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Agrobacterium-mediated Transformation of Chrysanthemum (*Dendranthema grandiflora*) Plants with a Disease Resistance Gene (*pac*1)

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Abstract

We constructed a binary vector to improve the transformation efficiency and transgene expression in chrysanthemum plants in an *Agrobacterium*-mediated system. Most of the sequences unnecessary for maintaining plasmids in bacteria were removed from the pBI121 vector, and an expression cassette of the neomycin phosphotransferase II gene, in which a point mutation was repaired, and an expression cassette of β-glucuronidase (GUS) with a short 5' un-translated sequence of β-glucanase from soybean, were set within a transferred DNA. This vector was used to transform leaf discs of chrysanthemum plants, resulting in the transformation efficiency increasing several times compared with pBI121 and the activity of GUS increasing prominently. We also introduced a double-stranded RNA-specific ribonuclease (*pac*1) from *Schizosaccharomyces pombe* by using this novel vector to confer simultaneous tolerance against virus and viroid diseases. Using this technique we obtained more than 80 transgenic chrysanthemum plants, and found that half of them expressed a moderate amount of *pac*1 protein as detected by Western blot analysis.

Key words: *Dendranthema grandiflora*, disease resistance, *Schizosaccharomyces pombe*, transformation.

Abbreviations

GUS, β-glucuronidase; MS medium, Murashige and Skoog medium; neomycin phosphotransferase II, NPTII.

Introduction

Traditional breeding methods in flowers have produced a large number of cultivars with improved floral characteristics and tolerances to environmental and biotic stresses. Recently, genetic transformation techniques for engineering crops have made it possible to introduce target genes without requiring the long-term period of selection that is characteristic of conventional breeding programs. There have been some reports on genetic transformation of chrysanthemum (*Dendranthema grandiflora*) plants via *Agrobacterium*-mediated processes (Ledger, *et al.*, 1991; Courtney-Gutterson, *et al.*, 1994; Urban, *et al.*, 1994; Fukai, *et al.*, 1995) and biolistic techniques (Yepes, *et al.*, 1995). However, the expression of introduced gene was reportedly low for both a reporter gene, β-glucuronidase (GUS), and a pathogen-tolerant gene in chrysanthemum plants (Yepes, *et al.*, 1995; Sherman, *et al.*, 1998a). Takatsu *et al.* (2000) recently reported transgene inactivation after a 1-year period of transgenic plant cultivation. Moreover, full-length sequence analysis of pBin19 revealed that there are unexpected sequences inside the T-DNA that are not necessary for maintaining plasmids in bacteria (Frisch, *et al.*, 1995). The above data suggest that the transformation efficiency would be improved if these defects are remedied.

In greenhouse crops, viruses assigned to the genus *Tospovirus*, such as tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INSV),

which are easily vectored by western flower thrips, have become major pathogens since they can infect many kinds of important flowers such as chrysanthemum, petunia, impatiens, and snapdragon in North America and Europe (Daughtrey, *et al.*, 1997). There are other diseases caused by viroids (small single-stranded RNA molecules), which are found in several vegetatively propagated plants including chrysanthemum, and they severely damage the growth rate so as to destroy the commercial value of infected plants (Diener and Lawson, 1973). These diseases are caused by pathogens with the RNA genome, which we assumed might be destroyed by introducing a double-stranded RNA-specific ribonuclease such as *pac*1 enzyme from *Schizosaccharomyces pombe*. Using transgenic approaches in tobacco plants (Watanabe, *et al.*, 1995) and potato plants (Sano, *et al.*, 1997), we found that the *pac*1 enzyme suppressed multiple viral and viroid diseases. These results encouraged us to produce transgenic chrysanthemum plants with resistances to dangerous pathogens such as the chrysanthemum stunt viroid and TSWV.

In the present study, we modified a standard binary vector to improve the efficiency of transformation and to increase the expression level of transgene, and demonstrated a high level of *pac*1 enzyme as detected by Western blot analysis.

Materials and Methods

Plant material

In vitro plants of a spray-type cut flower cultivar of chrysanthemum (*Dendranthema grandiflora* cv. Reagan), and those of tobacco (*Nicotiana tobacum* cv. Xanthi) were aseptically maintained and propagated by cuttings on MS agar medium (Murashige and Skoog, 1962) without growth regulators in a growth chamber maintained at 25 ℃ and illuminated for 16 h per day with fluorescent lights at an intensity of 10-20 $\mu Em^{-2} s^{-1}$.

