) shoots were moved to roots induce medium at 45 dpi, (E) roots were induced at 75 dpi, (F) transgenic plants were generated at 90 dpi.

Selection of GM chrysanthemums by PCR

Although GM chrysanthemums were selected on medium containing barstar, it is necessary to confirm GM chrysanthemums by other method. For that, PCR was performed using T-DNA specific primers (507 bp of T-DNA detection forward and reverse primers). As a result, a total of 39 GM chrysanthemum lines (Table. 4). In particular, 18 and 14 GM lines were developed for anti-sense-3 and sense-3 constructs, respectively.

Chrysanthemum transformation efficiency

Chrysanthemum transformation efficiency was examined for each construct. From the sense-1 construct, four GM lines were generated from 173 leaf discs resulting in 2.31% transformation efficiency. From the anti-sense-2 construct, three GM lines were generated from 126 leaf discs resulting in 2.38% transformation efficiency. From the sense-3 construct, 14 GM lines were generated among 680 leaf discs resulting in 2.05% transformation efficiency. From the anti-sense-3 construct, 18 GM lines were generated from 740 leaf discs resulting in 2.43% transformation efficiency.

Propagation of chrysanthemums

To prepare plants for CSVd resistance test, wild type (WT) and GM chrysanthemums were propagated in culture room and green house in National

Institute of Horticultural Herbal Science (NIHHS), Suwon, Korea (Fig. 8). A total of 145 transgenic plants composed of 18 lines (sense-1), 15 lines (anti-sense-2), 69 lines (sense-3), and 43 lines (anti-sense 3) were propagated (Table 5).

Table 4. Summary of generated GM chrysanthemums from each construct.

Constructs	No. of transgenic plants
Sense-1	4
Anti-Sense-2	3
Anti-Sense-3	18
Sense-3	14
Total	39

Table 5. Summary of propagated chrysanthemums from each construct.

Constructs	No. of propagated transgenic plants
Sense-1	18
Anti-Sense-2	15
Anti-Sense-3	43
Sense-3	69
Total	145

Fig. 8. Propagation of WT and GM chrysanthemums in culture room and green house in NIHHS, RDA.

CSVd resistance test by sap inoculation and RT-PCR

CSVd infected chrysanthemum leaves were used to prepare sap. WT and GM chrysanthemums were infected with CSVd by sap inoculation. A total of 48 GM plants composed of 6 (sense-1), 6 (anti-sense-2), 21 (sense-3), and 15 (anti-sense-3) lines were inoculated (Table. 6). At 21 dpi, four different leaves from two inoculated and two non-inoculated leaves, respectively, were harvested in a chrysanthemum plant and total RNAs were extracted.

Examination of GM chrysanthemum lines for CSVd resistance by RT–PCR

To identify GM chrysanthemum lines resistant to CSVd, RT-PCR was performed using CSVd specific primers (CSVd forward primer information: AAAGAAATGAGGCGAAGAAGTCC, CSVd reverse primer information: TTCTTTCAAAGCAGCAGGGTCAG). Unfortunately, RT-PCR found that the chrysanthemum in this study was infected by CSVd. However, the level of CSVd RNA was very low. After CSVd infection in WT chrysanthemums, the level of CSVd RNA was strongly induced in both inoculated and non-inoculated leaves. In contrast, RT-PCR data indicated that GM lines showed strong reduction in the level of CSVd RNA as compared to WT. In particular, RT-PCR could not detect CSVd RNAs in the non-inoculated upper leaves of many GM lines. CSVd resistance was measured with at least three different plants for a single GM line

(Table 7). Of tested 16 GM lines from four different constructs, RT-PCR revealed that four GM lines from the anti-sense-3 displayed strong resistance against CSVd infection

Table 6. Number of propagated chrysanthemums and CSVd inoculated chrysanthemums for each GM line.

