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Variation amongst *Brassica juncea* cultivars for regeneration from hypocotyl explants and optimization of conditions for *Agrobacterium*-mediated genetic transformation

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Summary. Twelve cultivars of *Brassica juncea* grown in different agroclimatic regions of the world were tested for their ability to regenerate *in vitro* from hypocotyl explants and, accordingly, were divided into three groups. One group of cultivars regenerated on MS medium supplemented with 2,4-D, BAP and with NAA, BAP combinations; another group regenerated only on MS with 2,4-D, BAP; and the third group showed very low regeneration on both of these combinations. Inclusion of silver nitrate in the medium was essential for high frequency of regeneration. In general, Indian cultivars were more responsive than the cultivars of CIS and Australian origin. Using the media optimal for regeneration and an *Agrobacterium*-based binary vector carrying *hpt* and *gus*-intron genes, conditions for genetic transformation of *B. juncea* hypocotyl explants were optimized. Transformation frequencies, identified by GUS staining at the initial stages of growth, were lower on MS medium with 2,4-D, BAP than on MS with NAA, BAP. Plants resistant to 20 μg/ml hygromycin were regenerated at a frequency of 11-36% from hypocotyl explants and were shown to be transformed by Southern blotting, GUS staining and progeny analysis.

Key words : *Brassica juncea*, hypocotyl, plant regeneration, *Agrobacterium*, genetic transformation

Introduction.

Brassica juncea (L.) Czern and Coss is a major oilseed crop of the Indian subcontinent and is also grown in Australia, China, CIS and Canada. Extensive variation in morphological and agronomic characters has been observed amongst *B. juncea* cultivars (Vaughan et al., 1963; Pradhan et al., unpublished). The cultivars (cvs) also differ in their ability to regenerate shoots *in vitro* from cotyledons (Fazekas et al., 1986), hypocotyl (Kirti and Chopra, 1989) and stem cortex explants (Bonfils et al., 1992). Furthermore some of the important cultivars have been shown to be recalcitrant to regeneration.

Cotyledonary-petioles of *B. juncea* are a good source of explants for shoot morphogenesis (George and Rao, 1980; Fazekas et al., 1986; Jain et al., 1988; Chi et al., 1990; Sharma et al., 1990). However, this regeneration system is not amenable to high frequency *Agrobacterium* mediated genetic transformation (Mathews et al., 1990). Amongst *Brassica* species only successful report of high frequency transformation of cotyledonary-petiole explants relates to *B. napus* (Moloney et al., 1989). In *B. campestris* cotyledonary-petiole explants were shown to be refractory to transformation and only in rare cases some chimeric transformed plants were obtained (Mukhopadhyay et al., 1992). In contrast, hypocotyl explants could be readily transformed with *Agrobacterium*-based vector systems (Mukhopadhyay et al., 1992; Radke et al., 1992). Effective transformation systems with hypocotyl explants have also been reported for *B.napus*, *B. oleracea* (DeBlock et al., 1989) and *B. juncea* (Barfield and Pua, 1991).

For regeneration and transformation of *B. juncea*, a condiment type cv named India mustard was used (Barfield and Pua, 1991). The present study was undertaken to explore extent of variation in shoot morphogenesis in hypocotyl explants of some agronomically important Indian and exotic oilseed cultivars of *B. juncea*. This information was

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subsequently used to optimize conditions for genetic transformation of *B. juncea* cultivars by an *Agrobacterium* based binary vector carrying *hpt* gene and *gus*-intron gene.

