Transgenic Plant Journal ©2007 Global Science Books

Optimization of *Agrobacterium*-Mediated Overexpression of Osmotin-Ferritin Genes in *Brassica juncea*

N. Nirupa • M. N. V. Prasad* • S. K. Jami • P. B. Kirti

Department of Plant Sciences, University of Hyderabad, Hyderabad-500 046, India

Corresponding author: * **mnvsl@uohyd.ernet.in**

ABSTRACT

The binary vector p35SGUSINT mobilized into *Agrobacterium* strain GV2260 was used for transformation of cotyledonary petiole explants of *Brassica juncea* (L.) Czern cv. 'Pusa Jaikisan' and transient GUS expression was used as the basis for identifying the most appropriate conditions for transformation. Genetic transformation is influenced by a number of factors which affect the efficiency. Some of the crucial factors like explant age, preculture period, and bacterial density and use of non-ethylene agent like silver nitrate were evaluated to optimize a protocol for cotyledonary petiole explants. The efficiency of the method optimized with the GUS construct has also been applied for introducing the osmotin-ferritin construct into *B. juncea*. The transfer of the foreign GUS gene into *B. juncea* was demonstrated through GUS assays, PCR and Southern blot analyses. The osmotin-ferritin transformants were confirmed in the T_0 and T_1 generations for the integration and expression of the osmotin and ferritin transgenes through PCR, Southern and Western blots.

Keywords: beta-glucuronidase, *GUS*, mustard**,** neomycin phosphotransferase, *nptII*, transformation, transgenic

INTRODUCTION

Brassica juncea (L.) Czern (*Brassicaceae*), commonly known as Indian mustard, is given considerable attention by present day researchers (Prasad and Freitas 2003) as it is an important economical plant in India (Batra 2001) and also known throughout the world for its oil production and phytoremediation. 'Pusa Jaikisan', a somaclone of *B. juncea* was released in 1994 for commercial cultivation (Katiyar and Chopra 1995). Its performance over a period of three years in all-India coordinated trials displayed distinct superiority (20% increase in seed yield 36 Q/hectatre and 40% increase in oil production) over the best check 'Varuna' variety. This cultivar has been immensely popular among the farmers even in the zones for which it was not recommended. It is early maturing and pod-shattering resistant. A simple and quick transformation protocol hence would be helpful in genetic manipulation with various genes of interest.

Vector-less procedures may eliminate *Agrobacterium*-related variables but introducing new methods may lead to inferior results. *A. tumefaciens*-mediated transformation is most widely used for *Brassica* and it is generally quite efficient and practical for most species in the genus. Transformation has improved *Brassica* species and was mostly concentrated upon *B. napus* for many traits like oil quality, eliminating glucosinates, and herbicide tolerance (Poulsen 1996; Earle and Knauf 1999; Mehra *et al*. 2000; Cardoza and Stewart 2004). There have been recent reports on increasing transformation efficiencies in *Brassica* such as *B. oleracea* var. *italica* (Henzi *et al*. 2000) and *B. napus* (Cardoza and Stewart 2003; Khan 2003), however, there have been fewer reports on *Agrobacterium*-mediated transformation of *B. juncea* (Mathews *et al*. 1990; Barfield and Pua 1991; Kanrar 2002, 2005) and there is still a need for developing efficient transformation methods in *B. juncea* for overcoming genotype dependency as this plays an important role in efficiency (Pental *et al*. 1993; Ono *et al*. 1994). Selection of the appropriate explant and optimized culture conditions are important for improved transformation efficiency (Uranbey *et al*. 2005).

The *E. coli β-Glucuronidase* gene (*gusA*, *uidA*) (Jefferson *et al*. 1986, 1987), when regulated by a constitutive promoter, serves as a useful tool for primary screening, promoter analysis, and histological studies. The GUS assay however can be biased by the presence of contaminating *Agrobacterium* that remains endogenously in the plant material from cocultivation and which some how can express the *β-Glucuronidase* gene resulting in false positive reactions. This problem is coped with by inserting an intron sequence into the *gusA* gene which then no longer expresses in *Agrobacterium*, due to lack of an eukaryotic RNA splicing apparatus (Vancanneyt *et al*. 1990). p35S GUSINT was used in the present study.

In the present study efforts have been made to standardize a simple and swift transformation protocol with cotyledonary petioles as explants. Various factors influencing the efficiency of T-DNA delivery were evaluated in preliminary experiments. These factors included *A. tumefaciens* cell density for inoculation, co-culture period and the use of non-ethylene agents in the shoot induction medium. The selection of the appropriate explant and optimized culture conditions are important for improved transformation efficiency. These parameters were optimized using transient GUS percentage in one-week-old cultures. The standardized protocol was then used to mobilize an osmotin and ferritin construct to confirm the efficacy of the method. The ferritin gene, coding for an iron-chelating protein apart from improving the nutritional content also appears to help the plant with defense against pathogenic fungi like *Alternaria*, *Botrytis* and also *Tobacco necrosis virus* (TNV) (Deak *et al*. 1999). Such a natural scavenger molecule of iron in combination with a defense gene like osmotin may further enhance the plant resistance against abiotic and biotic stresses. Osmotin belongs to PR-5 (Pathogenesis Related) family of proteins. Transgenic plants overexpressing PR-5 proteins showed resistance or delayed development of disease symptoms and protection against phytopathogenic fungi (Liu *et al*. 1994, 1996; Chen *et al*. 1999; Datta *et al*. 1999). It has been shown that osmotin induces a cascade of events of

Received: 12 April, 2007. Accepted: 23 August, 2007.

Original Research Paper

Mitogen-activated protein kinase (MAPK) pathway genes in yeast and enhances its cytotoxicity by stimulating the changes in the cell wall, which result in the access of osmotin to the plasma membrane causing cell death (Yun *et al*. 1998).

A full-length 910 bp cDNA encoding osmotin-like protein with an open reading frame of 744 bp encoding a protein of 247 amino acids with a calculated molecular mass of 26.8 kDa cloned from *Solanum nigrum* (SniOLP) was used in the present study. Recombinant protein purified from overexpressed *E. coli* cells showed hyphal growth inhibition in *Rhizoctonia batiticola* and *Sclerotinia sclerotiorum* (Jami *et al*. 2007). The optimized transformation conditions using GUS gene were thus validated by transforming the osmotin-ferritin gene construct in *B. juncea* assuming the rationale that these plants might confer tolerance to both biotic and abiotic stress.

