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Original Paper

An improved method for *Agrobacterium*-mediated genetic transformation from cotyledon explants of *Brassica juncea*

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Abstract An efficient *Agrobacterium*-mediated genetic transformation method was established for *Brassica juncea* by investigating several factors responsible for successful gene transfer. Four-day-old cotyledon explants from *in vitro* grown seedlings were co-cultivated with *Agrobacterium* strain GV3101 harboring the binary vector EnPCAMBIA1302-YCF1, which contained the hygromycin phosphotransferase (*HPT*) gene as a selectable marker and the yeast cadmium factor 1 (*YCF1*) gene. Two days co-cultivation period on shoot induction medium (MS medium supplemented with 0.1 mg l^{-1} α-naphthaleneacetic acid, 1.0 mg l^{-1} 6-benzyladenine, and 2.0 mg l^{-1} silver nitrate) containing 20 mg l^{-1} acetosyringone and five days delaying exposure of explants to selective agent enhanced transformation efficiency significantly. A three-step selection strategy was developed to select hygromycin resistant shoots. Hygromycin-resistant shoots were subsequently rooted on root induction medium. Rooted plantlets were transferred to pot-soil, hardened, and grown in a greenhouse until maturity. Using the optimized transformation procedure, transformation efficiency reached at 16.2% in this study. Southern blot analysis was performed to confirm that transgenes (*HPT* and *YCF1*) were stably integrated into the plant genome. All transgenic plants showed single-copy of transgene integration in the host genome. Segregation analysis of T_1 progeny showed that the transgenes were stably integrated and transmitted to the progeny in a Mendelian fashion.

Key words: Cotyledon explants, co-cultivation, genetic transformation, hygromycin, Indian mustard.

Indian mustard (*Brassica juncea* L.) is an important oilseed crop throughout the world. Recently, it has drawn attention of researchers due to its exceptional capacity of tolerating and accumulating heavy metals such as As, Cd, Ni, and Pb from soil (Clemente at al. 2005; Gasic and Korban, 2007; Pilon-Smits et al. 2005). The ability of heavy metal tolerance and accumulation, however, could be significantly increased through transformation with specific genes capable of conferring the ability to detoxify heavy metals. Therefore, an efficient and reproducible genetic transformation system of *B. juncea* is required. *Agrobacterium*-mediated gene transfer is known as the preferable method mainly because of the increased chance for single copy transfer of target genes to transgenic plants (Hiei et al. 1997). Multiple copy transfer often leads to gene silencing (Bhalla et al. 2008).

There are several reports on *B. juncea* transformation with the respect to the introduction of various new traits such as modified oil composition (Das et al. 2006; Kanrar et al. 2006), pod shatter-resistance (Qstergaard et al. 2006), salt tolerance (Prasad et al. 2000), herbicide tolerance (Bisht et al. 2004), insect resistance (Cao et al. 2008; Dutta et al. 2005), and heavy metal resistance (Gasic and Korban 2007; Pilon-Smits 2005; Zhu et al. 1999). However, most of these reports were based on hypocotyl explants with the only exception of leaf segment explants (Dutta et al. 2008), in which transformation efficiency was very low. Although cotyledon explants responded better for high frequency shoot regeneration than hypocotyl and leaf explants of *B. juncea* in tissue culture (Bhuiyan et al. 2009), *Agrobacterium*-mediated transformation based on cotyledon explants was lacking.

In this report, we describe a consistent and efficient

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Agrobacterium-mediated genetic transformation system for *B. juncea* using cotyledon explants from *in vitro* grown seedlings. Using this standardized protocol, the yeast cadmium factor 1 (*YCF1*), an important ATP-binding cassette (ABC) transporter gene was successfully introduced into *B. juncea* with high transformation efficiencies.

Materials and methods

Plasmid vector construction

The recombinant plasmid vector EnPCAMBIA1302-YCF1 containing hygromycin phosphotransferase (*HPT*) and yeast cadmium factor 1 (*YCF1*) genes (Song et al. 2003) was introduced into *Agrobacterium tumefaciens* strain GV3101 by heat-shock method (Bowyer 2001). The *HPT* gene confers resistance to the antibiotic hygromycin as plant selection marker and the *YCF1* gene was introduced into the vector as a target gene with the aim of enhancing heavy metal tolerance and accumulation.