Materials and methods

Vector construction. Agrobacterium based binary vector pGSFR780A (DeBlock et al., 1989) was modified to introduce the *hpt* and *gus*-intron genes (Vancanneyt et al., 1990). Plasmid pGSFR780A was cut with *Sna* BI and *Nru* I to remove a 3994 bp fragment carrying both the *bar* and the *npt* genes. Plasmid pRT100HPT (Topfer et al., 1987) was cut with *Hind* III to release a 1694 bp *hpt* gene fragment that included both the CaMV35S promoter and CaMV polyadenylation signal sequences. This fragment was made blunt-end with DNA polymerase I and ligated to an 8330 bp *Sna* BI, *Nru* I fragment of pGSFR780A. The ligated mixture was used to transform *E. coli* HB101 with calcium chloride treatment. Recombinant clones were picked and characterized for the presence of the insert by digesting DNAs with *Hind* III and *Eco* RV. Recombinant plasmid with *hpt* gene, named pJB90, is about 10 kb in length. A restriction map of pJB90 for the region between border sequences is shown in Fig. 1. The plasmid p35S-GUS INT (Vancanneyt et al., 1990) was cut with *Hind* III to release *gus*-intron gene including the CaMV35S promoter and CaMV poly-adenylation signal sequences. This fragment was ligated at the *Hind* III site of plasmid pJB90 to construct plasmid pJB90GI which is about 12.7kb in size. This plasmid was used to transform *A. tumefaciens* strain pGV2260 (Deblaere et al., 1985) by electroporation (Mattanovich et al., 1989). The transformed strain was checked for the presence of the correct binary vector.

Plant material and tissue culture. B. juncea cultivars (RLM 198, RLM 514, RLM 619, RC781, Kranti, RLC 1359, Varuna, Pusa Bold, RH30 from India; Skorospieka II, Donskaja IV from CIS and Zem II from Australia) were maintained by selfing. Seeds of different cultivars selfed for 4-5 generations were germinated aseptically in glass bottles (10-12 seeds per bottle) on MS (Murashige and Skoog, 1962) medium in dark for 2 d, then transferred to light (200μEm-2S^{-1}, 10h light, 14h dark) and maintained at 25±1°C. Hypocotyl segments (0.5-1.0 cm) were obtained from 5 d old seedlings and 45-50 explants were laid lengthwise in 90mm plastic petridishes (Tarsons, Calcutta) containing various media solidified with 0.8% agar (HiMedia, Bombay). Silver nitrate was filter sterilized and added to the autoclaved media. Regeneration frequencies were observed at different time intervals.

Agrobacterium mediated transformation. Agrobacterium tumefaciens strain pGV2260 carrying the plasmid pJB90GI was grown overnight in 5 ml of liquid YEB medium containing 50 mg/l rifampicin and 100 mg/l spectinomycin. Five ml of fresh medium was added to this culture which was incubated for another 3-4 h. Bacteria were pelleted (2100 g) and suspended in liquid MS medium. The OD of the bacterial suspension was adjusted to around A_{600}=0.6. Explants plated on the medium were removed and incubated in the bacterial suspension for 10 min. Excess fluid was removed by placing explants on a filter paper and subsequently explants were replated. Silver nitrate was not included in co-cultivation media. After 1 d, explants were transferred to media containing silver nitrate and 200 μg/ml claforan. After 3-4 d of growth explants were plated on shoot regeneration media with 20 μg/ml hygromycin and 20 μM $AgNO_3$. Green plantlets that differentiated on selection media were transferred to MS with 2 mg/l IBA for rooting.

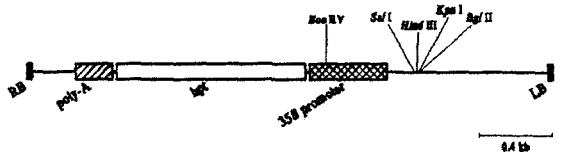

Fig. 1 : Map of the plasmid pJB90 for the region between right border and left border. Unique restriction sites in the plasmid for cloning of a passenger gene are marked.

Confirmation of genetic transformation. For studying the extent of transformation at early stages, 14-16 d old cultured explants were stained for GUS activity (Jefferson, 1987). Leaves and internodes of putatively transformed shoots were also hand-sectioned and stained for GUS activity. Transformed plants were grown in soil in a plant growth chamber. Total DNA was isolated from leaves of putative transformants following Dellaporta et al. (1983). DNAs were purified on cesium chloride density gradients. For Southern hybridization, DNAs were digested with *Hind* III in the presence of 0.01 M spermidine. Digested DNAs were run on 0.8% agarose gels (20 μg DNA in each lane) and were processed as reported earlier (Mukhopadhyay et al., 1991). For detecting *hpt* gene in transformed plants, a *Bam* HI fragment of *hpt* gene was used as a probe (Mukhopadhyay et al., 1991).

