Plant Cell Reports (1991) 10:195 -199

Plant Cell Reports

Regeneration and Agrobacterium-mediated transformation of chrysanthemum

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Received November 30, 1990/Revised version received March 14, 1991 – Communicated by R. B. Horsch

Abstract. A method has been developed to regenerate shoots directly from leaf pieces of the autumn flowering chrysanthemum *Dendranthema indicum* (L.) Des Moul (genotype Korean). Transgenic plants of this genotype were generated using transformation mediated by the disarmed strain of *Agrobacterium tumefaciens* LBA4404, containing either pKIWI110 or pGA643. Both pKIWI110 and pGA643 contain the selectable marker gene neomycin phosphotransferase II (NPTII) and pKIWI110 also contains the reporter gene β-D-glucuronidase. Leaf pieces inoculated with pKIWI110 produced zones of blue cells two days after inoculation. Shoots from leaf pieces inoculated with pGA643 were selected on kanamycin. PCR and Southern analysis of shoots that were able to root on kanamycin confirmed the presence of the NPTII gene in the plant genome.

INTRODUCTION

Chrysanthemum is one of the world's major cut flower crops. Variation and improved qualities have been introduced into chrysanthemum by conventional breeding and selection and more recently through mutagenesis (Jung-Heiliger and Horn 1980, Broertjes and Lock 1985). An alternative method of creating variation is by introducing specific characteristics via genetic engineering, mediated by *Agrobacterium tumefaciens*.

An efficient shoot regeneration system is a prerequisite for the production of non-chimeric transformed shoots. Early reports of adventitious shoot regeneration of chrysanthemum often describe an initial callus phase (Hill 1969, Roest and Bokelmann 1975, Bush et al 1976, Sutter and Langhans 1981) which may increase the chance of somaclonal variation and the production of chimeric shoots. Recently a protocol for regenerating shoots directly from leaf and stem explants of chrysanthemum has been published (Kaul et al. 1990), however there were large cultivar differences. Although the susceptibility of chrysanthemum to *Agrobacterium tumefaciens* infection is widely reported (see Miller et al. 1975) there have been no previously published reports of successful transformation of chrysanthemum.

We screened a number of cultivars of chrysanthemum to identify a genotype that regenerated adventitious shoots efficiently. We incorporated the regeneration system with *Agrobacterium tumefaciens*-mediated transformation to produce transformed shoots using β-D-glucuronidase (GUS) as a reporter gene and neomycin phosphotransferase II (NPTII) gene as a selectable marker for kanamycin resistance. Southern analysis showed the presence of the NPTII gene in the kanamycin resistant plants.

MATERIALS AND METHODS

Plant material Seven genotypes of chrysanthemum (six *Dendranthema morifolium* (Ramat.) Tzvelev (Webb et al. 1988) and one *Dendranthema indicum)* were screened on a wide range of shoot regeneration media to select the most efficient regenerating genotype for transformation. The genotypes used were *D. morifolium*: "Refour", "Red Minstrel", "Inferno", "Peach Margaret", "Red Cassandra", "Yellow Lucondra" and *D. indicum*: "Korean" and were obtained from local nurseries as cuttings.

Tissue culture media and growth conditions The basal medium (BM) used for tissue culture comprised of Murashige and Skoog (MS) salts (1962), 0.4 mg/l thiamine-HCl, 100 mg/l inositol, 30 g/l sucrose and 8.0 g/l Davis agar. When half BM was used only the MS salts were reduced. All media were adjusted to pH 5.7 with NaOH before autoclaving at 103 kPa for 15 mins. Growth regulators used were benzyladenine (BA), gibberellic acid (GA_3), indoleacetic acid (IAA), kinetin (Kin), napthalene acetic acid (NAA) and thidiazuron (TDZ). When used, antibiotics IAA, GA_3 and TDZ were filtered sterilised into autoclaved media.