Media preparation

The media used in this study except YEP were based on MS (Murashige and Skoog 1962) medium with 3% (v/v) sucrose. The pH of the medium was adjusted to 5.7 before the addition of Phytagar ($6\,g\,l^{-1}$) and autoclaved at 121°C for 15 min. Acetosyringone (AS), silver nitrate ($AgNO_3$) and antibiotics, such as kanamycin, hygromycin, and cefotaxime were filter-sterilized and added to media cooled to 50–60°C after autoclaving.

Explant preparation

Brassica juncea L. cv. Rai-5 seeds were provided by Mr. Nazim-ud-Dowla (Scientific officer, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh). Seeds were surface-sterilized according to Bhuiyan et al. (2009). Approximately, 15 seeds were placed in 100×40 mm Petri dishes, each containing 50 ml germination medium (1/2MS agar medium containing $20\,g\,l^{-1}$ sucrose) and maintained at 25°C in 16-h light/8-h dark photoperiods at a light intensity of approximately $30\,\mu mol\,m^{-2}\,s^{-1}$ from cool-white fluorescent lamps. Cotyledons including 1–2 mm petioles were carefully excised from 4-day-old seedlings and used as explants in transformation experiments.

Suitable concentration of selective agent (hygromycin) for transformant selection

To determine the effect of hygromycin concentrations on shoot regeneration, cotyledon explants from 4-day-old *in vitro*-grown seedlings were placed on shoot induction medium (MS medium supplemented with $0.1\,mg\,l^{-1}$ NAA, $1.0\,mg\,l^{-1}$ BA, and $2.0\,mg\,l^{-1}$ $AgNO_3$) (Bhuiyan et al. 2009) with hygromycin (0, 5, 10, 15, 20, or $30\,mg\,l^{-1}$) in Petri dishes (90×15 mm; 30 ml medium) and the cultures were maintained at previously described conditions. Two replicates with 30 explants each were used. The regeneration response were evaluated under the selection conditions after 3 weeks of culture *in vitro*.

Agrobacterium-mediated transformation and regeneration

A single colony of *A. tumefacien*s strain GV3101 containing *HPT* and *YCF1* genes was cultured in 3 ml liquid YEP medium containing $50\,mg\,l^{-1}$ kanamycin and $100\,mg\,l^{-1}$ rifampicin at 28ºC overnight with an agitation of 150 rpm. Cultured bacteria with an OD_{600} of 0.6–0.8 were collected by centrifugation at 5,000 rpm for 2 min and gently suspended in 15 ml *Agrobacterium*-inoculation medium (MS medium supplemented with $20\,mg\,l^{-1}$ AS. Approximately, 50 explants were incubated with 15 ml *Agrobacterium* suspension (with touching the cotyledonay petiole into *Agrobacterium* suspension) for 15 min with occasional gentle shaking at dark conditions. After infection, the bacterial suspensions were removed and the explants were blotted dry on sterilized paper towels and subsequently cultured on co-cultivation medium (MS medium supplemented with $0.1\,mg\,l^{-1}$ NAA, $1.0\,mg\,l^{-1}$ BA, and $2.0\,mg\,l^{-1}$ $AgNO_3$, $20\,mg\,l^{-1}$ AS) for 0–4 days in a controlled growth room at dark conditions. Following co-cultivation, the explants were washed twice for 2 min in washing medium (sterilized distilled water with $500\,mg\,l^{-1}$ cefotaxime) to remove the bacteria attached to the explants and then blotted dry on sterilized paper towels. Ten explants were placed in each Petri dish (90×15 mm) containing *Agrobacterium*-elimination medium (shoot-induction medium with $300\,mg\,l^{-1}$ cefotaxime) and cultured for 0–6 days. After this time, the explants were transferred to a primary selection medium (shoot induction medium with $15\,mg\,l^{-1}$ hygromycin, and $300\,mg\,l^{-1}$ cefotaxime) and cultured for 14 days. Subsequently, explants were subcultured onto secondary selection medium (shoot-induction medium with $20\,mg\,l^{-1}$ hygromycin, and $300\,mg\,l^{-1}$ cefotaxime) at 10 days intervals until shoot buds developed. Hygromycin-resistant shoot buds were excised from the explants and transferred to shoot-elongation medium (MS medium supplemented with $0.1\,mg\,l^{-1}$ BA, $30\,mg\,l^{-1}$ hygromycin, and $300\,mg\,l^{-1}$ cefotaxime) for 10–15 days. Selected hygromycin-resistant shoots of 1–2 cm in length possessing distinct nodes, internodes, leaves and apical buds were then transferred to root-induction medium (MS medium supplemented with $0.1\,mg\,l^{-1}$ NAA, $30\,mg\,l^{-1}$ hygromycin, and $300\,mg\,l^{-1}$ cefotaxime). The transformation efficiency was calculated as the percentage of total number of hygromycin-selected shoots out of total number of *Agrobacterium*-infected explants. Regenerated plantlets with well-developed roots were washed thoroughly in tap water to remove Phytagar, transferred to pots containing soil and grown for 5 days in the same environmentally-controlled growth chamber as described above. Acclimatized plantlets were then transferred to the greenhouse until mature seeds were harvested.