MATERIALS AND METHODS

Plant material and culture conditions

Mature seeds of 'Pusa Jaikisan' (Indian Council of Agricultural Research, New Delhi) were sterilized with 70% ethanol for half a min followed with 7 min in 0.1% mercuric chloride. Then seeds were rinsed 5 times in sterile double distilled water. Seeds were germinated in half strength Murashige and Skoog (1962; MS) salts with 0.8% agar (Sigma, USA) as a solidifying agent. Six-day-old seedlings were used to isolate the cotyledonary petiole explants. MS was the basal medium used for all experiments. MS basal medium with 2% sucrose (w/v) was modified with different types of plant growth regulators like 6-benzylaminopurine (BAP) 2, 4-dichloro-phenoxyacetic acid (2,4-D) and or α-naphthaleneacetic acid (NAA) at different concentrations and combinations. The pH of the medium was adjusted to 5.7 ± 0.1 prior to gelling with 0.8% (w/v) tissue culture grade agar or 0.5% agar gel (Sigma, USA). Cultures were done in Petri dishes, bottles and magenta boxes and were incubated at 27 ± 1ºC with 16/8 hrs light/dark photoperiod provided by white fluorescent tube light with an intensity of 120 μmol $m^{-2}s^{-1}$ (400-700 nm).

Bacterial strains and binary plasmid vectors

Agrobacterium tumefaciens strain GV2260 (Deblaere *et al*. 1985) harboring plasmid p35S GUSINT (Vancanneyt *et al*. 1990, **Fig. 1**) was used for the optimization of transformation conditions transiently. The binary plasmid p35S GUSINT contains a neomycin phosphotransferase II (*npt*II) gene driven by a nopaline synthase (NOS) promoter and the β-glucuronidase (GUS) gene controlled by the cauliflower mosaic virus (CaMV35S) promoter. For validation of the protocol, *Agrobacterium* GV2260 strain carrying an osmotin-ferritin construct (described in detail below) was used. The strains were grown overnight in a liquid Luria Bertani (LB) medium containing 50 mgL^{-1} kanamycin and 100 mgL^{-1} rifampicin along with 50 mgL^{-1} carbenecillin for the osmotin-ferritin carrying strain at 28°C in a rotary shaker at 200 rpm and used for transformation of *Brassica* cotyledonary petioles.

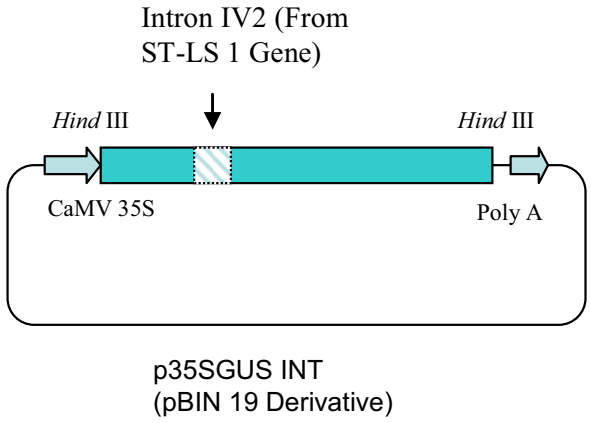

Fig. 1 Schematic diagram representing p35SGUSINT, a BIN 19 derivative (12.4 Kb) used in the present study. Source: Vancanneyt *et al*. (1990). The ST-LS1 gene derived portable intron (IV2) is cloned in the β-glucuronidase (GUS) gene.

Osmotin-ferritin vector construction

Osmotin (SniOLP from *Solanum nigrum*, Jami *et al*. 2007) and ferritin were cloned into pCAMBIA 2300 to have the ferritin gene (van Wuytswinkel *et al*. 1995) and osmotin in the same vector. Both genes were placed under the CaMV35S promoter for constitutive expression. To make the construct, the osmotin gene present in the PRT-100 vector was excised out by digesting with *Hind*III (MBI Fermentas, Germany). The fragment was gel eluted and end filled with Klenow enzyme (MBI Fermentas, Germany) to generate blunt ends. Simultaneously binary vector pCAMBIA 2300 vector digested with *Eco*RI (MBI Fermentas, Germany) was also end-filled with Klenow enzyme to fill up the 3′ recessed ends. The end-filled cassette with the osmotin gene was ligated to the end-filled pCAMBIA 2300 vector using T4 DNA ligase. Competent *E. coli* cells were transformed using the construct and recombinant clones were selected on 50.0 mgL^{-1} kanamycin on Luria Agar (LA) medium and 4.0 mgL^{-1} X-gal and 40 mgL^{-1} IPTG. Plasmid DNA was isolated from recombinant clones using an alkaline lysis method (Birnboim and Dolly 1979) and the construct was confirmed by digesting the isolated vector DNA with *Bam*HI and *Sma*I while the PRT-100 ferritin clone was partially digested with *Hind*III for 2½ min to get the intact cassette with the ferritin sequence. This fragment was ligated at the *Hind*III site of pCAMBIA 2300 osmotin vector. This pCAMBIA 2300 osmotin-ferritin construct was used to transform *E. coli*-competent cells and recombinant clones were selected on LA agar medium with 50.0 mgL^{-1} kanamycin. Plasmids were isolated from the clones and digested with *Bam*HI to confirm the presence of the construct. The plasmids isolated from the confirmed *E. coli* colonies were used to transform *Agrobacterium* strain GV2260 and the presence of both genes was confirmed.

Plant transformation using cotyledonary petioles

The transformation parameters were optimized for cotyledonary petiole explants using the bacterial strain GV2260 harboring the p35SGUSINT binary plasmid vector. Parameters were tested, one at a time, in a sequential order. The optimized conditions determined in this experiment were used in subsequent experiments. The parameters tested were density of bacterial culture (OD_{600}), seedling age, duration of preculture and use of silver nitrate in shoot induction medium. Mature seeds of cv. 'Pusa Jaikisan' were surface sterilized, then germinated on solid medium with half-strength MS salts. Cotyledonary petioles of six-day-old seedlings were used as explants. Explants were precultured on 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} NAA. After preculture, the explants were used for transformation by *A. tumefaciens* strain GV2260 harboring the p35SGUSINT binary plasmid. When the bacterial suspension OD_{600} reached 0.8-0.9 it was centrifuged at 5000 rpm for 5 min and the pellet was resuspended in sterile half strength MS salts. After 72 hours of co-cultivation in light the explants were subcultured on shoot induction medium supplemented with 250 mg L^{-1} cefotaxime. After 3-4 days they were transferred to selection medium having 20.0 mgL^{-1} kanamycin with the same growth regulators. Subcultures were done at 10-day intervals. Explants developing shoots were transferred to shoot elongation media containing 0.5 mgL^{-1} BAP, 250 mgL^{-1} cefotaxime and 15.0 mgL^{-1} kanamycin. After 5-6 subcultures on shoot elongation medium, shoots with 2 internodes were cut and rooted on root induction medium having half-strength MS medium with 1.0% sucrose and 0.5% agar gel as an solidifying agent along with 250 mgL^{-1} cefotaxime and 10 mgL^{-1} kanamycin.