Index	GM lines	Total No. of plants	No. of inoculated plants
1	S3_B2_17	13	3
2	S3_B2_7	12	3
3	S3_B2_6	6	3
4	S3_B2_5	9	3
5	S3_B2_12	1	0
6	S3_B2_9	5	3
7	S3_B2_3	2	0
8	S3_D2_12	11	3
9	S3_D2_24	7	3
10	S3_D2_27	1	0
11	S3_D2_9	2	0
12	AS3_B1_2	6	3
13	AS3_B1_4	10	3
14	AS3_B1_35	8	3
15	AS3_C2_17	1	0
16	AS3_B1_1	6	3
17	AS3_B1_9	4	0
18	AS3_D1_10	8	3
19	S1_A1_1	9	3
20	S1_A1_2	9	3
21	AS2_A2_9	6	3
22	AS2_A2_19	9	3
Total	22	145	48

Table 7. CSVd resistance test of GM chrysanthemums by RT–PCR.

Index	Name	CSVd resistance in upper leaves	CSVd resistance in inoculated leaves
1	S3 B2 5	++	+++
2	S3 B2 6	+++	+++
3	S3 B2 7	+++	++
4	S3 B2 9	++	+++
5	S3 B2 17	+++	+++
6	S3 D2 12	+++	+
7	S3 D2 24	+++	++
8	AS3 B1 1	+++	+++
9	AS3 B1 2	+++	+++
10	AS3 B1 4	+++	+++
11	AS3 B1 35	+++	+++
12	AS3 D1 10	+++	+++
13	S1 A1 1	++	+++
14	S1 A1 2	+++	+++
15	AS2 A2 9	++	+++
16	AS2 A2 19	+++	+++

+++:Very strong, ++: Strong, +: Weak

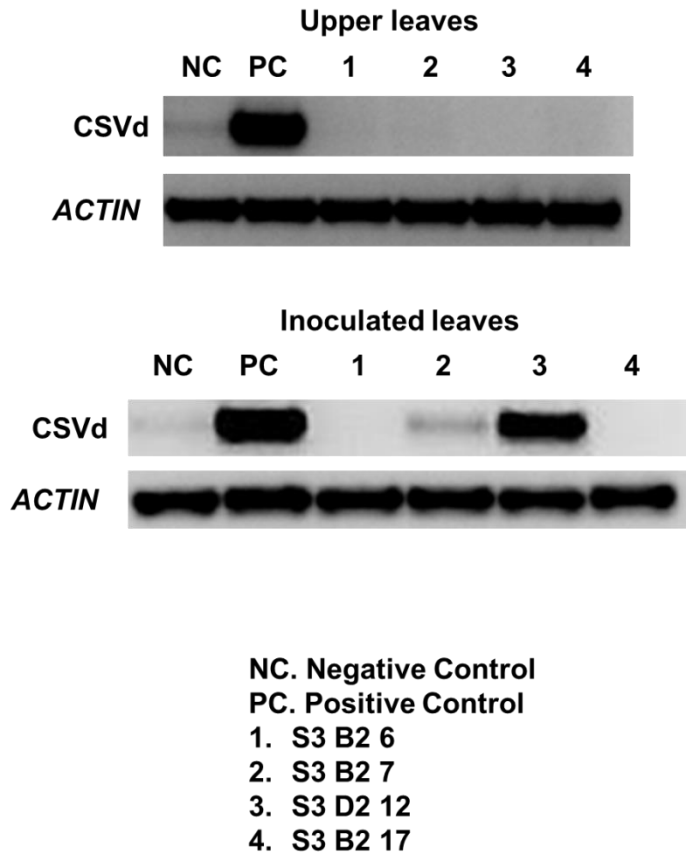

Fig. 9. RT-PCR results using CSVd specific primers to test CSVd resistance for sense-3 line.

Total RNA was isolated by Qiagen RNeasy plant mini kit (Qiagen, Hilden, Germany), concentration of RNA samples was 100 ~ 500 ng/µl. Size of amplified band was 354 bp. Actin was used as positive control. In first RT – PCR results for Sense-3 GM line, Line 1 and 2 are wild type and lines 3 to 6 are GM chrysanthemum. Line 2 to 6 samples are infected with CSVd. Line 2 is CSVd inoculated leaf of wild type chrysanthemums.