Results

Media optimization and extent of variability for regeneration

Hypocotyl explants of two cvs, RLM 198 and Pusa Bold, the former reported to be amenable and the latter recalcitrant to regeneration(Kirti and Chorpa, 1989), were inoculated on a number of media (Kirti and Chopra, 1989; Chi et al., 1990; Table 1). Hormone combinations were tested with and without 20 μM silver nitrate (Table 1). Inclusion of silver nitrate in the media considerably enhanced the extent of regeneration in all the hormone combinations (Table 1). Medium containing BAP 1mg/l and NAA 1mg/l was optimal for cv RLM 198 but combinations of these two hormones consistently gave very low frequencies of regeneration from cv Pusa Bold. In comparison, the BAP 1 mg/l, 2,4-D 0.05 mg/l combination induced high frequency of shoot morphogenesis in both the cultivars. Inclusion of silver nitrate in MS medium containing 2,4-D 0.25 mg/l, NAA 1 mg/l, BAP-R (6-benzylaminopurine riboside) 0.5 mg/l led to regeneration of shoots directly on this medium in cv RLM 198; transfer to MS, BAP 0.2 mg/l medium as reported earlier (Kirti and Chopra,1989) was not required. However, these shoots remained stunted. Regeneration frequency for Pusa Bold was poor on this medium, even in the presence of silver nitrate.

Two of the media given in Table 1, one with BAP 1mg/l, NAA 1mg/l (MSB1N1) and the other with BAP 1mg/l, 2,4-D 0.05 mg/l (MSB1D.05), were chosen for

Table 1. Shoot regeneration frequency of hypocotyl explants of two *Brassica juncea* cvs. RLM 198 and Pusa Bold on MS medium with different adjuvants. Observations were taken 30d after the initiation of experiments.

Media : MS+hormones(mg/l)+$AgNO_3$(μM)	cv RLM 198		cv Pusa Bold	
	No. of explants	Regeneration %	No. of explants	Regeneration%
BAP 1 + NAA 1	101	9.8	43	0
BAP 1 + NAA 1 + $AgNO_3$ 20	84	72.6	87	5.7
BAP 2 + NAA 1	89	5.6	39	0
BAP 2 + NAA 1 + $AgNO_3$ 20	35	68.6	80	10
BAP 4 + NAA 1	95	4.2	42	0
BAP 4 + NAA 1 + $AgNO_3$ 20	54	55.6	107	5.6
BAP 1 + 2,4-D 0.05	87	0	103	0
BAP 1 + 2,4-D 0.05 + $AgNO_3$ 20	44	95.5	80	92.5
BAP 5 + NAA 1 + 2,4-D 0.25	92	0	40	0
BAP-R 0.5 + NAA 1 + 2,4-D 0.25 + $AgNO_3$ 20	92	57.6	43	13.9

Table 2. Frequency of shoot regeneration from the hypocotyl explants of twelve cultivars of *Brassica juncea* on two different media. Observations were taken 30 d after the initiation of experiments

Cultivar	Medium MSB1N1[a]				Medium MSB1D.05[a]			
	-$AgNO_3$		+$AgNO_3$20μM		-$AgNO_3$		+$AgNO_3$20μM	
	No of explants	regeneration (%)	No of explants	regeneration (%)	No of explants	regeneration (%)	No of explants	regeneration (%)
RLM 198[b]	453	9.3	469	77.4	87	0	44	95.5
RC 781	NT	-	118	44.1	NT	-	87	71.3
Kranti	82	2.4	249	24.9	NT	-	123	41.5
RLC 1359	118	0	172	37.2	96	0	164	39.6
RLM 514	40	0	204	35.3	78	0	239	25.1
RLM 619	250	0.4	246	66.7	78	0	197	30.5
Varuna	71	9.9	162	28.4	NT	-	122	81.1
Pusa Bold	43	0	126	7.1	103	0	80	92.5
RH 30	83	0	42	4.8	NT	-	114	60.5
Skorospieka II	87	0	282	20.9	NT	-	117	49.6
Donskaja IV	NT	-	41	4.9	NT	-	115	53.9
Zem	85	0	135	5.9	NT	-	189	6.3