When the root system was well developed with 2-3 branched roots, the plants were removed from the magenta box and the agar was carefully removed from the roots by hand. The plants were transferred into a magenta box containing 20 ml of 1/10 liquid MS-salt solution. Magenta boxes with perforated lids were used to facilitate gas diffusion. Sterility was no longer necessary since the root system had formed and submerged in nutrient solution. The

magenta box was placed in the growth chamber (27°C, 16/8 hrs photoperiod and 50% relative humidity) for 2 days and then the plants were transferred into 10 cm diameter plastic pots containing a sterilized 1:1:1 mixture of soil: vermiculite: manure. The plantlets were covered with polythene bags. Two days later, holes were made in the polythene covers and the plantlets were watered daily with approximately 50 ml of distilled water for the first week. When the plants had four true leaves, they were transplanted into 20 cm diameter plastic pots and placed in the greenhouse. These plants were subsequently identified as the T_0 generation since they were developed entirely by tissue culture. Each plant that was produced represented an individual T_0 line. For p35SGUSINT lines, they were named Tg1, Tg2, etc. For osmotin-ferritin lines, they were named Tof1, Tof2, etc. The T_1 progeny osmotin-ferritin lines are represented by Tof 1.1, 2.1, 3.1, etc.

Assay for β-Glucuronidase (GUS)

GUS analysis was conducted according to the protocol by Jefferson *et al*. (1987). T_1 transgenic seedlings were incubated overnight at 37°C in X-gluc solution consisting of 10 mM EDTA, 100 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide and 0.1% X-gluc. Chlorophyll was removed by treating the tissue for 15 min in methanol before microscopic examination. Segregation of the GUS gene was determined by counting the number of GUS-positive versus GUS-negative seedlings.

Molecular analyses of transformants

Polymerase chain reaction

Total plant genomic DNA was isolated from leaves according to the method described by Doyle and Doyle (1990). The PCR was performed to screen putative transformant plants for the presence of the *gus* or *npt*II gene by using gene specific primers. The PCR reactions were carried out using 100 ng of purified genomic DNA as template and 2.5 U of recombinant *Taq* DNA Polymerase (Invitrogen, São Paulo). The 700 bp of the *npt*II fragment was amplified by using 21-mer oligonucleotide primers (*npt*II forward 5′-GAGG CTATTCGGCTATGACTG-3′ and *npt*II reverse 5′-ATCGGGAGC GGCGATACGTA-3′). The cycling conditions comprised an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min and a final extension of 3 min at 72°C. The 469 bp GUS fragment was amplified by using 22-mer oligonucleotide primers (GUS forward 5′-TACCTCGCAT TACCATTACGCG-3′ and GUS reverse 5′-TTCTCTGCCGTTTC CAAATCG-3′). Cycling conditions were similar as in PCR using *npt*II primers except for the annealing temperature at 62°C for 55 s. The amplified products were electrophoresed on 1.0% agarose gels (Invitrogen, CA) and visualized with ethidium bromide.

Southern hybridization

Genomic DNA (15 μg) from T_1 plants was separately digested to completion with *Hin*dIII or *Sma*I or *Eco*RI (MBI Fermentas, Germany) which release a cassette from the T-DNA region and the restriction fragments were resolved by electrophoresis on 0.8% agarose gels and blotted by capillary method onto Hybond N+ membrane (Amersham Pharmacia, UK) using 20X SSC as a transfer buffer. Probes with α-32P dATP labeled 700 bp *npt*II or gus or osmotin probes were made using Random Primer Labeling kit (MBI Fermentas, Germany) according to manufacturers instructions. Southern hybridization was carried out with a [^{32}P] dATP-labeled PCR amplified fragment of GUS or osmotin or ferritin. Following 16 h of hybridization at 65°C, membranes were washed for 20 min each at 65°C in 2X SSC, 0.1% SDS, 1X SSC, 0.1% SDS and finally with 0.1X SSC, 0.1% SDS for 10 min. The washed membranes were wrapped in saran wrap and subjected to autoradiography (Sambrook *et al*. 1989).

Western blotting

Total cellular protein (50 µg) from putatively transformed and untransformed control plants and were fractionated in SDS-PAGE (15% polyacrylamide) and transferred onto a PVDF membrane (Amersham, UK) using an electro-blotting apparatus as per the method described by the manufacturer (Bio Rad, UK). The membrane was blocked for 1 h (37°C) using 2% BSA in Tris buffered saline (TBS), pH 7.0. Further, it was incubated with rabbit anti-ferritin serum (1: 12000 dilution) overnight at room temperature. The membrane was washed with washing solution containing 0.1% Tween-20 in TBS for 10 min after each stage. Next the membrane was incubated in goat-rabbit IgG coupled to alkaline phosphatase (Bangalore Genei, India) for 1 h (37°C). Finally the membrane was developed in BCIP/NBT (Bangalore Genei, India) until the bands became visible.

RESULTS

Transformation of *B. juncea* with p35SGUS INT construct

This protocol describes an increase in transformation efficiency by optimizing parameters like bacterial OD, seedling age, preconditioning, co-cultivation periods and use of silver nitrate in the medium. *A. tumefaciens*-mediated transformation is described using cotyledonary petioles as explant tissue. Kanamycin was used a selectable marker, since the selectable marker gene in this work is *npt*II, which confers resistance to kanamycin. The binary vector p35SGUSINT was mobilized into *Agrobacterium* strain GV2260 and used for transformation and transient GUS expression was used as the basis for identifying the most appropriate conditions for transformation. Explant mortality

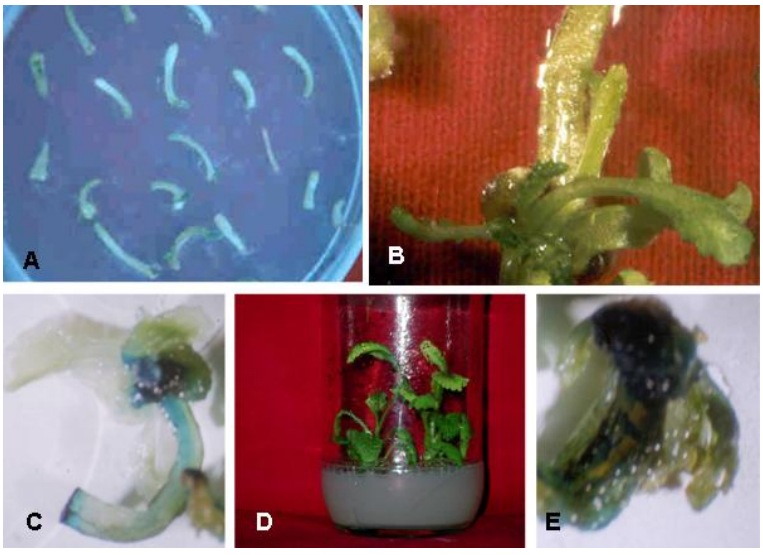

Fig. 2 Regeneration and *Agrobacterium* mediated transformation with cotyledonary petioles in *B. juncea* cv. 'Pusa Jaikisan' with p35SGUSINT. (**A**) Explants in shoot induction medium (2.0 BAP mgL^{-1} and 0.1 mgL^{-1} NAA). (**B**) Direct shoot regeneration from a cotyledonary explant. (**C**) Elongated shoots of the putative GUS transformants. (**D, E**) Transient GUS expression in the one week old cotyledonary explants.