Polymerase chain reaction (PCR) analysis

The putative transgenic plants and wild-type plants (WT) were analyzed by the polymerase chain reaction (PCR) to confirm the presence of the transgenes. Genomic DNA extraction was carried out according to Edwards et al. (1991). Young leaves from WT and transgenic lines were placed in a 1.5-ml microcentrifuge tube, and then homogenized with a pellet pestle with 400 µl of genomic DNA extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% sodium

dodecyl sulfate). The primer pairs used for DNA amplification were 5′-CAT GAG TGC GTT CTA TCC CTC TAT-3′ (forward) and 5′-CCA CCT TCG GTT AGT TGG GCA TCT-3′ (reverse) for the *YCF1* gene; and 5′-CAT GTG TAT CAC TGG CAA ACT GT-3′ (forward) and 5′-GTA CTT CTA CAC AGC CAT CGG TC-3′ (reverse) for the *HPT* gene. Total volume of PCR reaction mixture was 20 μl which contained 10 μl of SUN PCR blend (SUNGENETICS Co. Ltd., Korea), 7 μl of distilled sterile water, 10 ng of genomic DNA, and 1 μl of each primer (10 pmol). Thermocycling was performed as follows: one step of 5 min at 94ºC (for initial denaturation) followed by 29 cycles of 30 sec at 94ºC (denaturing), 30 sec at 55°C (annealing) and 30 sec at 72ºC (extension). Thermocycling ended with a final extension cycle of 5 min at 72°C. The amplified products were separated on 1% agarose gels containing 0.5 μg ml^{-1} ethidium bromide.

For specific detection of the bacterial contamination in the plant samples (DNA), the primers were designed to target specifically the junction between the *HPT* gene of the T-DNA and the non-transferable vector sequences of the plasmid used. The defined region would not be multiplied by PCR in samples containing the T-DNA inserted plant chromosome/genome. The following primer pairs were used: 5′-TGA TGG GCT GCC TGT ATC GA-3′ (forward) and 5′-CAT GTG TAT CAC TGG CAA ACT GT-3′ (reverse), resulting in a 939-bp PCR product. The same PCR cycling conditions described above were utilized for this reaction.

Southern blot analysis

Leaves of the fully-grown putative transgenic and WT plants were used to extract total genomic DNA by the methods described by Dellaporta et al. (1983). For each sample, fifty μg of DNA was digested overnight at 37ºC with *Bgl*II or *Eco*RV and separated by electrophoresis on 1% agarose gel. DNA fragments were denatured and transferred to Hybond-N$^+$ membrane (Amersham Biosciences, London, UK) following the manufacturer's instruction. The 0.5-kb PCR amplified *HPT* gene and 0.7-kb PCR amplified *YCF1* gene were labeled with α-^{32}P dCTP using the Rediprime II random prime labeling system (Amersham Biosciences) and used as hybridization probes. Hybridization and subsequent washing of the blots were performed at 65°C according to Sambrook et al. (1989).