Bacterial strains and culture conditions

A strain of Agrobacterium tumefaciens, LBA4404, harboring a disarmed vector (pBI121) was used as a control strain (Clontech Laboratories, Palo Alto, CA), and another strain, AGL0 (generous gift from Dr. R. Ludwig, University of California, USA), was used in most transformation experiments. Bacteria were cultured in yeast extract peptone (YEP) medium containing 25 $\mu g\ ml^{-1}$ of both streptomycin and kanamycin (Km).

Gene manipulation

The molecular manipulations were carried out according to standard methods unless otherwise stated. In early transformation experiments, the combination of plasmid pBI121 (Gelvin and Schilperoort, 1994) and bacterial strain LBA4404 was used. This plasmid contains two expression cassettes: (1) GUS gene driven by 35S promoter, and (2) neomycin phosphotransferase II (NPTII) driven by nopaline synthase (Nos) promoter in the transfer region between two border sequences (T-DNA). Besides, only half of the sequences outside the T-DNA sequence are prerequisite for replication of the plasmid and its transference between bacteria (Frisch *et al.*, 1995). To improve the plasmid by removing the unnecessary sequence located outside T-DNA, a small plasmid retaining the region with minimal function for maintenance in *E. coli* and *Agrobacterium*, such as RK2 Ori V, TrfA, and the NPTIII gene, was recovered by double digestion of pBI121 with a combination of restriction enzymes *Not*I and *Bgl*II (at positions 92 and 5606, respectively, in **Fig. 1** of Frisch *et al.*, 1995) followed by fill-in reaction by Klenow fragment, and then a polylinker sequence of pBluescriptII was inserted between these restriction sites by ligation (pNB2 in **Fig. 1**). The NPTII expression cassette in the plants was prepared after repairing a point mutation that caused a decrease in this enzyme activity (from GAG to GAT at Glu 182) by site-directed mutagenesis, and a 5' untranslated (leader) sequence from soybean β-1,3-glucanase (5'-TCT AGA CTT CTT TCC TCA ACC TTC TTT CTT CTT ATA TAT TCG AAC GAT CCC CCT ACT AGT) was introduced between the Nos promoter and the NTPII coding sequence by PCR reaction. This marker cassette was inserted between right and left border sequences that were cloned in pBluescriptII SK+ plasmid with the same direction. The GUS expression cassette was made based on a pIG121Hm plasmid (generous gift from Dr. K. Nakamura, Nagoya University, Japan), in which the GUS coding sequence contains an intron from castor bean catalase to block its expression in bacteria (Ohta, *et al.*, 1990), and a 5' untranslated sequence from *psaDb* gene (Yamamoto, et al, 1995) and that of β-glucanase were sequentially introduced by PCR reaction between the 35S promoter and the intron containing GUS with Nos terminator. This GUS expression cassette was integrated into the above pBluescriptII plasmid containing the NPTII gene, and the GUS cassette was located beside the right border. These two genes with both borders were then cut by digestion with *Kpn*I and *Spe*I, and the resulting fragment was transferred into a small pNB2 to produce pKT2 (**Fig. 1**). To make the *pac*1 gene-expression cassette, a soybean leader