[a] MSB1N1, MS medium with BAP 1mg/l and NAA 1mg/l MSB1D.05, MS medium with BAP 1mg/l and 2,4-D 0.05mg/l.

[b] Regeneration from cv RLM 198 was studied in three experiments. The number of explants in each experiment was 84, 133 and 252 (total 453). Percentage regeneration in each experiment was 73, 75 and 80 giving an average percentage of 76.0±3.6. Rest of the cultivars were tested in one or two independent experiments. Cultivar RLM 198 was grown as a control in each of the experiments.

NT, not tested.

studying shoot regeneration from hypocotyl explants of twelve Indian and exotic cultivars (Table 2). All the cultivars regenerated very poorly on these two media in the absence of silver nitrate (Table 2). Of all the tested cultivars, RLM 198 regenerated at highest frequencies on both BAP, NAA and BAP, 2,4-D media. Cultivars RC 781, Kranti, RLC 1359, Varuna, RLM 514, RLM 619 and Skorospieka II also showed high levels of regenration on both NAA and 2,4-D containing media (Table 2). Pusa Bold,RH 30 and Donskaja IV showed high frequencies of regeneration on BAP, 2,4-D combination and regenerated only at a low frequency on BAP, NAA combination (Table 2). Shoot regeneration on BAP,NAA medium occurred only from the basal segment (nearest to the root) of the hypocotyl. In three independent experiments only the basal segments of cv Pusa Bold were plated and were observed to regenerate at a frequency of 39% (10 out of 22, 6 out of 12 and 8 out of 36 explants) as compared to regeneration frequency of 7.1% (Table 2) when all the hypocotyl segments of a seedling were cultured. Cultivar Zem regenerated poorly on both the media (Table 2).

Table 3. Transformation frequency as detected by GUS staining of hypocotyl explants for *gus*-intron gene in three cultivars of *Brassica juncea*

Cultivar	Exp. No.[a]	Medium[b]	No.of cut ends	Staining (%)
RLM 198	1	MSB1N1	86	60.5
		MSB1D.05	82	3.0
	2	MSB1N1	110	43.6
		MSB1D.05	258	7.0
	3	MSB1N1	180	45.0
		MSB1D.05	252	11.9
	4	MSB1N1	348	16.7
		MSB1D.05	374	0.8
Pusa Bold	1	MSB1N1	266	19.2
		MSB1D.05	220	5.9
	2	MSB1N1	278	34.9
		MSB1D.05	318	1.9
	3	MSB1N1	240	18.3
		MSB1D.05	228	3.1
	4	MSB1N1	248	15.3
		MSB1D.05	374	4.5
Skorospieka II	1	MSB1N1	130	16.9
		MSB1D.05	122	0.8

[a] Observations were taken 15-16 d after co-cultivation with *Agrobacterium*

[b] MSB1N1, MS medium with BAP 1mg/l and NAA 1 mg/l; MSB1D.05, MS medium with BAP 1 mg/l and 2,4-D 0.05 mg/lA

Table 4. Frequency of transformation of *Brassica juncea* cv RLM 198 measured as the percentage of hypocotyl explant cut-ends showing expression of *gus*-intron gene on two selection media and frequency of explants producing transformed shoots

Exp. no.	Medium for selection*	No. of cut-ends	% of cut -ends staining +ve for GUS	No. of explants	% of explants producing transformed shoots
1.	MSB1N1	110	43.6	67	36
	MSB1D.05	96	36.5	74	24
2.	MSB1N1	96	49.0	74	11
	MSB1D.05	110	58.2	293	20
3.	MSB1N1	84	40.5	--	--
	MSB1D.05	92	37.0	--	--