Table 1 Effect of transformation conditions on mortality and GUS expression in cotyledonary explants exposed to GV2260 with p35SGUSINT.

Transformation parameters	Explant mortality % after 1 week (counting from transformation)	% Explants showing GUS after 1 week (counting from transformation)	Response of the explants
Bacterial culture OD at 1:10 dilution for 20 min			
OD_{600} = 0.7	59.6 ± 3.055 c	11.1	Bacterial overgrowth
Vacuum Infiltration for 10 min at OD_{600} = 0.6	82.7 ± 4.333 a	-	Bacterial overgrowth with tissue necrosis
OD_{600} = 0.4	27.3 ± 2.333 b	13.4	Reduced necrosis
Bacterial culture OD at 1:20 dilution for 20 min			
OD_{600} = 0.7	56.8 ± 4.372 a	12.3 ± 3.055 a	Bacterial overgrowth and subsequent mortality of the remaining healthy explants.
Vacuum infiltration for 10 min at OD_{600} = 0.6	76.2 ± 6.360 a	-	Tissue necrosis and mortality after 1 week
OD_{600} = 0.4	26.1 ± 2.404 b	14.6 ± 2.848 a	Very less bacterial overgrowth reduced necrosis.
Seedling age (days)			
3	89.0 ± 5.196 a	-	Hypersensitive response and mortality
6	23.0 ± 4.256 c	14.1 ± 1.528 a	Explants were firm with developing cells.
8	11.8 ± 1.764 b	9.2 ± 1.856 a	Explants were firm with developing cells.
Preculture period (days)			
0	64.0 ± 6.438 a	7.6 ± 1.202 b	Hypersensitive response and mortality
3	22.6 ± 3.283 c	15.1 ± 2.646 a	Explants were firm with developing cells.
5	5.3 ± 0.882 b	5.6 ± 2.186 c	Multiple shoots prior to *Agrobacterium* infection
Co-cultivation period (days)			
2	22.9 ± 3.786 b	10.1 ± 1.528 a	Decreased bacterial over growth.
3	24.1 ± 3.283 c	13.9 ± 1.155 b	Explant vigor with low necrosis.
4	58.0 ± 6.566 a	-	Bacterial overgrowth and subsequent mortality of the remaining healthy explants.
AgNO3			
3.3 $mgL^{-1}AgNO_3$ in the recovery medium after co cultivation	15.6 ± 3.283 a	14.9 ± 2.309 a	Explants vigor
Without $AgNO_3$	16.2 ± 2.186 a	14.6 ± 3.055 a	No difference in the explant vigor

Each mean value was an average calculated from three experiments ± SEM. Optimum growth regulator combination has been shown in bold face. Means followed by similar letters do not differ significantly at 95% confidence level (Sigma Stat Version 3.5).

and the number of explants expressing GUS were considered while standardizing the transformation parameters. Further, the optimized protocol was used to evaluate efficiency with another gene construct.

Exposure of cotyledonary petiole explants to an undiluted culture of *Agrobacterium* (>OD_{600} = 0.4) resulted in 100% severe necrosis of the explants. A diluted culture (1: 10 and 1: 20 dilution) reduced necrosis to a great extent compared to the exposure to undiluted culture (**Fig. 2A**). With respect to seedling age, it was observed that explants from 6-day-old seedlings performed better than 8-day-old seedling explants. The maximum GUS response was observed with 6-day-old seedlings (14.1%). A preconditioning time of 72 h was found to be optimal for high transformation efficiency. There was no improvement in transformation efficiency when silver nitrate was not used in the shoot induction medium with 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} NAA with 250 mgL^{-1} cefotaxime (**Table 1**).

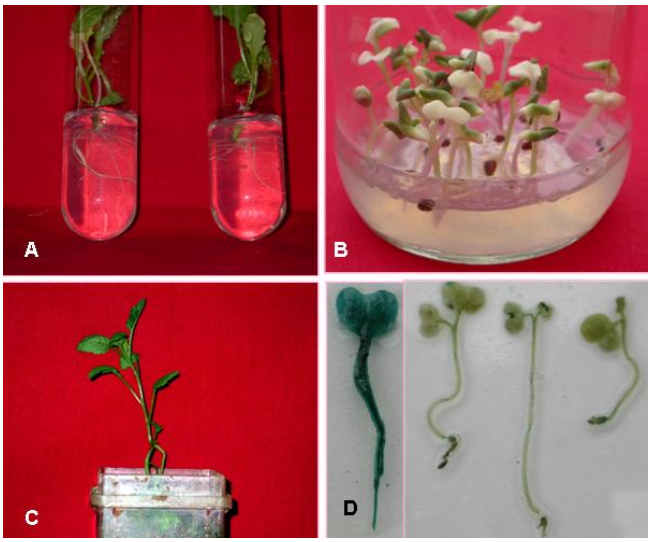

Fig. 3 ***B. juncea*** **(T_1) p35SGUS INT transformants showing GUS expression.** (**A**) Rooting of p35SGUS INT shoots. (**B**) Acclimatized transformant. (**C**) Progeny test of T_1 GUS seedlings D. Gus analysis on the p 35SGUS INT T_1 seedlings.

Out of the 500 explants infected with p35SGUSINT, 144 explants regenerated giving shoots (overall frequency of around 23%). Rooting is very efficient using half-strength MS medium vs. full-strength MS medium and reducing the sucrose concentration from 30 gL^{-1} to 10 gL^{-1} (**Fig. 3A**). The rooting medium resulted in 100% rooting in a short period of time (1-2 weeks). When full-strength medium was used the plants grew tall instead of producing roots, hence a low strength, low sugar medium that facilitates rooting was used. Of the regenerating explants, 8 putative transgenics with p35SGUS INT construct transferred to the greenhouse grew and flowered normally and set copious (approx. 50-60) seeds like non-transformed plants (**Fig. 3C**). Stable kanamycin-resistant lines were similarly recovered within 20 weeks' selection (maximum after 12-15 weeks) after *Agrobacterium*-mediated transformation. The optimized protocol was then used to mobilize the osmotin-ferritin gene into *B. juncea*.