In vitro stock plants were used as the source of explants for all regeneration and transformation experiments. The stock plants were maintained by subculturing shoot tips at six-weekly intervals on half strength BM and were grown at 25 °C under a 16/8h light/dark photoperiod using one Thorn 40W CoolWhite, three Thorn 55W Daylight and one Sylvania Grolux F40T12 tubes giving 118 μmoles/m^2/s at culture level.

Explants for shoot regeneration and transformation experiments were screened on a range of media containing BM plus a number of auxin and cytokinin types and concentrations (Tables 1 and 2). Newly formed expanding leaves were removed from stock plants, cut into 4 - 5 mm square segments and placed

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with the abaxial surface down on the medium. Petioles and internodal stem segments were cut into 5 mm lengths and placed horizontally on the shoot regeneration media. Explants were grown under the conditions described above except for the light intensity which was reduced to 35 μmoles/m^2/s (one Thorn 40W Cool White tube). Shoots that formed were proliferated by sub-culturing the tips on half strength BM.

Agrobacterium strains The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al, 1983) was used in the transformation experiments and contained either pGA643 (An et al 1988) or pKIWI110 (Janssen and Gardner 1990). The structures of the T-DNA regions of pGA643 and pKIWI110 are shown in Fig 1a and b. The avirulent strain A2002 containing pKIWI110 was used as a negative control. A2002 contains a mutation in the *vir*B gene (Klee et al. 1983) which prevents the transfer of the T-DNA to plant cells.

Transformation Experiments to produce transformed chrysanthemum shoots were carried out with the Korean genotype due to its superior regeneration ability. The regeneration media (RM) comprised of BM supplemented with 0.2 mg/l IAA and 3 mg/l BA.

Leaf pieces were inoculated using a technique based on that of Horsch et al. (1985). Leaf pieces were cut on the day of inoculation and placed onto RM. The inoculum was prepared by growing an overnight culture of *Agrobacterium* in YN broth (beef extract 3 g/l, Bacto-peptone 5 g/l, NaCl 8 g/l, yeast extract 10 g/l) containing 25 mg/l kanamycin at 28 °C and 310 rpm. The cells were then pelleted and resuspended in YN broth. Leaf pieces were submerged in the inoculum then blotted dry between two pieces of sterile filter paper and placed onto RM. After two days of cocultivation the leaf pieces were transferred to selection media (RMS) containing RM plus 500 mg/l ticarcillin and 10 mg/l kanamycin and grown under conditions described above for shoot regeneration. In subsequent experiments (see `b' below) the kanamycin level in RMS was raised to 20 mg/l.

Three to four weeks after inoculation shoots that formed in the presence of kanamycin were removed from the leaf piece and placed on half strength BM containing 500 mg/l ticarcillin to allow the shoots to enlarge. After 2-3 weeks roots had developed and the shoots were 3-4 cm high. The shoots were rechallenged in the following two ways: (a) the upper 2 cm (apex) of the shoot was removed and placed on half strength BM containing 500 mg/l ticarcillin and 25 mg/l kanamycin to test for rooting in the presence of kanamycin. (b) leaf pieces were removed from each shoot and placed on RMS to test for shoot regeneration in the presence of kanamycin. After 3 to 4 weeks shoots that formed roots (a above) and regenerated shoots from leaf pieces (b above) were transplanted into pumice and grown under high humidity in a growth cabinet. After a further 2-3 weeks the rooted plants were repotted in soil and induced to flower under short day conditions (13/11hr light/dark) with 700 μmoles/m^2/s at 25 °C.

GUS assay Histochemical GUS assays of leaf pieces inoculated with pKIWI110 were carried out using a technique based on that of Jefferson (1987). Leaf pieces were incubated overnight at 37 °C in a substrate solution of 0.3 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) dissolved in 0.003% v/v of dimethyl formamide and buffered with 50 mM $NaPO_4$ (pH 7.0). Cells expressing GUS were expected to produce a blue precipitate (Jefferson 1987).