* Explants were co-cultivated with *Agrobacterium* on MSB1N1 medium for 1 d, transferred to MSB1N1 with claforan for 3 d and subsequently transferred to either MSB1N1 or MSB1D.05 medium with 20μg/ml hygromycin

Different cultivars also showed variation in their regeneration pattern and in number of shoots emerging from each explant. In cultivars Zem, Donskaja IV, Skorospieka II and RH 30, explants regenerated 1-3 shoots from unorganized growth at the cut end. In

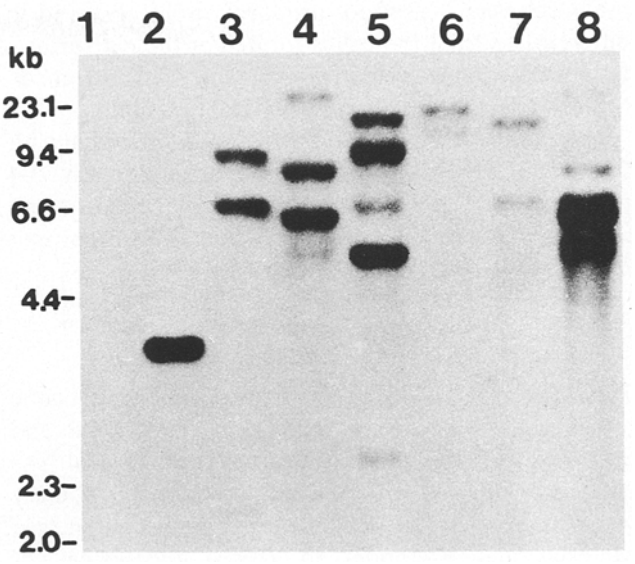

Fig. 2 : Southern blot analysis of total DNAs isolated from seven independent transformed plants of *Brassica juncea* cv RLM 198. DNAs were digested with *Hind* III and probed with structural part of *hpt* gene. Lane 1, DNA from a non-transformed plant; lane 2-8, DNAs of transformed plants. Only one transformed plant had a single insertion site (Lane 2)

Table 5. Segregation of hygromycin resistant (R) and sensitive (S) plants in the selfed progeny of transformed *B. juncea* cv RLM 198 plants

Plant No.	No. of vector DNA insertions	No. of germinated seeds[b]	Seedling type R	Seedling type S	χ^2 value for 3:1 ratio
Culture	None	135	0	135	
Hm3[a]	1	10	4	6	6.53*
Hm7	NA	21	16	5	0.02
Hm11	NA	24	22	2	3.56
Hm17[a]	2	27	26	1	6.53*
Hm18	NA	77	62	15	1.25
Hm20[a]	3	44	44	0	14.67*
Hm21[a]	5	13	11	2	0.64
Hm30	NA	8	5	3	0.67

NA Not analysed by Southern hybridization

[a] Southern blot analysis for number of vector DNA insertions in transformants - Hm3, Hm17, Hm20 and Hm21 is shown in lanes 2,3,4 and 5, respectively, of Fig. 2

[b] Media used : MS+20μg/ml hygromycin

* Shows significant deviation from 3:1 ratio at the 0.05 level of probability

other cultivars 1-12 shoot primordia were observed to differentiate from the callus formed at the cut surface. In general, regenerated shoots elongated better on MSB1N1 medium than on MSB1D.05 medium.

Regenerated shoots were rooted on MS medium with 2 mg/l IBA. A number of passages (usually 3-4 passages of 3 wk duration) on this medium were required to produce sturdy shoots with proper unvitrified leaves.

Genetic transformation.