Progeny analysis

The progeny of putative transformed plants were analyzed using PCR and Southern analysis for the presence of transgene(s). A total of five well established T_0 GUS transformants isolated through the described selection regime were subjected to molecular analyses. Transgene segregation of the five T_1 seedlings was noted by a progeny test. Seeds from primary transformants germinated in culture bottles in MS media grew normally with green color and produced secondary and tertiary leaves where as sensitive seedlings bleached at 100 mgL^{-1} kanamycin (**Fig. 3B**). A 3:1 Mendelian ratio was observed in the marker inheritance. GUS ana-

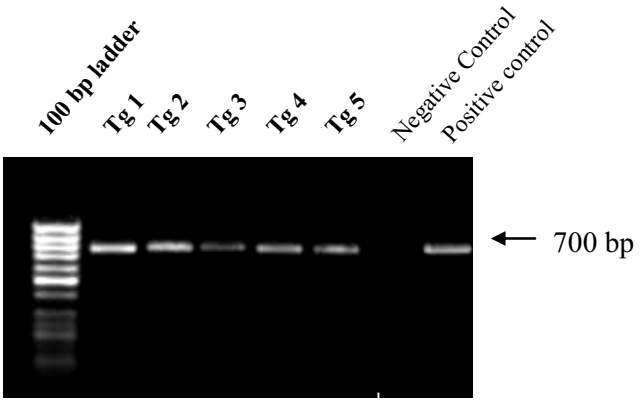

Fig. 4 PCR analysis of kanamycin resistant p35SGUSINT transformed, T_0 plants with *npt*II primers. Lane 1: 100 bp ladder. Lanes Tg 1, 2, 3, 4, 5: Transformed *B. juncea.* Lane 5: Negative control (non-transformed plant). Lane 6: Positive control (p35SGUSINT plasmid).

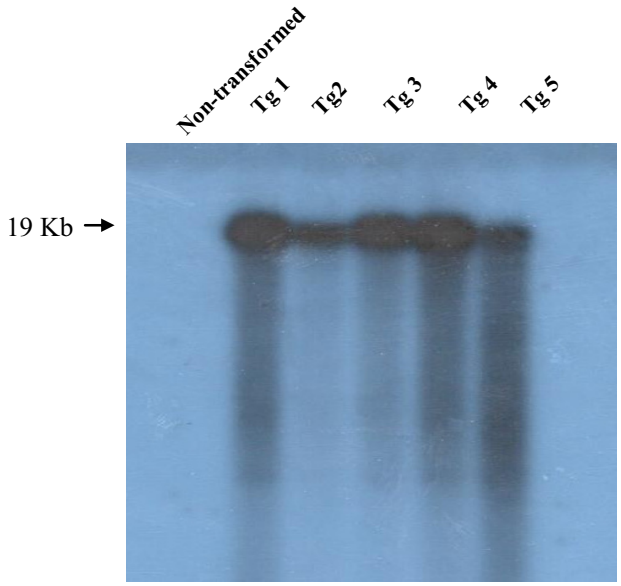

Fig. 5 Southern blotting hybridization pattern of DNA from T_0 transformed and non-transformed *B. juncea* with p35SGUSINT construct. Genomic DNAs (12 µg) were digested with *Hind*III restriction enzyme and probed with ^{32}P-labeled GUS fragment. λ DNA *Eco*RI/*Hind*III double digest was used as a molecular size marker. Lane 1: Negative control (non-transformed plant). Lanes Tg 1, 2, 3, 4, 5: Transformed *B. juncea* plants.

lysis of the progeny of the resistant seedlings on kanamycin medium indicated the expression of the GUS gene in the T_1 generation. T_1 transgenic seedlings showed blue coloration when incubated overnight at 37°C in X-gluc solution (**Fig. 3D**). PCR with *npt*II gene-specific primers amplified a 700 bp fragment respectively, from genomic DNA of kanamycin resistant shoots, thereby indicating the presence of the transgene in the regenerated plants (**Fig. 4**). Transformed plants of p35SGUS INT were examined for the presence of the T-DNA by Southern blot analysis. The PCR-amplified GUS fragment (700 bp) was used as a probe on the *Hind*III-digested genomic DNA detected a 19 Kb band in the transformants. The *Hind*III site present in the p35SGUS INT MCS releases the *Hind*III cassette thus excising out the GUS fragment as an intact band. The high molecular weight of the fragment detected might be due to the partial digestion of the genomic DNA (**Fig. 5**).

Osmotin-ferritin transformation

Transformation of *B. juncea* was done with *Agrobacterium* strain GV2260 (Deblaere *et al*. 1985) harboring the pCAMBIA 2300 osmotin-ferritin gene construct (**Fig. 6**) under optimized conditions. The optimized transformation parameters of the cotyledonary petioles were extended to the transformation of *B. juncea* with the osmotin-ferritin gene construct. pCAMBIA 2300 contains a kanamycin resistance (*npt*II) gene thus kanamycin was used as a selection marker for the transformants. Most of the shoots that regenerated turned white under kanamycin (20 mgL^{-1}) selection. Some shoots that developed fully with internodes on selection medium were transferred to rooting medium (half-strength MS medium) with 10 mgL^{-1} kanamycin. These shoots developed roots. Rooted plants were transferred to vermiculite and soil mix in a 1:1 ratio (**Fig. 7A-D**).

Hardened, well-rooted putative transgenic plants were transferred to soil, manure and vermiculite in a 1:1:1 ratio in magenta boxes and were transferred to bigger pots in the greenhouse (**Fig. 7C, 7D**). Four T_0 plants were grown in the greenhouse. Selfing of plants resulted in the progenies as the transgenic plants were fertile and normal in appearance. 10 seeds from each putative T_0 transformant and a control were cultured on solid half strength basal medium with 100 mgL^{-1} kanamycin. Seeds from T_0 transformants germinated and grew normally, green in color and produced secondary and tertiary leaves whereas sensitive seedlings became bleached. The analysis of the progeny for most of the transformants showed Mendelian inheritance of the resistance trait. Resistant seedlings were transferred to soil for subsequent studies.

Molecular analysis

From the DNA of T_0 *B. juncea* plants transformed with osmotin and ferritin genes a 700 bp fragment, corresponding to the expected size of *npt*II, was amplified in the transformants, including the positive control (pCAMBIA 2300 osmotin-ferritin) except (**Fig. 8**) in the control DNA from the untransformed plant. T_1 osmotin-ferritin transformed *B. juncea* and control plants were examined for the presence of the T-DNA by Southern blot analysis. Genomic DNA digested with *Sma*I when probed with the PCR-amplified osmotin fragment detected a 700 bp osmotin band (**Fig. 9**). *Eco*RI-digested genomic DNA probed with the PCR-amplified ferritin fragment detected a 900 bp ferritin band in the