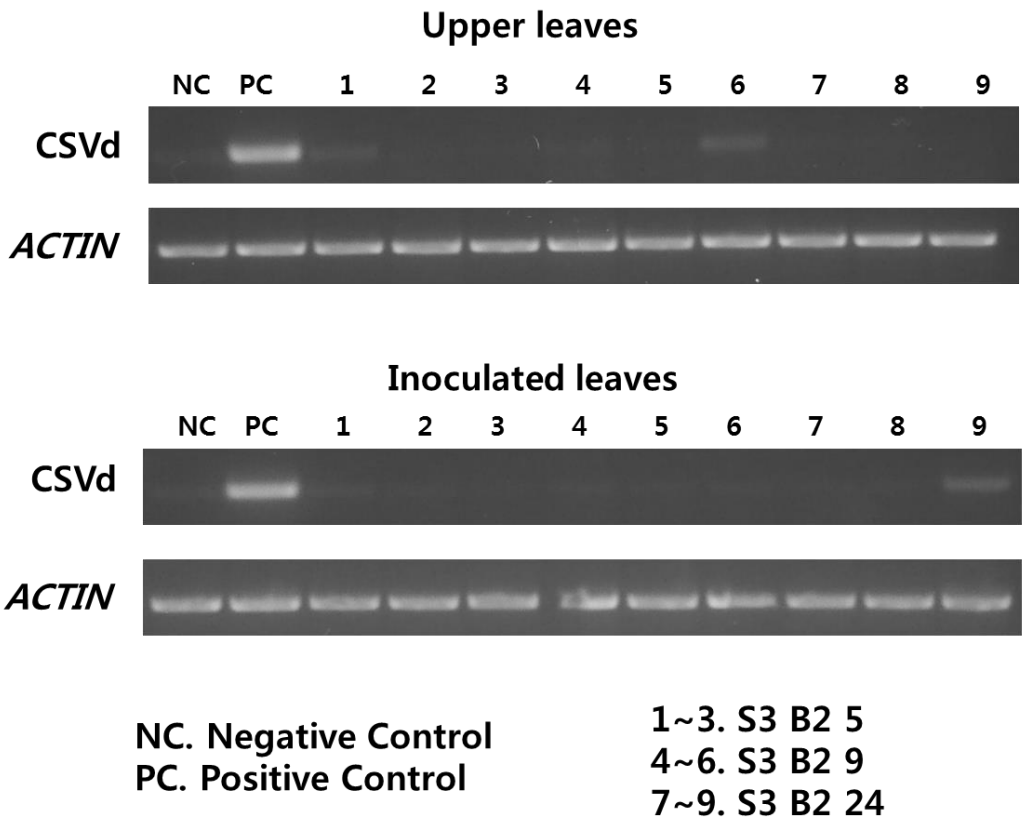

Fig. 10. RT-PCR results using CSVd specific primers to test CSVd resistance for sense-3 line.