To develop conditions for high frequency genetic transformation, cultivars RLM 198, Pusa Bold and Skorospieka II were used as test material. Explants co-cultivated with the *Agrobacterium* strain carrying binary vector pJB90GI were incubated in x-gluc solution (see Material and Methods) to record the extent of transformation in the early stages of growth. In the three cultivars frequency of transformation as revealed by GUS staining was considerably higher in explants grown on MSB1N1 medium than those grown on MSB1D.05 medium (Table 3). However, if the explants were co-cultivated with the bacterial strain on MSB1N1 medium, followed by transfer to MSB1N1 with claforan for 3 d and subsequently to MSB1D.05 medium, the transformation frequencies were comparable to those recorded on MSB1N1 medium (Table 4).

Cultivar RLM 198 showed higher frequency of regeneration of transformed plants with vector pJB90GI (Table 4) when MSB1N1 medium was used for continuous culture or at least for the initial infection stages followed by culture on MSB1D.05 (Table 4). A total of 30 independent transformed shoots (one shoot from each explant), obtained from transformation experiments with cv RLM 198, were transferred to MS medium with 2 mg/l IBA and 20 μg/ml hygromycin (lethal concentration for nontransformed shoots). Rooted plants were subsequently grown on MS with IBA 2mg/l and finally transferred to soil in a growth chamber. Southern blotting of seven plants showed the presence of *hpt* gene in all the tested plants (Fig.2). Transformed plants were grown to maturity and were fertile. Progeny analysis for resistance to hygromycin (20μg/ml) was done with selfed seed of eight independent transformed plants. All the plants,with the exception of one that only showed resistant types, segregated for resistant and sensitive types. In a number of transformed plants segregation values deviated from 3:1 ratios expected for single gene inheritance (Table 5). These differences could be either due to presence of multiple transcriptionally active insertions (eg. Hm 17 and Hm 20 plants) or due to small sample size (eg. Hm3 plant).

Discussion

Our results show that there is extensive variation within *B. juncea* cultivars for regeneration from hypocotyl explants. On the basis of their qualitative response to two auxins, *B. juncea* cultivars can be divided into two groups: one group of cultivars (RLM 198, RC 781, Kranti, RLC 1359, RLM 514, RLM 619, Varuna, Skorospieka II) is responsive to both NAA and 2,4-D and the other group (Pusa Bold, RH 30, Donskaja IV) is only responsive to 2,4-D. There may be another group of cultivars represented by Zem in this study that show very low regeneration on either of the two auxins.

Explants of cv RLM 198, Pusa Bold and Skorospieka II show significant differences in transformation frequencies on MSB1N1 and MSB1D.05 medium (Table 3). The problem of low infectivity on MSB1D.05 medium for genetic transformation of cultivars like Pusa Bold, that only regenerate on this medium, can be circumvented by initial co-cultivation on MSB1N1 medium followed by the transfer of explants to MSB1D.05 medium for selection. Another way around the problem is to use the basal segment of hypocotyl for both co-cultivation and selection on MSB1N1 medium. Using the latter approach for Pusa Bold, transformed shoots were regenerated from explants at a frequency of 6.6% (unpublished results).

Both *npt* (Barfield and Pua, 1991) and *hpt* (this study) genes can be used to recover transformed plants in *B. juncea*. However, the transformation frequencies reported with pJB90GI using *hpt* as selectable marker gene are higher (11-36%) than those reported earlier (Barfield and Pua, 1991) using *npt* gene (7.1%). In transformations with *npt* gene (Barfield and Pua, 1991) non transformed escapes occurred at a very high frequency of 68%. No such escapes were observed in transformation with *hpt* gene.

Methods of genetic transformation established in this study could be used to develop transgenics of agronomically important *B. juncea* cultivars for improved oil quality and for resistance to diseases and insect pests. Some of the cultivars tested for regeneration in this study are very good combiners for yield eg. Skorospieka II (CIS) x RH30 (India) and Donskaja IV (CIS) x Varuna (India) (data not reported here). These cultivars could be transformed with barnase, barstar constructs (Mariani et al., 1990, 1992) for heterosis breeding. Further work may be needed on cultivars such as Zem in which regeneration frequencies are probably too low for efficient recovery of transformed plants.

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