DNA isolation and analysis DNA was isolated from non-transformed shoots and kanamycin resistant shoots. The apex and youngest leaves (0.5-1.0 g) were excised and ground in liquid nitrogen. The tissue was suspended in extraction buffer (26 mM NaOAc pH 7.0, 4 M guanidinium isothiocyanate, 1.5% w/v Na lauryl sarcosine) with 2-mercaptoethanol (2.5% v/v), extracted with an equal volume of phenol/chloroform (1:1, v:v) followed by an equal volume of chloroform. The DNA was precipitated with 0.8 volumes of isopropanol and resuspended in TE buffer (10 mM Tris-Hcl, 1 mM EDTA).

For analysis by the polymerase chain reaction (PCR), two 16 nucleotide primers for the 5' and 3' ends of a 800 bp fragment of the NPTII gene were used with the `GeneAmp' Kit (Perkin Elmer Cetus). DNA was amplified by 30 cycles of 1 min at 94 °C, 2 min at 55 °C and 3 min at 74 °C.

For Southern analysis plant genomic DNA was cut with Hind III and Bam HI, treated with RNase A and separated electrophoretically on a 0.7% agarose gel. The DNA was then transferred onto Amersham `Hybond' N^+ blotting membrane under alkaline conditions, following the manufacturer's instructions. Control plasmid DNA was included in all blots. Probe DNA was labelled using BRL's nick translation kit with $\alpha^{32}P$ [dCTP]. Unincorporated label was removed using the method of Maniatis et al. (1982).

To test for the presence of the kanamycin resistance gene, the probe consisted of the coding region of the NPTII gene isolated as a Hind III/Bam HI fragment from pGA643 (Fig 1a).

To test for integration of the T-DNA into the plant genome the probe consisted of the left border region isolated as a Xba I/Sal I fragment from pGA643 (Fig 1a).

RESULTS AND DISCUSSION

Regeneration

D. indicum (Korean) was the most responsive genotype on the range of shoot regeneration media screened (Tables 1 and 2). All leaf pieces from this genotype produced at least 1-2 shoot loci on 7 out of the 14 media assessed. The most effective medium for the Korean genotype was BM supplemented with 0.2 mg/l IAA plus either 3 or 5 mg/l BA. Shoots were obvious after 2 weeks and were removed 3-4 weeks after initiation when they were approximately 5 mm in length. In comparison, the other six genotypes (the *D. morifolium* cultivars) regenerated poorly on the media screened. The most effective media produced 1-2 shoot loci on only some of the leaf pieces or internodal segments. A recent report showed that eight out of eleven *D. morifolium* cultivars regenerated shoots on media containing 1 mg/l NAA and 1 mg/l BA (Kaul et al. 1990). The *D. morifolium* and *D. indicum* genotypes used in the present study did not produce shoots from leaf pieces on this medium. We support the findings of Kaul et al. that shoot regeneration in chrysanthemum is cultivar dependent. We also suggest that *D. indicum* genotype Korean is superior to *D. morifolium* cultivars for shoot regeneration.

Table 1. The response of chrysanthemum leaf pieces on shoot regeneration media, based on BM with a range of concentrations and types of plant growth regulators. (Data collected 3 to 4 weeks after initiation: 10 to 20 explants per treatment)

Growth regulator and concentration (mg/l)					Genotype			
IAA	NAA	BA	Kin	GA_3	Kor	Ref	Min	Inf
0.2		3			++++	+	+	++
0.2		3		0.3	++	+	+	+
0.2		3		2	+	+	+	+
0.2		5			++++	+	+	++
0.2		5		0.3	+	+	+	+
0.2		5		2	-	+	+	+
0.3		3			+++	nt	nt	+
1			3		+++	nt	nt	+
1		3			+++	nt	nt	++
2		3			+++	nt	nt	++
	0.3		3		++	nt	nt	+
	0.3	3			+++	nt	nt	++
	1		3		+	nt	nt	+
	1	1			nt	+	+	+
	1	2			nt	-	+	+
	1	3			+	nt	nt	+