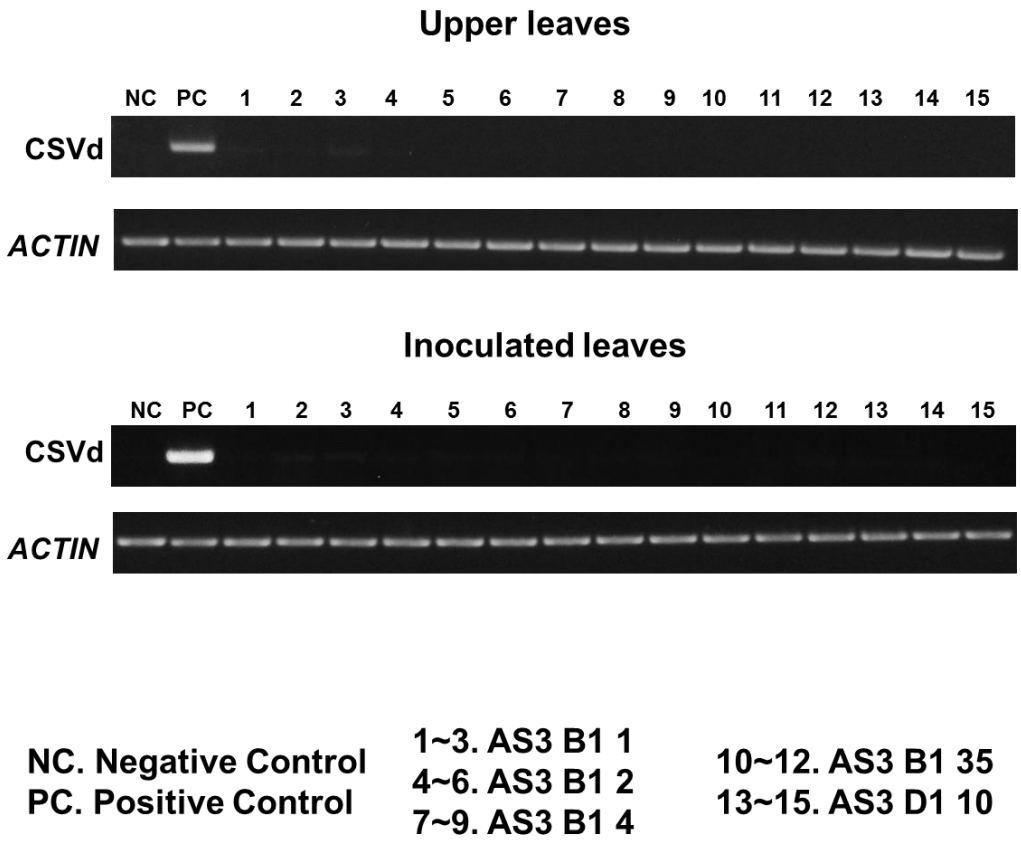

Fig. 11. RT-PCR results using CSVd specific primers to test CSVd resistance for anti-sense-3 line.

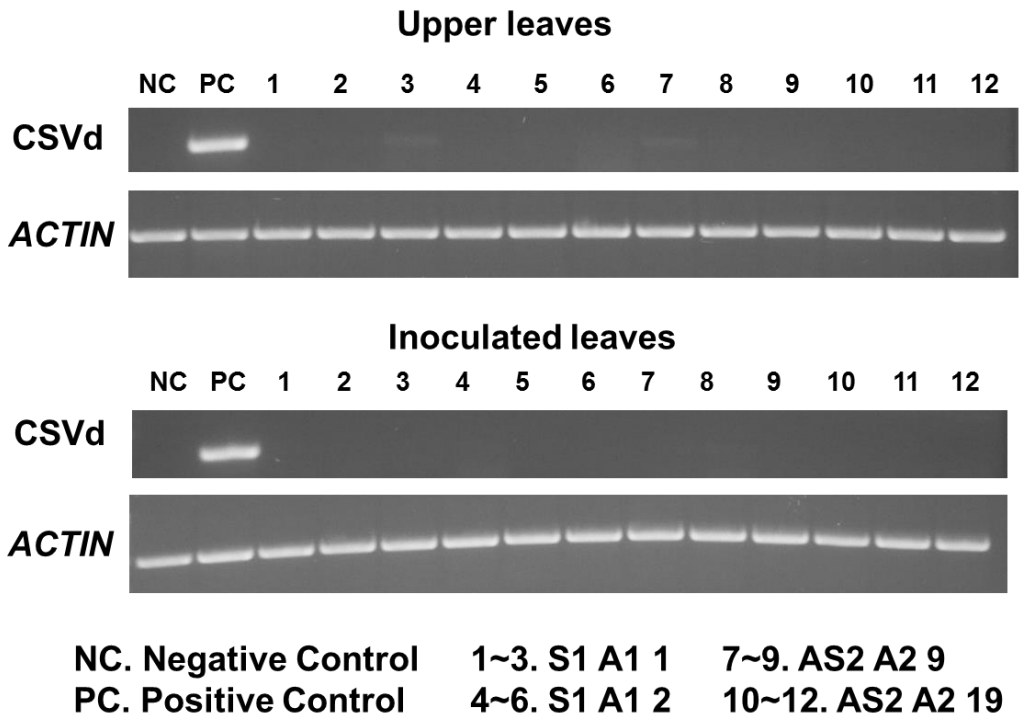

Fig. 12. RT-PCR results using CSVd specific primers to test CSVd resistance for sense-1 and anti-sense-2 lines.