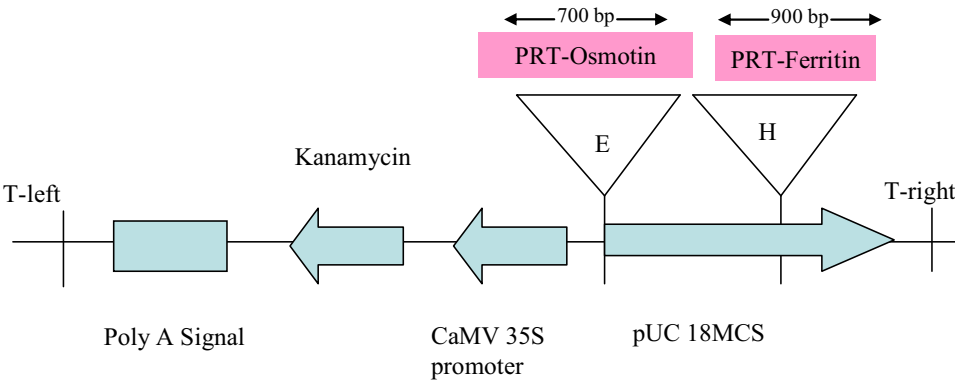

Fig. 6 Schematic representation of the plasmid pCAMBIA 2300 osmotin-ferritin employed in the study. The selective antibiotic resistant gene neomycin phosphotransferase (*npt*II) was driven by the constitutive CaMV 35S promoter. This plasmid pCAMBIA 2300 osmotin-ferritin was used for *Agrobacterium* mediated transformation of *B. juncea*. *Eco*RI site is disrupted due to the blunting of the cohesive ends of pCAMBIA 2300. E: *Eco*RI; H: *Hind*III.

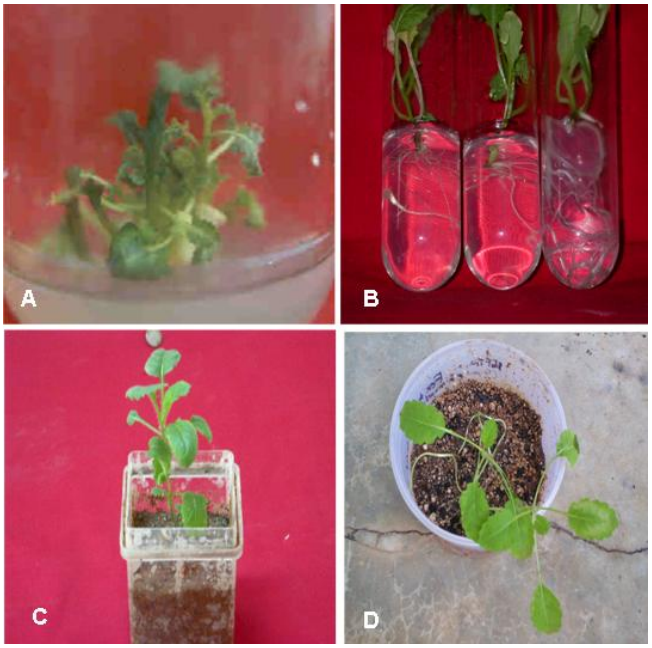

Fig. 7 *B. juncea* osmotin-ferritin transformants. (**A**) Elongated shoots developed from cotyledonary petiole explants after transformation on MS with 0.5 mgL^{-1} BAP. (**B**) Rooting of osmotin-ferritin transformed shoots. (**C, D**) Acclimatized osmotin-ferritin transformed plant.

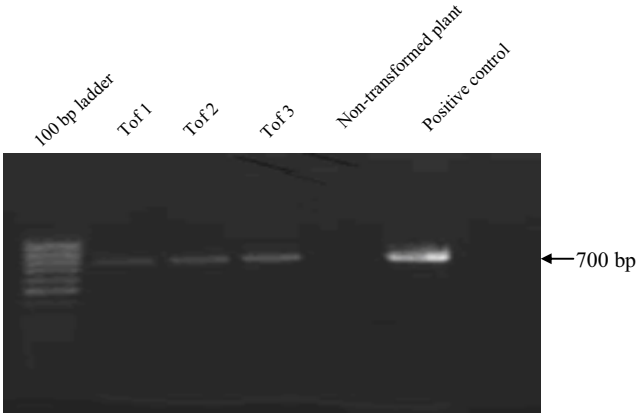

Fig. 8 PCR analysis of T_0 osmotin-ferritin transformed *B. juncea* showing the presence of 700 bp *npt*II fragment. Lane 1: 100 bp ladder. Lane Tof 1, 2, 3: Transformed *B. juncea* (Tof: Transformed osmotin ferritin). Lane 5: Negative control (non-transformed plant). Lane 6: Positive control (pCAMBIA2300 osmotin-ferritin).

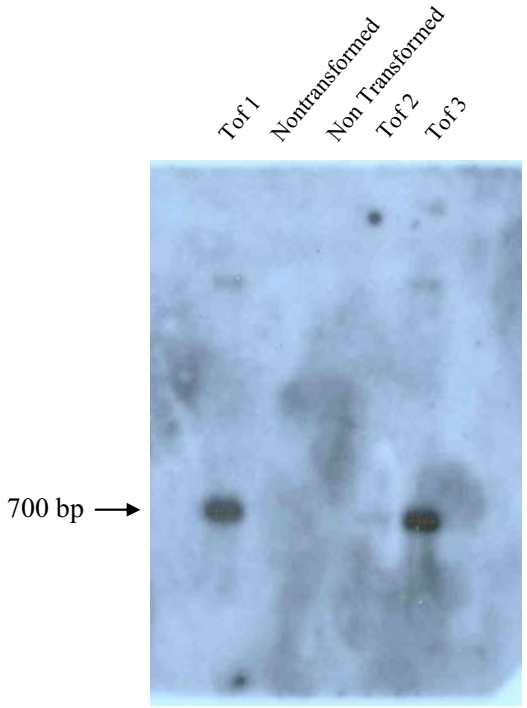

Fig. 9 Southern blotting hybridization pattern of DNA from T_1 transformed and a non-transformed *B. juncea* with osmotin-ferritin construct. Genomic DNAs (15 µg) were digested with *Sma* I restriction enzyme and probed with ^{32}P-labelled osmotin fragment. λ DNA *Eco*RI/*Hind*III double digest was used as a size marker. Lane 2, 3- Negative control (non-transformed plant). Lane Tof 1, 2, 3: Transformed *B. juncea* (Tof: Transformed osmotin ferritin).