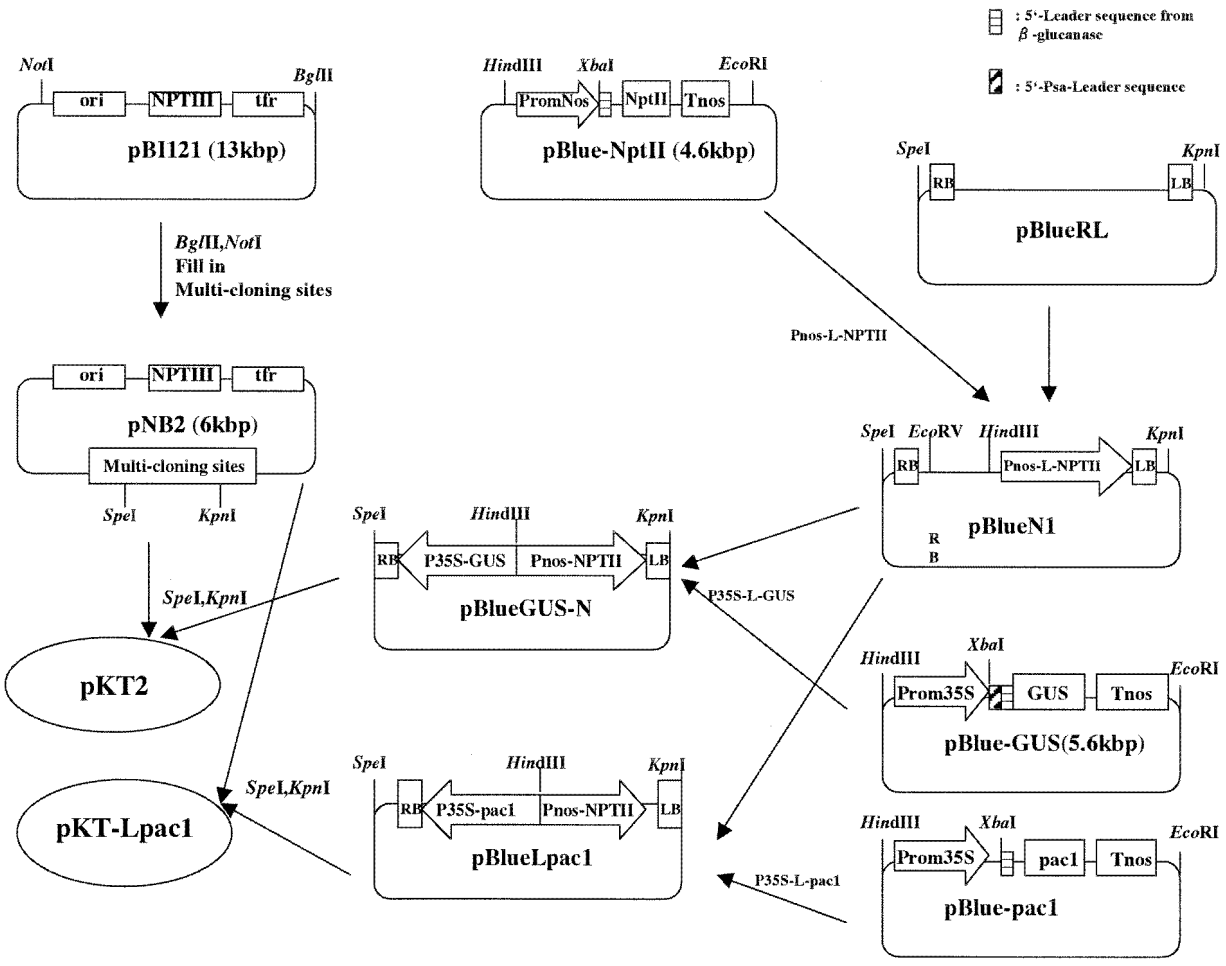

Fig. 1 Construction of binary vectors, pKT2 and pKT-Lpac1. Details are described in Materials and Methods.

sequence was inserted by PCR reaction into the expression cassette of *pac*1 driven by 35S promoter (Watanabe *et al.*, 1995), then transferred to a plasmid containing NPTII gene, and finally pKT-Lpac1 was constructed to introduce the *pac*1 gene into the plants.

Transformation

Leaves from aseptically cultivated plants were cut into small square pieces with a length of less than 5 mm, and inoculated immediately by *Agrobacterium* strains harboring binary plasmid previously grown overnight in YEP medium. They were then placed on MS medium containing 0.2 mM acetosyringone, 3% sucrose, and 0.8% agar in the dark. Co-cultivation was terminated after 3 days by transferring the leaf segments to the above MS agar medium supplemented with 5 mM MES (pH 5.7), 1 mg l^{-1} naphthaleneacetic acid, 2 mg l^{-1} benzyl adenine, 250 mg l^{-1} cefotaxime, and 12.5 mg l^{-1} Km, and these tissues were cultivated for 6 weeks. Regenerated shoots were cut and transferred to MS agar medium with antibiotics to induce roots by depleting growth regulators. In the case of tobacco plant transformation with pBI121, the concentration of benzyl adenine was reduced to 1 mg l^{-1}.

PCR and Southern blot analyses

For PCR analysis, leaves with diameters of approximately 5 mm were homogenized in 100 μl of a buffer comprising 50 mM Tris-HCl (pH 7.5), 20 mM EDTA, 0.3 M NaCl, 1% SDS, 5 M urea, and 5% phenol for 30 s in micro tubes. Equal volumes of phenol and chloroform were added to this solution and mixed by vortex. Debris and proteins were removed by centrifugation for 10 min, and the DNA pellet was obtained by ethanol precipitation. PCR reaction was carried out to detect the *pac*1 gene with a primer set (5'-TCC TCA TCC GCT TCC GAC TC-3', and 5'-CAA GGA CTT CGA GTG CTT GC-3'). For Southern blot analysis, 20 μg of DNA was digested with *Hin*dIII, separated by electrophoresis in a 0.8% agarose gel, and blotted on to HybondN^{+TM} membrane (Amersham Pharmacia Biotech). Hybridization was performed for 16 h at 65 ℃ with a ^{32}P-dCTP labeled *pac*1 coding sequence.