Phenotype analysis of GM chrysanthemum lines as compared to wild type

After CSVd infection, I compared the phenotypes of one GM chrysanthemum line named as S3 B2 17 as compared to wild type. As a control, I used non transgenic chrysanthemums. At the same time, a total of 10 GM and WT plants were also inoculated with CSVd (Fig. 13). After 50 dpi, the S3-B2-17 GM lines were much healthier than wild type plants (Fig. 14). In addition, GM chrysanthemums were uniformly grown (Fig. 15). I examined four different morphological characteristics including stem length, stem girth, number of leaves, and length of leaves with 10 plants of GM and WT plants, respectively. In stem length, GM lines (82.9 cm) were taller than WT plants (55.8 cm). In stem girth, GM lines (3.96 cm) were thicker than WT (1.95 cm). In number of leaves, GM lines (31.4) had more leaves than WT (21.9). In length of leaves, GM lines (9.88 cm) were larger than WT (6.28 cm) (Fig. 16). Although both GM and WT plants were infected by CSVd, GM chrysanthemums were healthier, taller, and stronger than WT and were grown straightly and uniformly (Fig. 17). But CSVd inoculated WT chrysanthemums were relatively poorly and unevenly grown and unhealthier as compared to GM lines (Fig. 18).

Fig. 13. Comparison of two GM chrysanthemum lines to non-transgenic chrysanthemums at the early developmental stage after CSVd infection.

Fig. 14. Comparison of two GM chrysanthemum lines to non-transgenic chrysanthemums after CSVd infection.

Fig. 15. GM chrysanthemums evenly grown.