Segregation analysis of T_1 progeny

For studying segregation of the transgene, putative transgenic plants were self-pollinated and seeds of each plant were collected separately. A random sample of approximately 50 seeds from each plant was germinated on 1/2MS agar medium containing 30 mg l^{-1} hygromycin. Non-germinated seeds and brown seedlings were considered sensitive, whereas 14-day-old green and vigorous seedlings were scored as resistant. The hygromycin-resistant seedlings were transferred to soil and further grown in a greenhouse.

Results and discussion

Determination of the optimal hygromycin concentration

Although shoot organogenesis and plant regeneration in *B. juncea* were achieved with cotyledon and hypocotyls explants, cotyledon explants responded better (Bhuiyan et al. 2009). Therefore, cotyledon explants were used for *Agrobacterium*-mediated transformation in this study, which led to an efficient transformation protocol.

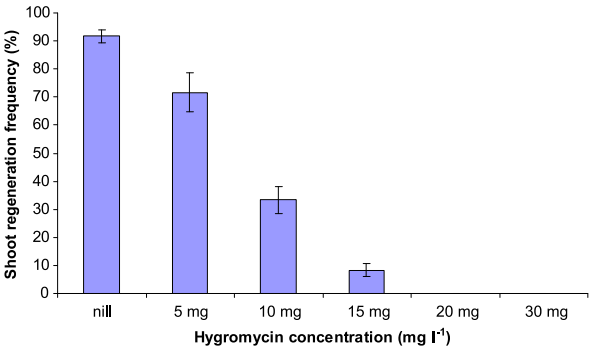

Figure 1. Effect of hygromycin concentrations on shoot regeneration from cotyledon explants of *B. juncea* cv. Rai-5. Values represent mean and SD (n=2).

An optimized concentration of a suitable selection agent can efficiently inhibit growth of non-transformed tissues and increase the acquisition of transgenic plants (Khan et al. 2003). Hygromycin was tested as a selective agent for transgenic *B. juncea*. Cotyledon explants were cultured on shoot induction medium containing various concentrations (0, 5, 10, 15, 20, or 30 mg l^{-1}) of hygromycin. Shoot regeneration was greatly inhibited by the increased concentration of hygromycin (Figure 1). Only 8.3% of the explants regenerated shoots in the presence of 15 mg l^{-1} hygromycin and there was no shoot regeneration with 20 mg l^{-1} or greater concentrations of hygromycin. Therefore, 15 mg l^{-1} hygromycin was used for the primary selection of transgenic shoots and subsequently was increased to 20 mg l^{-1} for the secondary subculturing steps. To eliminate false-transgenic shoots, hygromycin concentrations were further increased to 30 mg l^{-1} for shoot-elongation and root-induction steps.

Transformant regeneration

We found that the time of co-cultivation was critical for high efficiency transformation. In this study, a co-cultivation time of 2 days gave the highest transformation efficiency (Table 1). Longer period of co-cultivation (more than 2 days) proved to be detrimental as the plant tissue died resulting in no or transgenic shoots. An interesting result found in this study was that delaying the transfer of explants into selection media after co-cultivation until certain periods (3 to 5 days) dramatically enhanced the transformation efficiency (Table 2). This positive effect of delayed exposure of the explants to selective agent suggested that delayed selection permitted the division of transformed cells, which conferred greater protection against the selective

agent. Visser et al. (1989) also reported that delayed application of the selective agent in potato enhanced transformation efficiency significantly. We proposed that this strategy could be useful for plant species that tend to regenerate rapidly under tissue culture conditions. To increase the transformation efficiency, we adopted a three-stage hygromycin selection process. Initially, we exposed the explants to a low concentration of hygromycin (15 mg l^{-1}) and then, in the next subculturing step in shoot-induction medium, the hygromycin level was increased. The initial low levels of hygromycin would potentially allow shoot regeneration of both transformed and untransformed explants. However, the higher concentration of hygromycin (20–30 mg l^{-1}) in subsequent steps retarded the division of untransformed cells and increased the division of transformed cells. Hygromycin-resistant shoot buds were formed on the cut edges of the cotyledonary petioles after 2–4 weeks in culture on selection medium (Figure 2A–D). The majority of the initially-green shoots turned white or purple at high concentration of hygromycin (30 mg l^{-1}) in the shoot-elongation medium, only a few remained green and produced additional green shoots (Figure 2E). Green shoots were excised and transferred to the rooting medium. Roots developed from all shoots after 10–15 days of culture (Figure 2F). A total of 32 putative transgenic shoots (transformation efficiency of 16.2%) were obtained. Putative transformants were transferred to soil and acclimatized for 5 days at controlled environment and subsequently transferred to the greenhouse. All transformants showed normal morphology and flowering characteristics (Figure 2G, H). All plants were fertile, and seeds were collected from individual transgenic lines separately after maturation of the pods.