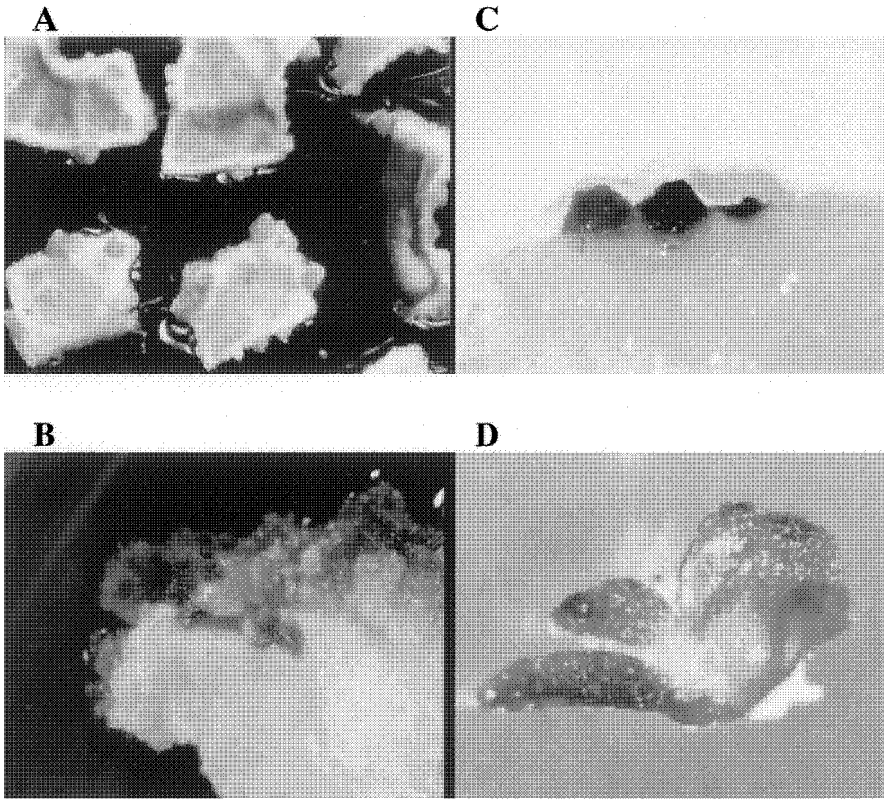

Fig. 2 GUS staining of transformed cali and regenerated shoots.
(A) callus proliferation around leaf discs, (B) shoot regeneration from a leaf disc, (C) GUS staining of calli, (D) GUS staining of a shoot.

GUS assays and Western blot analysis of transformed plants

GUS histochemical assays were carried out by staining plant tissues with 5-bromo-4-chloro-3-indolyl-D-glucuronidase (X-gluc) overnight, followed by washing with absolute methanol. To measure GUS activity, tissues were ground in a solution of 50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 1 mM DTT, 10 mM 4-methylumbelliferyl-beta-D-glucuronide, and 0.1% Triton® X-100. Homogenates were spun for 10 min to collect cleared supernatant. The activity was normalized according to protein concentration, and assayed fluorometrically by measuring the production of 4-methylumbelliferone (4-MU).

Western blot analysis was performed on the homogenized leaves, and proteins were separated in SDS-PAGE with an acrylamide concentration gradient from 4 to 20%, and blotted onto Immobilon™ (Millipore). Membranes were reacted with *pac*1 antiserum raised in rabbits, and bands were visualized with alkaline phosphatase reaction pigments.

Results

Previous studies of chrysanthemum transformation used pBin19 and its derivatives, such as pBI121 and pIG121Hm. However, detailed sequence information of the region transferred into plant genome (T-DNA) revealed several superfluous insertions, and a functional selective marker was not located near the right border of the T-DNA region. Moreover, half of the non-T-DNA sequence was predicted to be unnecessary for maintaining plasmids in bacteria. Therefore, pKT2 plasmid was constructed by solving these problems, and used to examine its capability for chrysanthemum transformation (**Fig. 1**).