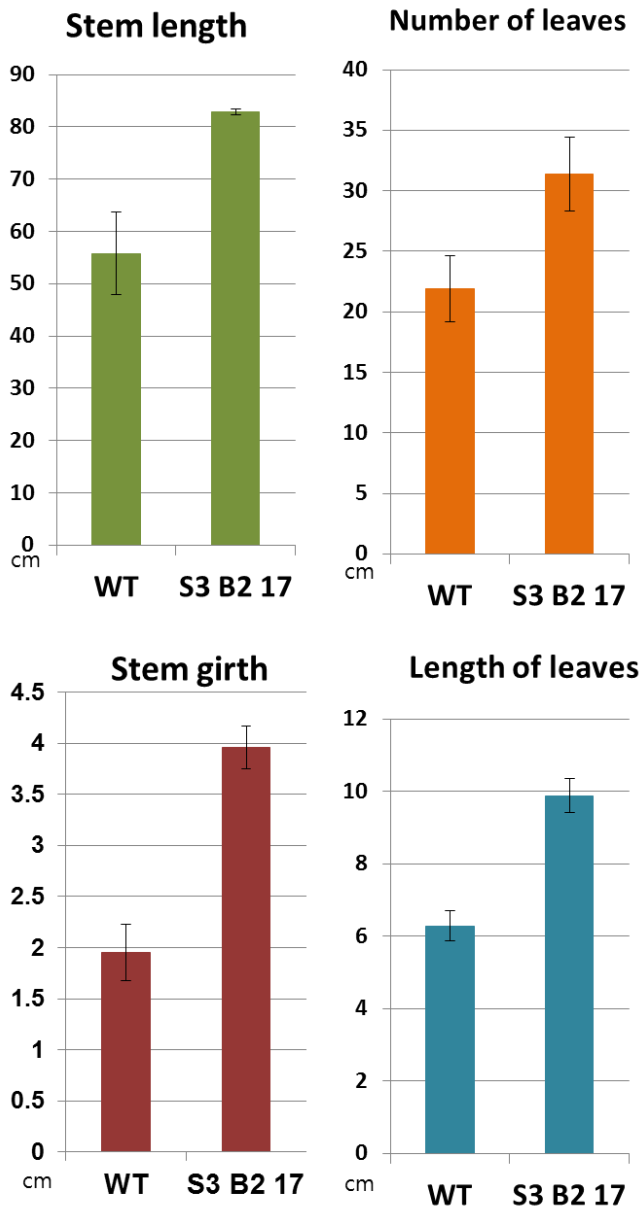

Fig. 16. Phenotype analysis of three GM chrysanthemum lines as compared to wild type.

Fig. 17. Healthy grown CSVd inoculated GM chrysanthemums in greenhouse.

Fig. 18. Poorly grown CSVd inoculated WT chrysanthemums in greenhouse.

Discussion

Development of GM chrysanthemums resistant to viruses

In case of chrysanthemums, many GM plants have been developed because chrysanthemums are one of comfortable plants for tissue culture and transformation. Previous studies demonstrated that leaves, stems, flowers, and petioles could be good materials for chrysanthemum transformation (da Silva, 2004; Kumar et al., 2012). According to recent studies, many viruses and viroids infecting chrysanthemums have been identified (Cho et al., 2013). Of them, *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), and *Chrysanthemum virus B* (CVB) are regarded as serious viruses in chrysanthemum production. Therefore, several research groups have developed GM chrysanthemums using viral genes. For example, a gene encoding capsid protein (CP) of CMV, full-length or partial sequences of nucleocapsid gene of TSWV, a CP of CVB were successfully used to make GM chrysanthemums resistant to each virus (Nakajima et al., 1993; Sherman et al., 1998; Singh et al., 2007).

GM plants resistant to viroids

So far, a limited number of GM plants resistant to viroids have been developed. For instance, a previous study has developed GM potato plants using antisense

RNA of PSTVd (Matoušek et al., 1994). The GM plants showed strong antisense RNA mediated an inhibitory effect suppressing PSTVd replication against both plus and minus-strands (Matoušek et al., 1994). Another study has generated tomato plants expressing antisense RNA of *Citrus exocortis viroid* (Atkins et al., 1995). Interestingly, the accumulation of negative-strand RNA was slightly reduced whereas the accumulation of positive-strand RNA was increased in GM tomato. This data demonstrated that there was a high degree of variability among different plant genotypes and even between different plants derived from the same vector (Atkins et al., 1995).

Development of GM chrysanthemums resistant to CSVd

The viroid has been shown to be responsible for the reduction of quality and quantity in chrysanthemum production. Of two viroids infecting chrysanthemums, the natural occurrence of CSVd on chrysanthemum cultivars has been frequently reported in the world including Korea. Recently, I examined CSVd infection rate in commercial chrysanthemum cultivars resulting in high rate of CSVd infection. This data indicates that CSVd is currently the most serious pathogen in chrysanthemums grown in Korea. Therefore, it is necessary to develop viroid resistant chrysanthemum plants. Using viroid derived antisense RNAs, previous studies have successfully demonstrated that the genetically engineered plants carrying antisense RNAs of the viroid were resistant to viroids such as PSTVd

(da Silva, 2004; Schwind et al., 2009). So far, CSVd resistant GM chrysanthemum has not been developed. For the first time, I generated GM chrysanthemums resistant to CSVd. The generated GM chrysanthemums could be commercial important for the production of chrysanthemums due to high rate of CSVd infection in commercial chrysanthemum cultivars.

CSVd resistance test

In this study, I generated GM chrysanthemums using a commercial cultivar named as vivid scarlet, however, this cultivar was slightly infected by CSVd. The level of CSVd was very low. To find a commercial cultivar without viroid infection, several plant stocks were obtained from RDA, however all of them were infected by CSVd indicating that chrysanthemum cultivar without viroid infection might be not available in Korea. Therefore, I selected a WT plant which was slightly infected by CSVd using RT-PCR and this plant was used for generation of GM chrysanthemums. Interestingly, the GM lines carrying sense or antisense RNA of CSVd showed strong CSVd resistance. For example, the level of CSVd RNA was strongly reduced in the inoculated leaves. Moreover, I could not detect any CSVd RNA in upper leaves of many GM lines.