Table 1. Effect of co-cultivation period on transformation efficiency from cotyledon explants of *B. juncea* cv. Rai-5

Co-cultivation period (day)	Number of explants tested	No. of explants regenerated shoots	Transformation efficiency (%)
0	89	0	0
1	102	0	0
2	197	32	16.2
3	120	2	1.6
4	115	0	0

Table 2. Effect of delayed exposure the explants to selective agent (15 mg l^{-1} hygromycin) on transformation efficiency of *B. juncea* cv. Rai-5

Period (d)	Total number of explants tested	Number of explants regenerated shoots	Transformation efficiency (%)
0	115	0	0
1	102	0	0
2	145	0	0
3	157	1	0.6
4	135	3	2.2
5	197	32	16.2
6	105	0	0

Confirmation of the presence of transgene by PCR and Southern blot analyses

All hygromycin-resistant plants were subjected to PCR analysis with the primers specific for *YCF1* and *HPT* genes to confirm the insertion of transgenes into the *B. juncea* host genome. Expectedly, all transgenic lines showed 734-bp band representing the *YCF1* fragment and a 501-bp band representing the *HPT* fragment. No PCR band was observed with the WT (data not shown).

Transgenic plants obtained by *Agrobacterium*-mediated transformation could have been contaminated by latent *Agrobacteria* even with the use of high

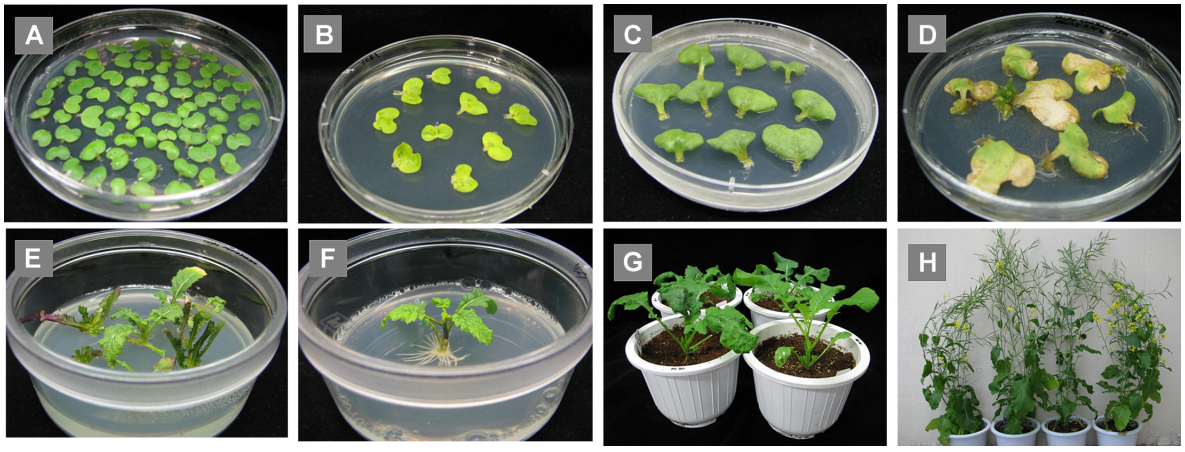

Figure 2. Development of transgenic *B. juncea* cv. Rai-5 plants. (A) 4-d-old cotyledon explants. (B) explants after 2 days culture on co-cultivation medium, (C) explants on shoot induction medium supplemented with 300 mg l^{-1} cefotaxime on day 5. (D) Shoot bud formation on selection medium (shoot induction medium plus 20 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime). (E) shoot elongation on MS medium supplemented with 0.1 mg l^{-1} BA, 30 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime. (F) *in vitro* rooting of hygromycin-resistant shoots on MS medium supplemented with 0.1 mg l^{-1} NAA, 30 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime. (G) Acclimatized plants in pot soil. (H) Flowering and fruiting (pod development) of transgenic plants in the greenhouse.