Among the regenerated plantlets obtained by *Agrobacterium* infection, transgenic plants were identified by the presence of GUS gene. The efficiency of transformation, based on the number of transgenic shoots in 100 leaf discs, was about 0.4% when pBI121 plasmid in AGL0 was used. This efficiency increased to 2% when using pKT2 in AGL0.

When GUS activity was monitored visually in 20 pBI121 transgenic plantlets, we could not detect a significant amount of the pigment of GUS gene products. However, in the case of pKT2 plasmid, visible staining sectors were obvious in many calli and roots, and stainable and nonstainable calli were discriminated even at the stage of cell proliferation (**Fig. 2C**). Some of the pKT2-derived plantlets

Table 1 GUS activity in transgenic plants [nmoles 4-MU min^{-1} (mg $protein^{-1}$)].

Plasmid[1)]	Leaf		Root	
	Mean	Max.	Mean	Max.
pBI121	0.0	0.0	0.0	0.0
pKT2	11.5	47.4	7.8	28.4

[1)] Twenty plants were analyzed in both plasmids.

regenerated on an agar medium containing 12.5 mg l^{-1} Km showed uniform staining with GUS (**Fig. 2D**). When GUS activity was measured fluorometrically, leaves of the plants obtained by pBI121 transformation scarcely showed the activity, i.e., less than 0.1 nmoles 4-MU in all of the 20 transgenic plantlets (**Table 1**). In contrast, when pKT2 was used to introduce GUS gene, the activity was about 12 nmoles 4-MU on average.

Based on the results mentioned above using the GUS gene, we tried to introduce the *pac*1 gene using the pKT-Lpac1 plasmid, and selected putative transgenic shoots for 3 months in Km-containing media. As the result, we obtained 82 transgenic plants as judged from the presence of the *pac*1 gene by PCR analysis, and most of them showed rooting ability on medium containing 25 mg l^{-1} Km.

The genomic DNA was analyzed in several putative transgenic plants by Southern hybridization to confirm the presence of the *pac*1 gene and to check the number of copies. Most of the transgenic plants showed the integration of a small number of copies of the gene in their genome (**Fig. 3**).

Analysis of the proteins extracted from the leaves of transgenic plants by Western blotting using anti-*pac*1 serum showed that more than half of them (45 plantlets) expressed detectable *pac*1 protein (**Fig. 4**), with three of them showing relatively high expression. However, the plantlets contained much less *pac*1 protein than did transgenic tobacco leaves (Watanabe, *et al.* 1995), based on the same amount of leaf proteins analyzed. These transgenic plants were acclimated to grow in a greenhouse, propagated by cuttings, and used to check the stability of protein expression during propagation and under different growth conditions. We could not detect any remarkable changes in the expression level of *pac*1 protein during the course of propagation and growth for several seasons (data not shown).

Discussion

There have been many reports on the transformation of chrysanthemum plants, though the ex-

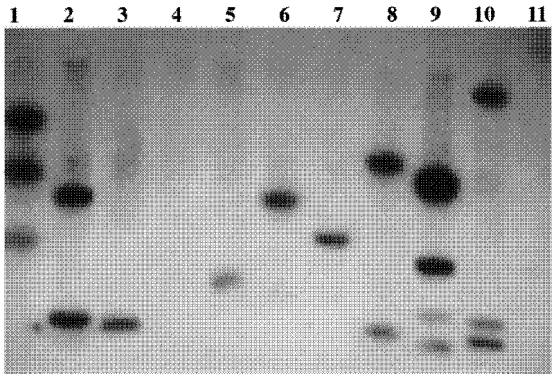

Fig. 3 Southern hybridization analysis of genomic DNA.
Genomic DNA was digested by *Hin*dIII and DNA transferred filter was hybridized with *pac*1 gene as a probe. Lane 1-10, transgenic plants, and lane 11, non-transgenic plant.