I performed sap inoculation for CSVd resistance in WT and GM plants. Quantitative RT-PCR results confirmed that a small amount of CSVd RNAs could be detected in inoculated leaves, but the level of CSVd RNA in upper leaves was

not detectable in many GM lines. This data suggest that sense or antisense RNA of CSVd plays a negative role in viroid replication or movement in the developed GM plants. Moreover, there was difference of CSVd resistance among generated GM chrysanthemum lines from the same construct. Out of four constructs, the GM lines obtained from antisense-3 displayed the strongest CSVd resistance. It seems that antisense RNA is better than sense RNA for the generation of GM plants resistant to viroid.

Several important points for chrysanthemum propagation, tissue culture, and transformation

There are several points to be careful during chrysanthemum tissue culture. The first is callus induced from leaf disc should be transfer to new medium every 10 days. The callus in the same medium for more than 14 days will be malnutrition and dead. Chrysanthemums in culture media should be propagated every 3 weeks. For example, the upper part of chrysanthemum will be cut using sterilized knife and place them on new culture medium. For propagation in green house, about 10 cm length of upper part of chrysanthemum could be cut and be transferred to moist bed soil. To keep optimal humid at early stage of propagation, plastic film is used to cover them for curing. Water should be provided to chrysanthemums 3 times for a day and the amount of water will be just enough to wet the leaf surface. When the plants are grown up to 60 cm tall, the

chrysanthemum should be propagated.

During *Agrobacterium*-mediated transformation, there were several problems. For example, *Agrobacterium* grew in transformed chrysanthemum tissues. So the upper stems of transgenic plants should be cut and washed with 1% sodium hypochlorite. For propagation or transformation, it is desirable to use a sharp surgical knife in order to facilitate for inoculation of *Agrobacterium* and induction of callus. In addition, the concentration of Basta is important for selection of transgenic plants. The optimized Basta concentration is 0.75 mg/L in this study.

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국화왜화바이로이드 저항성 유전자 조작 국화 개발

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초록

국화(*Dendranthema X grandiflorum*)는 국화 과에 속하는 식물로서, 아시아와 유럽에서 약3000년 동안 재배되어 왔다. 최근에는 가장 대중적이고 세계 화훼 산업에 중요한 부분을 차지하고 있다. 바이로이드는 가장 작은 병원체로서 원형의 RNA로 구성되어 있으며 식물을 기주로 삼아 병을 일으킨다. 현재까지 2개의 바이로이드*Chrysanthemum stunt viroid* (CSVd) 와 *Chrysanthemum chlorotic mottle viroid* (CChMVd) 가 국화에 감염된다고 보고되었다. 특히 CSVd 감염은 국화에 잎의 탈색, 왜화, 안토시아닌량 감소 와 같은 심각한 피해를 일으킨다. 그리하여 CSVd 유전자 조작 국화제작의 필요성이 있다. 바이

로이드 저항성 국화를 제작하기 위해 바이로이드 RNA 염기서열 절편을 이용한 RNAi mediated silencing system이 이용되었다. CSVd sense/antisense 방향의 RNA 염기서열이 포함된 각기 다른 4개의 벡터가 제작되었으며, 이것들은 Agrobacterium mediated transformation에 사용되었다. 처음에 개별적인 CSVd RNA 염기서열이 들어간 총 39개의 형질전환체 라인을 얻었다. CSVd 감염에 대한 저항성을 검정하기 위해 145개체중 60 개체의 형질전환 국화가 즙액접종 방법으로 CSVd를 접종하였다. 상대적 저항성이 접종엽과 상엽에서 추출한 total RNA 와 CSVd 특이적 primers 를 이용한 semi-quantitative RT-PCR 방법을 통해 확인되었다. 테스트된 16개의 형질전환 국화라인 중아홉 개의 형질전환 국화 라인에서 CSVd감염과 복제에 대한 매우강한 저항성을 보였다. 전반적으로 형질전환 국화가 비 형질전환 국화보다 고른 성장과 강한 수세를 보였다. 본 연구는 CSVd의 sense/antisense RNAs 를 이용한 CSVd 저항성 형질전환 국화를 개발한 첫 번째 사례이다.