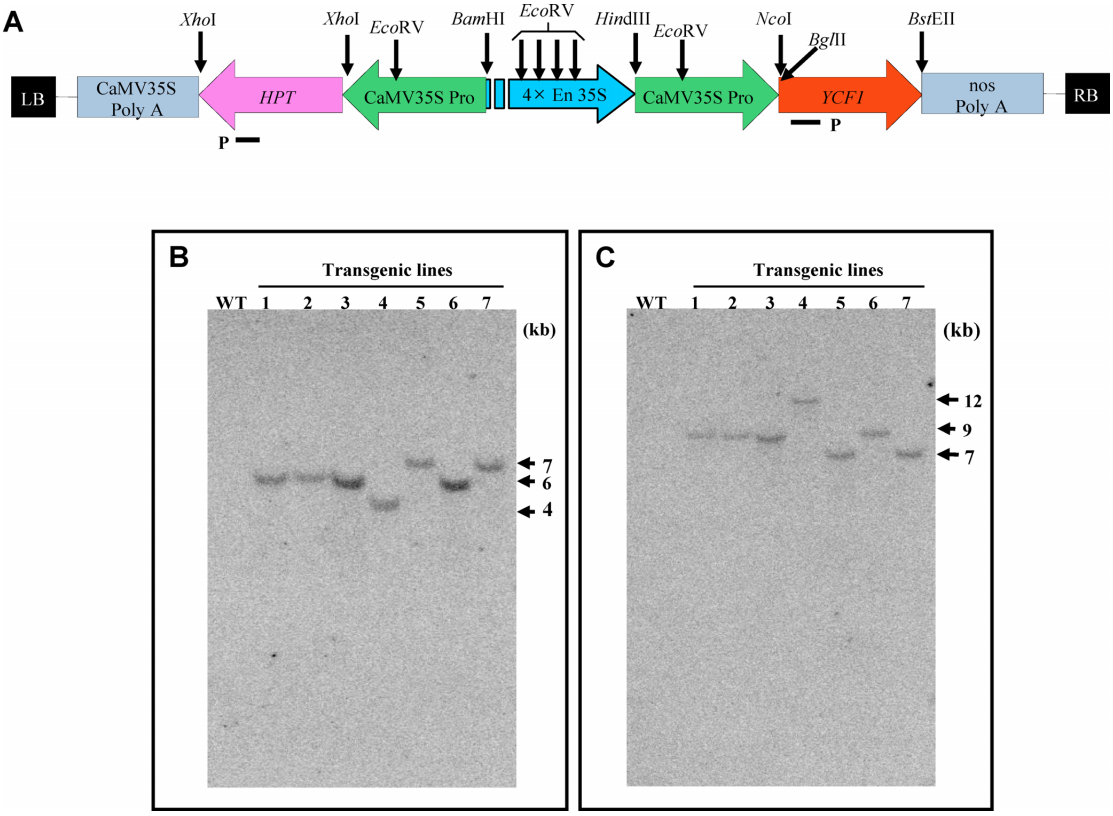

Figure 3. Plasmid vector map and Southern blot analysis. (A) Schematic representation of the T-DNA region of the EnPCAMBIA1302-YCF1 vector. LB, T-DNA left border; CAMV35S poly A, cauliflower mosaic virus 35S terminator; *HPT*, hygromycin phosphotransferase gene; CAMV35S Pro, cauliflower mosaic virus 35S promoter; 4×En 35S, four tandem copies of the cauliflower mosaic virus 35S enhancer; *YCF1*, yeast cadmium factor 1; nos ploy A, nopaline synthase terminator; RB, T-DNA right border; P, probe used for Southern blot analysis. Arrows indicate restriction sites. B Southern blot of *Bgl*II digested total DNA probed with a *HPT* gene. (C) Southern blot of *Eco*RV digested total DNA probed with a *YCF1* gene.

