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Genetic transformation and development of *Cucumber mosaic virus* resistant transgenic plants of *Chrysanthemum morifolium* cv. Kundan

S. Kumar[a,1], S.K. Raj[a,*], A.K. Sharma[b], H.N. Varma[c]

[a] Plant Molecular Virology, CSIR-National Botanical Research Institute, Lucknow 226001, India
[b] Tissue Culture Laboratory, CSIR-National Botanical Research Institute, Lucknow 226001, India
[c] School of Life and Basic Sciences, Jaipur National University, Jagatpura, Jaipur 302025, India

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ABSTRACT

Chrysanthemum morifolium Ramat. cv. Kundan has been found susceptible to infections of *Cucumber mosaic virus* (CMV) which drastically affects the quality and quantity of blooms and poses significant constraints in commercial cultivation of chrysanthemum in India. Therefore, development of inbuilt resistance in *C. morifolium* against CMV seems to be essential. *Agrobacterium*-mediated transformation of petiole explants of *C. morifolium* was attempted using p*RoK*2 binary vector harbouring coat protein (CP) gene of CMV under the control of CaMV 35S promoter. A total of 257 explants were transformed and 73 putative transgenic plants from seven independent co-cultivation events were obtained with ~6% transformation efficiency. Molecular analysis of these plants confirmed the successful integration of CP transgene in 63% plants, of which 12.3% plants were able to transcript and translate the transgene. Expression of coat protein did not evoke any abnormal phenotype. Transgenic plants showed delayed resistance when challenged by CMV-chrysanthemum strain which produced good quality blooms as compared to the susceptible ones.

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1. Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat., family *Asteraceae*) is the second commercially important floriculture crop after rose and well accepted globally as cut- and potted-flower plant (Teixeira da Silva, 2003). It accounts about 29% of the total cut-flower production contributing 299.6 million INR to Indian floriculture industry (Anonymous, 2005) and has high economic importance as a horticultural crop. Chrysanthemums are reported to be susceptible to viruses affecting its cultivation throughout India (Srivastava et al., 1992; Singh et al., 2006; Kumar et al., 2009; Raj et al., 2009). *Cucumber mosaic virus* (CMV) has been reported to cause severe mosaic, ringspots and flower deformations which drastically reduce the quantity and quality of the blooms in *C. morifolium* cv. Kundan (Kumar, 2009).

Cucumber mosaic virus (of genus *Cucumovirus*, family *Bromoviridae*) is a spherical, tripartite plus sense single-stranded RNA virus which is an important plant pathogen worldwide infecting more than 1200 plant species, including both monocots and dicots and causes significant yield losses. Its genome consists of three RNA species (RNA 1, 2 and 3) which encode five proteins. The coat protein (CP) is encoded by RNA 3 and expressed from a subgenomic RNA 4, required for host range, viral encapsulation, systemic movement and aphid transmission (Palukaitis and García-Arenal, 2003).

Coat protein being a multifunctional and essential protein for CMV, we utilized full-length CP gene to develop resistance against CMV in Kundan cultivar of chrysanthemum which is in high demand in local floriculture industry for its beautiful golden yellow blooms having more than two weeks long vase life.

2. Materials and methods

2.1. Explant source

Approximately 3.0 cm long apical shoots excised from healthy plants of *C. morifolium* cv. Kundan were surface-sterilized with 0.1% mercuric chloride solution, regenerated aseptically and maintained in half strength MS medium (Murashige and Skoog, 1962). The petiole segments of 5 mm length obtained from regenerated shoots having leaves of 16 mm × 7 mm size were excised and used as explants for transformation process. All cultures were maintained at 25 ± 2 °C temperature and 16 h photoperiod (50 μmol/m^2/s quantum flux density at culture level, Hangarter and Stansinpoulos, 1991).

* Corresponding author. Tel.: +91 522 2297950; fax: +91 522 3305836.
E-mail address: skraj2@rediffmail.com (S.K. Raj).
[1] Present address: Pohang University of Science and Technology, Pohang 790-784, South Korea.

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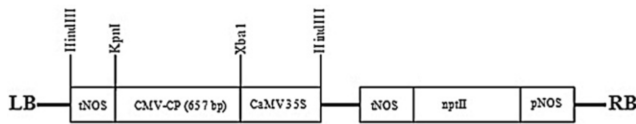

Fig. 1. Schematic representation of p*RoK*2-CMV-CP binary expression vector containing full length CP gene of 657 bp cloned at *Xba*I and *Kpn*I position under control of CaMV 35S promoter and *NOS* terminator and *NptII* gene for kanamycin resistance as selection marker.