Genotypes:- *D. indicum*: Kor = Korean. *D. morifolium*: Ref = Refour, Min = Red Minstrel, Inf = Inferno.
- No callus or shoots: + Callus but no shoots
++ 1-2 shoot loci on some leaf pieces
+++ 1-2 shoot loci on all leaf pieces
++++ Multiple (>3) shoot loci on all leaf pieces
nt Not tested.

Table 2. The response of different explants of three genotypes of *Dendranthema morifolium* on shoot regeneration media, based on BM with 0.2 mg/l IAA and a range of concentrations and types of cytokinins. (Data collected 3 to 4 weeks after initiation: 5 explants per treatment.)

Cytokinin (mg/l)	Genotype and Explant Type								
	Yellow Lucondra			Peach Margaret			Red Cassandra		
	LP	IS	PT	LP	IS	PT	LP	IS	PT
3 BA	+	++	+	+	++	+	+	+	+
5 BA	+	++	+	+	++	+	+	+	+
10 BA	+	++	+	+	+	+	+	+	+
0.1 TDZ	+	+	+	+	nt	+	+	+	+
0.3 TDZ	-	+	+	+	++	+	+	+	+
1 TDZ	+	+	+	+	+	+	+	+	+
3 TDZ	+	++	+	+	+	+	+	+	+

Explants: LP = leaf pieces, IS = internodal segments, PT = petioles.
- No callus or shoots: + Callus but no shoots
++ 1-2 shoot loci on some leaf pieces
nt Not tested.

GUS assay

Leaf pieces of the Korean genotype were assayed for expression of the GUS gene after 2 days of cocultivation with pKIWI110/LBA4404. A total of 90 leaf pieces, inoculated in 9 separate experiments, were assayed and 75 leaf pieces (83%) produced zones of blue cells. The zones varied in size from discrete loci of approximately 10 cells to more diffuse zones incorporating up to half of the circumference of the leaf piece. The zones tended to be localised near veins but with some scattered around the rest of the cut edge. Blue zones were occasionally seen on the surface of the leaf piece away from the cut edge and are thought to be sites of damage from handling the leaf piece during inoculation and transfers. The localisation of the zones of blue cells around veins in the leaf piece has also been reported by other workers in petunia (Janssen and Gardner 1990) and may indicate a region of cells that are actively dividing and are in the correct phase of cell division to enhance transformation (Chriqui et al. 1988). Janssen and Gardner (1990) suggest that the regions around the veins may include localised gradients of compounds, possibly plant growth regulators, that could stimulate cell division and enhance the transfer of T-DNA. The blue zones were considered to be specific expression of the GUS gene by cells in the leaf piece for two reasons. Firstly, the GUS gene in pKIWI110 lacks a bacterial ribosome binding site resulting in negligible expression in *Agrobacterium* (Janssen and Gardner 1990). Secondly, there were no zones of blue cells produced by the leaf pieces inoculated with pKIWI110/A2002 or by non-inoculated leaf pieces.

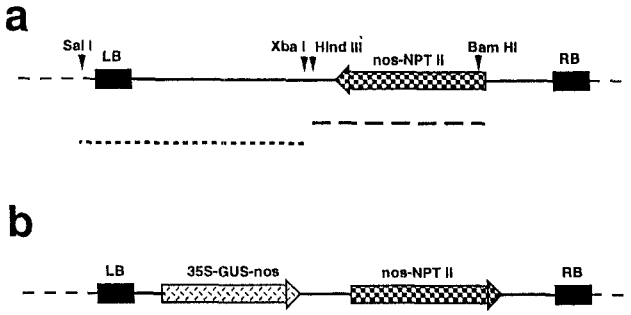

Fig 1. The T-DNA region of the binary vectors showing the left (LB) and right (RB) border regions and the neomycin phosphotransferase II gene (NPTII). (a) pGA643: ----- the Hind III/Bam HI fragment used as the probe for the kanamycin resistance gene. ..... the Xba I/Sal I fragment used as the probe for T-DNA/plant DNA junction fragments. (b) pKIWI110 also contains the β-D-glucuronidase gene (GUS). Diagram not drawn to scale.