concentrations of *Agrobacterium*-eliminating antibiotics (Shackelford and Chlan 1996; Ogawa and Mii 2007). The presence of *Agrobacterium* in plant tissues often leads to misleading results including environmental hazards. Therefore, getting transgenic plants without *Agrobacterium*-contamination are highly desirable. PCR is the most commonly used technique to screen putative transformants whether they contain *Agrobacterium*. Therefore, we attempted to check transgenic lines by PCR using *Agrobacterium*-specific primers. All transgenic lines were free of *Agrobacterium* contamination as they did not show any bands by primer specific PCR reaction (data not shown).

Randomly selected seven PCR-positive plants were subjected to Southern blot analysis using YCF1 and HPT probes (Figure 3A) to confirm the integration of the transgene and estimating the transgene copy number into the *B. juncea* genome. Interestingly, all transgenic lines showed a single locus for *HPT* and *YCF1* gene integration in the host genome (Figure 3B, C) which is considered most desirable for any *Agrobacterium*-mediated genetic transformation. Although transgenic lines were obtained from independent transformation events, some of these lines (particularly #1, #2, #3 and #6, and #5, #7) showed similar sizes of bands. Babic et al. (1998) and Leelavathi et al. (2004) also reported similar findings of same size bands of transgenes in different transgenic plants in Southern blot analysis, even transgenic plants were obtained from different transformation events of *Agrobacterium*-mediated genetic transformation of *B. carinata* and *Gossypium hirsutum*.

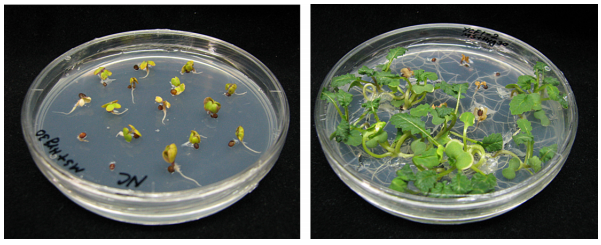

Figure 4. The growth of T_1 seedlings of WT (left) and YCF1-2 transgenic plants (right) on 1/2MS medium containing 30 mg l^{-1} hygromycin for 14 days.

Segregation of T_1 progeny

The putative transgenic plants were grown in an environmentally-controlled greenhouse until seed

Table 3. Segregation of hygromycin-resistant and-sensitive plants in the self-pollinated T_1 progeny of transformed *B. juncea* cv. Rai-5 plants. χ^2: In accordance with the expected Mendelian ratio of 3 : 1 at $\alpha=0.05$

Plant line	Number of seeds tested	No. of seedlings		χ^2 value	Probability ($\chi^2(0.05)=3.84$)
		Resistant	Sensitive		
WT	50	0	50	—	—
YCF1-1	48	37	11	0.1179	
YCF1-2	50	37	13	0.0259	<0.05
YCF1-3	51	37	14	0.15384	<0.05
YCF1-4	48	35	13	0.10549	<0.05
YCF1-5	48	37	11	0.1179	<0.05
YCF1-6	48	34	14	0.40336	<0.05
YCF1-7	50	40	10	0.78125	<0.05

setting. T_1 seeds were collected and grown on 1/2MS agar medium containing 30 mg l^{-1} hygromycin. In the presence of hygromycin, WT seeds showed initiation of germination by the fourth but subsequently day, turned yellow and dried up, whereas the transformed seeds continued to develop as green seedlings (Figure 4). All transgenic lines showed 3 : 1 Mendelian pattern on hygromycin containing media (Table 3), which further demonstrated that a single copy of T-DNA was integrated in all transgenic plants.

In the present transformation protocol using cotyledon explants, we obtained relatively high (16.2%) transformation efficiencies as compared with previous reports. This high-throughput transformation method should facilitate the use of this plant species for studies in gene manipulation and expression. We are currently using the transgenic *B. juncea* lines for heavy metal phytoremediation purposes.

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