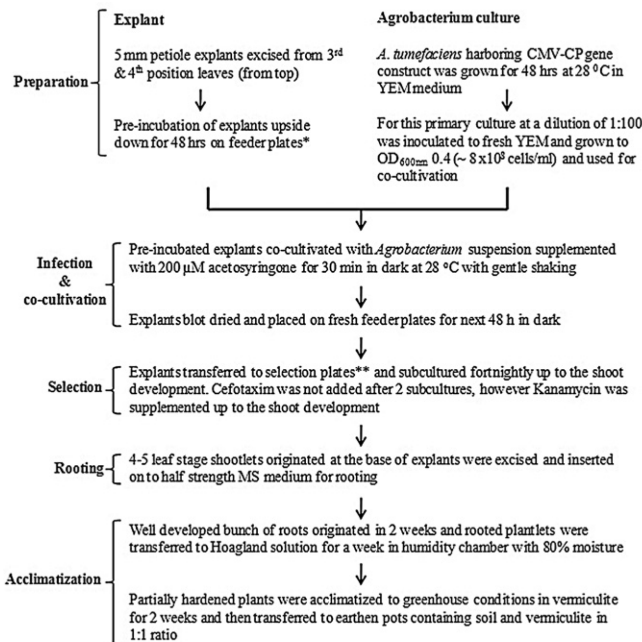

Fig. 2. Flow diagram of the co-cultivation and transformation procedure for chrysanthemum petiole explants with modification. *Feeder plate: MS + 5.0 mg/l BAP + 0.5 mg/l and **selection plate: MS + 5.0 mg/l BAP + 0.5 mg/l + 500 mg/l cefotaxim + 100 mg/l kanamycin.

2.2. Construct

For genetic transformation of petiole explants of *C. morifolium* cv. Kundan, *Agrobacterium tumefaciens* (strain LBA4404) harbouring *pRoK2* binary vector carrying the complete 657 bp long coat protein gene of CMV (Fig. 1) under the control of CaMV 35S constitutive promoter and nos terminator (Srivastava and Raj, 2008) was used.

2.3. Transformation of petiole explants

Co-cultivation and transformation of petiole explants of *C. morifolium* was carried out following the protocol of Renou et al. (1993) with some modifications as detailed in Fig. 2. The parameters for petiole transformation (described in Table 1) were optimized one by one and used as a guide in subsequent experiments. The parameters optimized were: concentration of antibiotic kanamycin (25, 50, 100 and 150 mg/l); cefotaxim (100, 250, 500, 750 and 1000 mg/l); bacterial inoculum density ($OD_{600\,nm}$ 0.4 and its dilutions: 1:10, 1:20); duration of pre-culture (0, 1, 2, 3 and 4 days); effect of acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone: 50, 100, 150 and 200 µM) and co-cultivation temperature (20, 25 and 28 °C). For each treatment 15 explants were considered and significance of the experiments was analysed using Duncan Multiple Range Tests (DMRT) at 95% confidence level, $P = 0.05$ (Kramer, 1956). The regenerated putative transgenic plants were maintained under controlled glasshouse conditions for evaluation of resistance to CMV by challenge inoculations.

Table 1
Effect of various parameters on transformation and regeneration efficiency of petiole explants of chrysanthemum.

Transformation parameters	Treatment[$]	Number of regenerated petiole explants[a]
Bacterial inoculum density (OD_{600} 0.4)	Undiluted, 30 min	4.3b
	1:10 dilution, 10 min	6.8a
	1:10 dilution, 30 min	9.0a
	1:20 dilution, 30 min	**12.9a**
Pre-incubation period (day)	0	2.7b
	1	8.6b
	2	**14.1a**
	3	11.5c
	4	5.7a
Acetosyringone (µM)	50	4.7c
	100	9.2c
	150	10.6a
	200	**13.8a**
Co-cultivation temperature	**28 °C**	**13.8a**
	25 °C	5.9a
	20 °C	2.1c
Co-cultivation duration (at 28 °C)	30 min	3.9b
	2 h	4.2a
	24 h	8.3b
	48 h	**12.3a**
	72 h	7.4b

[$] Each treatment experiments were repeated three times with 15 explants.
[a] The number of regenerated petiole explants is the mean value obtained from three replications for each treatment. Rate of mortality and regeneration are the average of three replicates each with 15 explants. Statistical analysis was carried out using one-way ANOVA and mean within same columns followed by the same letter were not significantly different according to DMRT ($P < 0.05$). Conditions selected for further transformation procedures have been shown in bold.

2.3.1. Validation of transgenic plants for CP transgene by PCR followed by Southern hybridization

Total genomic DNA from the transgenic and control plants was isolated by the method (Dellaporta et al., 1983) and used as the template for PCR employing CMV-CP gene specific primers (AM180922/AM180923). PCR was done in a 50 µl reaction volume containing ~10 ng of DNA as template, 100 pM each of forward and reverse primer, 0.2 M dNTPs and 1 U *Taq* DNA polymerase (Genei Pvt. Ltd., India) in a thermal cycler (PTC200 MJ Research, USA). The 25 cycle reaction, which consists of heat denaturation (95 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 60 s), was carried out and the reaction mixture was subjected to 1% agarose gel electrophoresis. Identity of amplicons was confirmed by Southern hybridization. PCR generated CP amplicons from a positive clone was used for homologous probe preparation by random primer labeling method (Fienberg and Vogelstein, 1983) using αP^{32} dCTP radio activity and pre-hybridization, hybridization and washing steps were performed according to standard methods (Sambrook et al., 1989).

2.3.2. Analysis of transgenic plants for copy number of CP transgene

Integration of CP gene and its copy number in the genome of transgenic plants was confirmed by Southern hybridization tests following the standard protocol described earlier (Sambrook et al., 1989). Briefly, 15 µg of total genomic DNA of transgenic lines along with positive and negative controls (p*RoK*2) were digested by *Hind*III restriction enzyme (as the CP gene contains this unique site at position 304), electrophoresed, blotted on nylon membrane and hybridized with CP specific probe (prepared by a positive clone of CMV-CP) to determine the actual copy numbers inserted in the genome of transgenic plants. The blots were washed as per manufacturer's instructions and exposed to X-ray films to observe

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