Production of kanamycin resistant shoots

Cocultivation with pKIWI110/LBA4404

Leaf pieces cocultivated with pKIWI110/LBA4404 produced 81 shoot loci from 264 leaf pieces. A total of 141 shoots

were removed and rechallenged with kanamycin as described previously. No shoots produced roots in the presence of kanamycin or formed shoots from leaf pieces on RMS.

Cocultivation with pGA643/LBA4404

Leaf pieces cocultivated with pGA643/LBA4404 produced shoot loci on more leaf pieces (155 out of 353) than when cocultivated with pKIWI110/LBA4404. Up to six shoot loci regenerated per leaf piece (Fig 2) and a total of 290 shoots were removed, each from a separate locus. The shoots were grown without selection for 2-3 weeks and then rechallenged with kanamycin as previously described. Explants from 3 of the 290 shoots were able to produce both roots (Fig 3) and shoots under kanamycin selection. No shoots were produced from 90 non-transformed leaf pieces tested in 9 separate experiments. In addition 50 non-transformed shoots, tested in four experiments, failed to root in the presence of kanamycin.

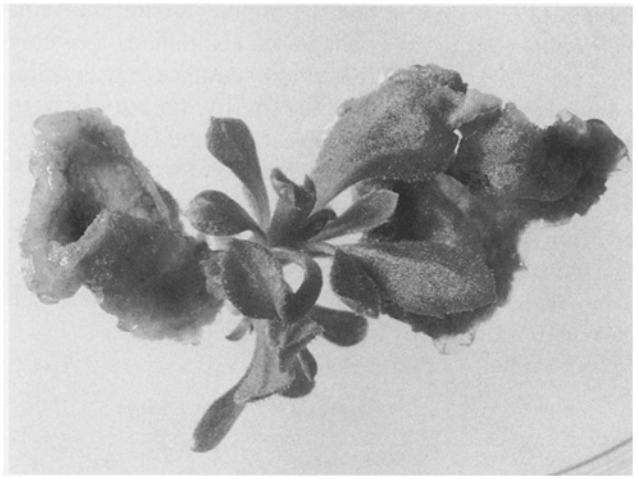

Fig 2. Shoot loci on leaf pieces cocultivated with pGA643/LBA4404 in the presence of kanamycin.

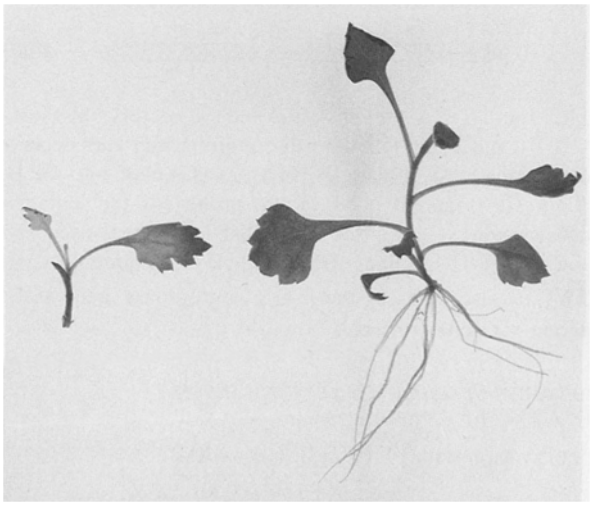

Fig 3. Root production from wildtype (left) and a kanamycin resistant shoot (right) in the presence of kanamycin.