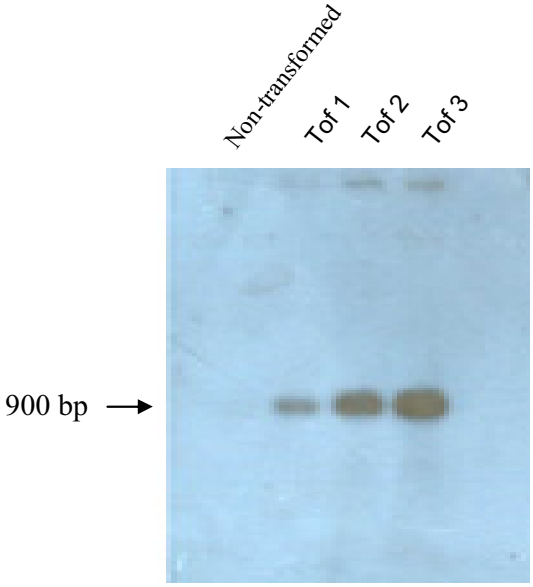

Fig. 10 Western blot analysis of osmotin from transformed leaves of *B. juncea* using osmotin antibody. Total soluble protein was extracted from leaves and resolved by SDS-PAGE and electro blotted onto a PVDF membrane. Lane 1: Negative control (non-transformed plant). Lanes 2, 3, 4: Transformed *B. juncea* (Tof 1.1, 2.1, 3.1; Tof: Transformed osmotin ferritin).

blot (**Fig. 10**). The *Eco*RI site is present in the pCAMBIA2300 MCS and in the *Hind*III cassette of PRT 100 thus excising out ferritin as an intact band. Total protein homogenates isolated from mature leaves of T_1 plants were analyzed by Western blot using anti-ferritin antibody and anti-osmotin antibody. The polyclonal antibody directed against the osmotin specifically detected a 25.0 kDa protein confirming the expression of the foreign *S. nigrum* osmotin-like protein in transformants (**Fig. 11**). The polyclonal antibody directed against the pea seed ferritin specifically detected 28.0 kDa and 26.5 kDa proteins confirming the expression of the foreign pea seed ferritin cDNA in transformants (**Fig. 12**). These proteins were not observed in the non-transformed control.

DISCUSSION

The concept of plant transformation combines components of plant tissue culture, regeneration and *Agrobacterium*-related parameters such as virulence induction, T-DNA activation, transfer and integration. With all these factors playing an important role it is obvious that establishing the optimal conditions for transformation are necessary (Poulsen 1996). Various factors influence the T-DNA delivery and stable transformation efficiency. Optimal conditions based on transient GUS expression were identified employing *Agrobacterium* strains carrying the binary vector p35SGUS-INT for transformation. A simple, swift, efficient transformation system using cotyledonary petioles has been optimized. Modifications to existing *Brassica* transformation protocols have been made and have resulted in improved transformation efficiencies. The use of binary vectors are

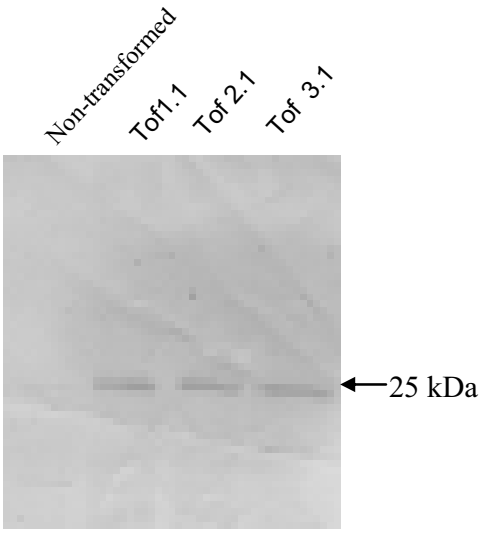

Fig. 11 Western blot analysis of osmotin from transformed leaves of *B. juncea* using osmotin antibody. Total soluble protein was extracted from leaves and resolved by SDS-PAGE and electro blotted onto a PVDF membrane. Lane 1: Negative control (non-transformed plant). Lanes 2, 3, 4: Transformed *B. juncea* (Tof 1.1, 2.1, 3.1; Tof: Transformed osmotin ferritin).

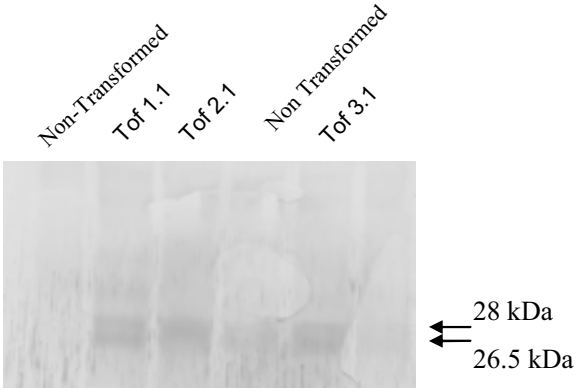

Fig. 12 Western blot analysis of ferritin from transformed leaves of *B. juncea* using pea seed ferritin. Total soluble protein was extracted from leaves and resolved by SDS-PAGE and electro blotted onto a PVDF membrane. Lane 1: Negative control (non-transformed plant), Lanes 2, 3, 5: Transformed *B. juncea* (Tof 1.1, 2.1, 3.1; Tof: Transformed osmotin ferritin).

often preferred to co-integrate vectors hence GV2260 harboring the pGUS35INT was employed. In *Brassica* transformation the most commonly used antibiotic resistance gene is neomycin transferase gene (*npt*II) from transposon *Tn5* which confers resistance towards some aminoglycosides such as kanamycin, neomycin, gentamycin and paromycin apart from genes like *hpt*II (hygromycin phosphotransferase), *dhfr* (resistance to methotrexate), *cat* (chloramphenicol acetyltransferase), *bar* (conferring resistance to bialophos and phosphoinothricin) and *pat* (phosphoinothricin acetyltransferase) genes. The level of selective agent applied depends on the type of explant and on the genotype to which it is applied. cv. 'Pusa Jaikisan' exhibited sensitivity at 20 mgL^{-1} kanamycin. A frequent observation is that lower levels of selectable markers result in more transformants but simultaneously allow more untransformed escapes. To ensure more number of the transformants after the initial stringent selection the concentration of the antibiotic was decreased in the shoot elongation medium to 15.0 mgL^{-1} and further in the rooting medium to 10.0 mgL^{-1}. The selected transformants were subjected to further analysis to confirm the transgenic nature. BAP was reported to be the most effective cytokinin in terms of the number of cultures forming shoots and the number of shoots forming per explant and is inversely proportional to the rooting response. The combination of auxin and cytokinin proved to be efficient in shoot formation. Thus both BAP (2.0 mgL^{-1}) and NAA (0.1 mgL^{-1}) were used for induction and then decreased BAP concentration (0.5 mgL^{-1}) for shoot elongation. Rooting was initiated in half strength MS medium without the use of auxins. It has been reported that the excised cotyledons of *B. juncea* may form adventitious shoots and roots at high frequencies under fairly simple conditions (Moloney *et al*. 1989; Sharma *et al*. 1990). So, cotyledonary petioles of cv. 'Pusa JaiKisan' were used to establish a simple transformation method. In most of the transformation studies, hypocotyls were used as a source of explants for achieving high frequency of transformation in *B. juncea.* The success rates in these investigations have varied but not very significantly. Barfield and Pua (1991) detected higher frequency of GUS activity in hypocotyls than in cotyledons while highest transformation frequency of the transgenic shoots was reported to be 9%. Mehra *et al*. (2000) transformed *B. juncea* var. RLM198 hypocotyl explants with *bar* constructs in MS medium containing NAA 1.0 mgL^{-1} and BAP 1.0 mgL^{-1} achieving a transformation percentage of 15%, 6% for different *bar* constructs used. Prasad *et al.* (2000) used pre-cultured hypocotyl segments of 5-day old *B. juncea* for transformation with bacterial *codA* gene. However in all the studies $AgNO_3$ was added as a prerequisite in shoot induction medium.