주요어: 국화왜화바이로이드, 바이로이드 저항성, 아그로박테리움을 이용한 형질전환, 유전자 조작 국화.

학번: 2011-23537

감사의 글

길지만 짧았던 2년간의 석사생활을 마치고 어느덧 졸업이 다가왔습니다. 처음 서울대 정문을 들어올 때 벅차고 기쁜 느낌은 아직도 잊지 못합니다. 저를 서울대에서 공부 할 수 있게 해주시고 인생의 선배님으로서 학문적인 스승님으로서 많은 것을 가르쳐 주신지도해주신 김국형 교수님께 깊은 감사를 드립니다. 또한 수업과 세미나를 통해 학문적 성장을 도와주신 이인원 교수님, 박은우 교수님, 김영호 교수님, 황인규 교수님, 가종억 교수님께 감사 드립니다. 교수님께서 아버지라면, 어머니처럼 곁에서 사랑과 정성으로 지도해주신 조원경 박사님께 진심으로 감사 드립니다, 많이 모자란 저지만 항상 믿어주시고 격려해 주셔서 석사학위를 마무리 지을 수 있었습니다. 또한 농촌진흥청 원예특작원에서 실험적인 면, 인생적인 면을 가르쳐 주시고 함께 울고 웃으며 같이해온 최승국 박사님께 감사 드립니다. 국화 형질전환의 노하우를 아낌없이 가르쳐주신 정봉남 박사님께도 감사 드립니다. 상냥한 웃음으로 힘을 주시는 박미리 박사님, 어려울 때 힘이 되어주신 박상호 박사님, 진심으로 저를 걱정

해주시고 챙겨주시던 상민형님, 카리스마 있는 왕고 경미 누나, 이쁘고 똑똑한 지숙 누나, 밝은 웃음과 격려로 힘을 주시는 문일형님, 실험실 고된 일 도맡아 하시는 정 많으시고 호성형님, 타지 와서 고생하는 착한 트리(Tri)와 미사(Misa), 부지런하고 성실한 리안센(Lian Sen), 동생이지만 친구 같은 진만이, 착하고 이쁜 민지, 앞으로 함께할 시간이 많은 털털한 동우형, 멋진 워킹맘 정민 선생님, 큰 힘이 되어주고 항상 고마운 연화누나, 조언과 든든한 힘이 되주tl는 효섭 형님 2년동안 동고동락 하고 많은 도움을 주신 저희 실험실 구성원 여러분께 진심으로 감사 드립니다. 함께 학문의 길을 걷고 있는 충구, 미사일 열심히 만들고 있는 전탱, 해킹 열심히 막고 있는 희훈, 쌀집아저씨 민호, 파워드라이버 구범, 녹색수호자 무누, 사회의 쓴맛을 보고 있는 동긔, 인터넷왕자 동구, 열공 하고 있는 새쿠, 학문의 길로 접어든 토머, 든든한 너희들이 있기에 인생 살아가는 힘이 난다. 난 가끔 너희들을 생각하면 난 참 복이 많은 놈이라 생각이 든다. 이 자릴 빌어 낯 부끄럽지만 고맙다는 말 전하고 싶다. 사랑하는 우리가족 아버지의 사랑과 남자로서의 존경하는 아빠,

아프시지만 우릴 위해 고생하시는 엄마, 맨날 툴툴대지만 사랑하는 동생 미수, 고양이만큼 도도한 프래도 모두 사랑하고, 마지막으로 내인생 최고의 선물 소담님 너무 감사하고 사랑합니다. 여러분들이 있었기에 지금의 제가 있다 생각됩니다. 진심으로 감사하고 사랑합니다.