In subsequent experiments with pGA643 the frequency of transformation was 1.7 shoot loci per leaf piece but 77 out of 122 shoots rooted in the presence of kanamycin. Investigations of the reasons for the improved rooting performance is in progress and will be published at a later date. A total of 80 kanamycin resistant plants have been produced using the vector pGA643.

Leaf pieces inoculated with pGA643/LBA4404 consistently regenerated a greater number of shoots than leaf pieces inoculated with pKIWI110/LBA4404. One explanation for the difference in transformed shoot yield between pKIWI110 and pGA643 is the presence of an overdrive sequence in the right border of pGA643 (An et al. 1988) which is responsible for high efficiency transfer of the T-DNA (Peralta et al. 1986). There have been no reports that the borders of pKIWI110 contain such a sequence.

Other workers (Horsch et al. 1985, Deroles 1988, Ulian et al. 1988) have also reported that high number of shoots that regenerate under kanamycin selection but fail to root in the presence of kanamycin. Two hypotheses have been put forward to account for the high number of 'escapes'. Cells with transient expression of the NPTII gene provide temporary protection from kanamycin allowing non transformed shoots to regenerate. Alternatively, escapes may have an integrated copy of the T-DNA which is initially expressed but is subsequently shut down (Horsch et al. 1985, Deroles 1988).

The regenerated transformed shoots were multiplied *in vitro* under kanamycin selection, transplanted into growth cabinets and induced to flower under short day conditions. All transgenic plants appeared morphologically normal when compared to the original plant material.

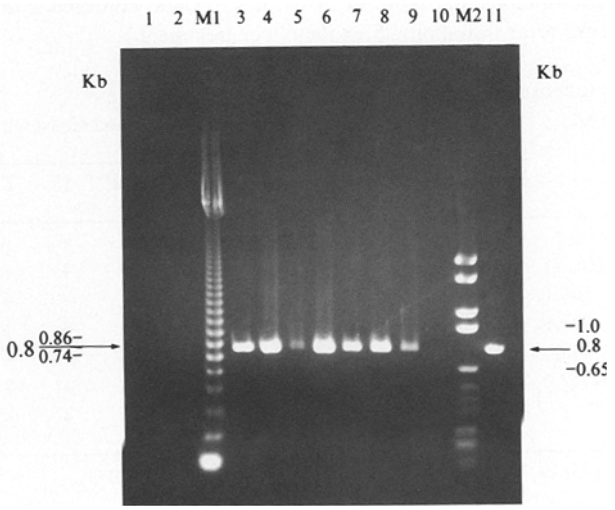

Fig 4. Polymerase chain reaction (PCR) analysis of kanamycin resistant shoots. Lanes 1 and 2: DNA from non-transformed shoots. Lanes 3 and 4: DNA from TC105. Lanes 5 and 11: DNA from TC123. Lanes 6 and 7: DNA from TC248. Lane M1: 123bp ladder (BRL). Lane M2: Boehringer Mannheim Marker VI. Lane 10: no template DNA.

Analysis of kanamycin resistant shoots

Initial screening of three original kanamycin resistant shoots by PCR analysis showed the presence of the expected 800 bp fragment of the NPTII gene (Fig 4).

Further investigation of DNA from these shoots by Southern analysis showed hybridisation to the NPTII probe (Fig 5a). The expected hybridisation pattern for an intact insertion event, when probed with the 2.5 kb Bam HI/Hind III fragment of pGA643, is a single band at 2.5 kb. Two of the plants, TC123 and TC248, showed the expected band. TC123 also contained a band at approximately 9 kb with a similar degree of homology as the 2.5 kb band. This suggests the presence of an additional T-DNA insert which may have been deleted from either the 5' or 3' region resulting in the loss of one of the expected restriction enzyme sites. The third plant, TC105, did not show a band of the expected size but contains a band at approximately 9 kb suggesting the presence of an altered T-DNA. As TC105 is kanamycin resistant, a possible deletion of the T-DNA is most likely from the left border region since a deletion from the right border to remove the Bam HI site would also result in the loss of the promoter for the NPTII gene. The band of approximately 9 kb in the positive control lane represents the remainder of pGA643 plasmid, which is 11.7 kb in total.