The efficiency of transformation using *Agrobacterium* is enhanced by preconditioning the explant on callus-inducing media before co-cultivation (Radke *et al*. 1988; Schroder *et al*. 1994). Since the GUS activity was higher in the 6-day explants, subsequent experiments were carried out with 6-day-old seedlings (**Table 1**). Cells could survive after *A. tumefaciens* infection with different bacterial ODs. Explants tend to become very sensitive to co-cultivation with a higher OD even to the extent that they become necrotic during subsequent cultivation. Co-cultivation period also plays a critical role in transformation with *Agrobacterium*. A co-cultivation period of 3 days was found to be optimal. Influence of co-cultivation period on *Agrobacterium*-mediated transformation has also been reported in a number of plant species (Mohan and Krishnamurthy 2003). Explant age, preculture period, bacterial strain and density were found to be some of the critical determinants of transformation efficiency. In general, our results confirmed earlier observations that lowering bacterial density (Henzi *et al*. 2000) and preculture of explant on callus inducing medium (Ovesna *et al*. 1993) help to improve transformation frequency. There was no enhanced transient GUS percentage when $AgNO_3$ is not used in the medium (**Table 1**). Many investigators reported the requirement of $AgNO_3$ for efficient regeneration and transformation (Pua and Chi 1993; Eapen and George 1997; Phogat *et al*. 2000). In the present study results corroborate that $AgNO_3$ is not always essential and does not always increase transformation and regeneration efficiency (Sethi *et al*. 1990; Yang *et al*. 1991; Radke *et al*. 1992). To evaluate the exact reason additional studies may be required but the cultivar specificity, its recalcitrant nature for transformation, explant source (Williams *et al*. 1990; Palmer 1992), age along with containers used for culture might play a role in this aspect. Increasing the agar concentration from 0.8% to 0.85% in the elongation medium, the water availability for the shoot is reduced which would otherwise make the plant hyperhydrated leading to decreased growth.

Eventually, an *A. tumefaciens*-mediated transformation system for *B. juncea* cv. 'Pusa Jaikisan' was developed using six-day old derived cotyledonary explants using GV2260 carrying a binary vector coding for *gus* and *npt*II. A total of 5 transgenic plants were produced in three independent experiments. In one of these experiments, starting with 100 explants, transgenic plants were recovered from three independent explants, giving a stable transformation frequency of 3% (stable transformation efficiency was cal-

culated based on the number of the Southern positive plants recovered to the initial number of explants taken). Also, it was found that constantly reducing the size of the calli by 2-3 millimeters during sub culturing and selection by chopping, enhanced the exposure to the selective agent kanamycin, improved the selection efficiency significantly. Molecular analyses established the transgenic status of the shoots recovered in selection medium. The T_0 transformants were genetically characterized by means of standard PCR, genomic DNA blot hybridization. Genomic DNA was digested with *Hind*III enzyme, which releases the GUS cassette (**Fig. 5**). The progeny analysis and GUS analysis of T_1 progeny also provided evidence for the incorporation of T-DNA into the *Brassica* genome. There was, however, no clear relation between number of transgene insertions and transgene expression *Brassica oleracea* botrytis var. Pusa Snowball K-1 (Chakrabarty 2002). The *npt*II and *gus* genes were inherited to the T_1 generations in a Mendelian fashion in most of the events. After self-pollination, GUS analysis of selected T_1 plants revealed that introduced marker genes were stably inherited to the next generation. These data demonstrate that morphologically normal, fertile transgenic plants of *B. juncea* cultivar can be achieved using cotyledonary petioles as explants without using $AgNO_3$. This protocol should have a broad application in the improvement of *B. juncea* cultivars by introduction of foreign genes.

Transient transformation and production of transgenic organisms are powerful ways in which to investigate the regulation of gene expression. Increasing transformation efficiency is desirable to decrease the amount of resources to produce transgenic plants, and to also potentially provide a higher baseline for subsequent transformation with other gene constructs. Various genes that improve the crops can be genetically engineered in Indian mustard using this transformation technique. The transient GUS assay approach was found to be an easy and reliable way of establishing optimal conditions for transformation. The results of transformation with an osmotin-ferritin gene construct have validated the contention that the transient GUS assay approach is a consistent method to optimize transformation conditions. Transgenic *B. juncea* plants over-expressing pea seed ferritin cDNA and *S. nigrum* osmotin like protein (SniOLP) under the control of CaMV35S promoter were generated (**Fig. 7C, 7D**). Genomic DNA was digested with *Sma*I, which releases the gene cassette in the transformant genomic DNA, the cassette was detected in transformants when probed with a PCR-amplified osmotin probe. A 900 bp cassette is detected in all transformants when genomic DNA is digested with *Eco*RI as the enzyme releases the intact ferritin fragment (**Fig. 9, 10**). Pea seed anti-ferritin antibody detected two sub-units whose predominant molecular weight was 28 and 26.5 kDa. Osmotin antibody detected a 25kDa band. The bands detected in the protein extracts of tranformants confirm that the pea seed cDNA in transgenic *B. juncea* plants is synthesized in the cytoplasm as a precursor whose N-terminal transit peptide is removed upon chloroplast uptake (van der Mark 1983b; Proudhon *et al*. 1989) and then the extension peptide is released (Ragland 1990; Lobreaux and Brait 1991). The bands detected in the protein extracts of T_1 transformants confirm that the pea seed ferritin cDNA and SniOLP are expressed in transgenic *Brassica* (**Figs. 11, 12**). The integration and expression of osmotin and ferritin genes in transgenic *Brassica* validated the transformation parameters that were optimized using GUS construct with cotyledonary petioles. The development of crops that have an in-built resistance to biotic and abiotic stress would help to stabilize annual production.

ACKNOWLEDGEMENTS

Financial assistance for green house facility from the Department of Biotechnology, Government of India, New Delhi Ref. BT/PR2273/PBD/17/117/2000 dt.7-9-01 (to MNVP as PI) and DST project A38/99 (to PBK) are gratefully acknowledged. The authors thank Prof. J. F. Briat, Laboratoire de Biochimie et Physiologie Moléculaire des Plantes, CNRS, INRA, France for the generous gift of many reprints, methodologies and pea seed ferritin cDNA that helped us to conduct research on phytoferritins.

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