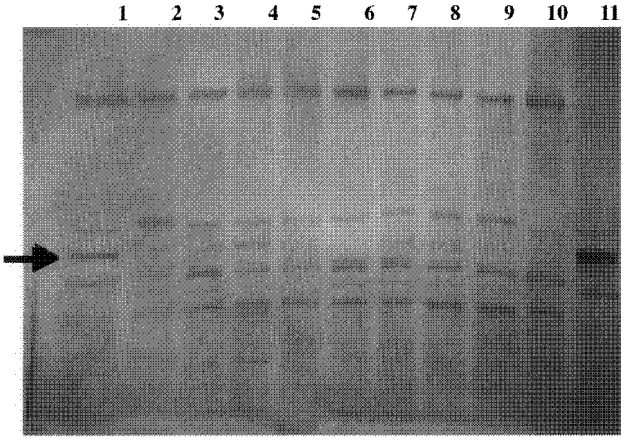

Fig. 4 Western blot analysis of *pac*1 protein
Leaf proteins were separated by SDS-PAGE and a filter was probed with anti-*pac*1 serum. Lane 1-9, transgenic plants, and lane 10, non-transgenic plant, lane 11, transgenic tobacco plant. Arrow indicates the position of *pac*1 protein.

pression level of introduced GUS gene has been very low [much less than 1 nmole 4-MU min^{-1} (mg $protein)^{-1}$] using pBin19-based plasmids including pBI121 and pIG121Hm (Pavingerová *et al.*, 1994; Sherman *et al.*, 1998b). To solve this problem, in the present study we made several modifications to the vector sequences. First, the selective marker gene (NPTII) was moved to near the left border with the expectation of stimulating the simultaneous introduction of GUS and NPTII genes, because T-DNA is mainly integrated into plant cells from the right border to the left side (**Fig. 1**). Second, a mutation of NPTII gene in pBin19-derived plasmids (Yenofsky *et al.*, 1990) was repaired by point mutagenesis to increase the tolerance level against

Km in transgenic chrysanthemum plants. De Jong *et al.* (1994) showed that repairing some of these defects increased the transformation efficiency in chrysanthemum plants. Moreover, detailed sequence information in this region shows several superfluous sequences within the T-DNA region, with less than half outside the T-DNA region being required as a binary vector (Frisch *et al.*, 1995). We deleted most of the superfluous sequences in order to reduce the size of the vector.

To increase the expression level, we utilized a 5' untranslated leader sequence, which was originally found in soybean β-glucanase and integrated just before the coding sequence of expressed genes such as GUS and *pac*1. The increase in the expression level was prominent in both genes. The average GUS activity of transgenic plant leaves from pKT2 was about 12 nmoles 4-MU, which is higher than in all other reported experiments on chrysanthemum plants (**Table 1**). However, its absolute value was lower than that of typical transgenic tobacco leaves obtained by pBI121 transformation in our laboratory (about 200 nmoles 4-MU). Although we succeeded in stabilizing gene expression by utilizing an untranslated sequence in the case of GUS and the *pac*1 gene, it is still necessary to increase the expression level of transgene in chrysanthemum plants (which is lower than those in other plant species).

In greenhouse crops, *Tospovirus* species such as TSWV and INSV, which are vectored by western flower thrips, have become major pathogens since they can infect many kinds of important flowers such as chrysanthemum, petunia, impatiens, and snapdragon in North America and Europe (Daughtrey, *et al.*, 1997). To overcome these diseases, some research groups have attempted to introduce the nucleocapsid gene into chrysanthemum plants, which has produced the promising result of resistance against some TSWV strains, though they could not detect the nucleocapsid protein by ELISA (Yepes, *et al.*, 1995; Sherman, *et al.*, 1998a). However, such strategies for inducing virus resistances based on the structural properties of each virus are sometimes ruined by the structural changes that occur from time to time.

Viroids also cause deleterious disease in chrysanthemum, causing severe damage such as reduced growth rate so as to destroy the commercial value of infected plants (Diener and Lauson, 1973). Since these virus and viroid diseases are caused by pathogens with an RNA genome, they are thought to be good targets for suppression by the double-stranded RNA-specific ribonuclease, *pac*1. We have analyzed the effects of *pac*1 ribonuclease on several plant diseases for the first time by using transgenic tobacco plants, which showed reduced symptoms after infection with several plant viruses such as the tobacco mosaic virus and the cucumber mosaic virus (Watanabe *et al.*, 1995). Moreover, the second experiments using transgenic potato plants showed reductions in infection rate and in the accumulation of potato spindle tuber viroid (Sano *et al.*, 1997). These positive results encouraged us to produce transgenic chrysanthemum plants that were protected from infection by dangerous pathogens possessing an RNA genome. In the present study, we have succeeded in producing transgenic chrysanthemum plants expressing the *pac*1 protein as detected by Western blot analysis. We believe that these transgenic plants will offer a straightforward solution against a major threat caused by these RNA-encoded pathogens in the commercial production of chrysanthemum plants, and we will challenge these transgenic plants with pathogens in the future.

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