In order to establish that the T-DNA region was integrated into the plant genome a further 6 kanamycin resistant plants were subjected to Southern analysis as described above (Fig 5b). The probe used was the Sal I/Xba I fragment spanning the left border region of the T-DNA in plasmid pGA643 (Fig 1a).

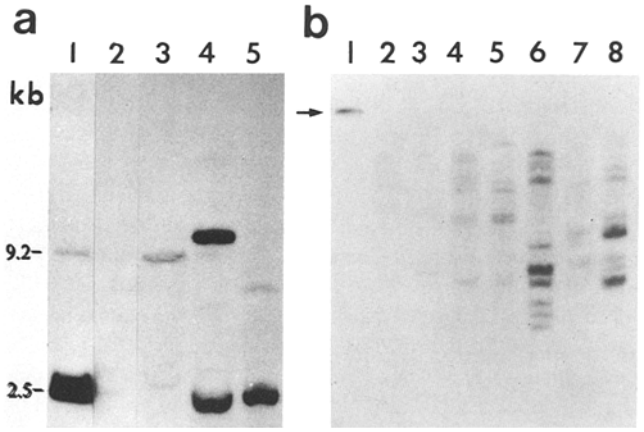

Fig 5. Southern analysis of kanamycin resistant shoots. **a)** Presence of the kanamycin resistance gene. Lane 1: 2.5 kb Bam HI/Hind III fragment (nos-NPTII gene) and a small amount of the 9 kb fragment of pGA643. Lane 2: DNA from a non-transformed shoot. Lanes 3, 4 and 5: three kanamycin resistant plants TC105, TC123 and TC248 respectively. **b)** Proof of integration into the plant genome. Lane 1: pGA643 digested with Bam HI and Hind III. Lane 2: DNA from a non-transformed shoot. Lanes 3 to 8: six kanamycin resistant shoots, TC494/7, TC491, TC492, TC533, TC572 and TC578 respectively. → band size = 8.35 kb.

In the intact pGA643 plasmid cut with Bam HI/Hind III this probe is homologous to the 8.35 kb band comprising the external sequences plus both border regions (See lane 1 Fig 5b).

All 6 kanamycin resistant plants tested did not show the presence of this band (lanes 3 to 8, Fig 5b) indicating that the external section of pGA643 was not present. Instead each plant showed a unique set of smaller bands indicating that the probe is binding to junction fragments containing both plant DNA and T-DNA. The banding patterns from this analysis indicates the presence of multiple copies of the T-DNA in each plant genome. Further analysis of kanamycin resistant chrysanthemums is in progress to establish the frequency of rearrangement and number of integration events per plant genome.

We have produced transformed shoots of the Korean genotype of *D. indicum* and have shown that they contain the NPTII gene by PCR and Southern analysis. Experiments are in progress to insert specific characteristics into chrysanthemum in an attempt to alter the pigmentation of the flowers.

Acknowledgements. We would like to thank Dr Gail Timmerman for the oligonucleotide primers for PCR, Dr Gynheung An for providing pGA643, Dr Richard Gardner and Dr Bart Janssen for pKIWI110 and valuable discussion and assistance, Foxy Flowers and Geerlings for the plant material, Ms Marie Bradley for teaching and assistance with PCR, Dr Cathie Martin and Mr Steve Butcher for discussions and support and Ms Robyn Miller, Mr David Manson and Mr Ray Rains for excellent technical assistance.

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