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GENETIC TRANSFORMATION OF *BRASSICA JUNCEA* WITH ANTIMICROBIAL *WASABI DEFENSIN* GENE

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Abstract

Brassica juncea is one of the most important oilseed crop. An efficient and reproducible protocol was developed for *Agrobacterium*-mediated transformation of *Brassica juncea* variety NIFA RAYA with *Wasabi defensin* gene to produce transgenic plants. This gene was isolated from the leaves of *Wasabia japonica.* The expression of *Wasabi defensin* gene; encoding antimicrobial protein, in plasmid *pEKH-WD* is driven by the constitutive 35S promoter. The role of a number of factors such as choice of explants, the age of explants, different ratios of growth regulators, various concentrations of growth hormones and chemicals which can directly or indirectly influence the process of transformation was evaluated. Hypocotyls and cotyledons from 4-7 days old seedlings, when used as explants for transformation, were found to be the best in terms of producing transgenic calli and shoots. Regeneration of the explants on solidified MS plates supplemented with different hormone ratios and concentrations showed that medium containing 2 mgL^{-1} BAP and 0.2 mgL^{-1} NAA was the best for callus initiation. Whereas, 3 mgL^{-1} BAP and 0.3 mgL^{-1} NAA was found to be the best hormone combination for callus formation. For shoot regeneration 3 mgL^{-1} BAP and 0.5 mgL^{-1} NAA suplemented with 20 µM $AgNO_3$ was the best combination. The transgenic nature of the transformed callus and regenerated shoots was confirmed via PCR analysis by using *Wasabi defensin* gene specific primers against their isolated genomic DNA.

Introduction

Brassica is economically the most important genus in the Brassicaceae family. Several species and types of Brassica are significant oilseed crops, forage crops, vegetables and are used in the production of condiments, such as mustard. In the genus *Brassica*, Indian mustard *(Brassica juncea)* is one of the most important species (Woods *et al*., 1991; Getinet *et al*., 1996; Rabbani *et al*., 1998; Farzinebrahimi *et al.,* 2012). In Pakistan, after cotton, rapeseed and mustard is the second most important source of oil sown from mid September to mid November (Abbas *et al.*, 2009). The cultivation of oilseed crops such as *Brassica* has gained elevated importance in countries like Pakistan which is one of the largest importer of edible oil in the world. The productivity of oilseed rape is limited by several abiotic and biotic stress factors (Grover & Pental 2003; Dutta *et al.,* 2005; Ullah *et al.,* 2012). Agrochemicals are commonly used to control many plant pests and diseases, however they are expensive and non-ecofriendly and the approach is particularly insufficient to combate the new emerging pathotypes. Conventional breeding has also limitations like time consuming, undesirable gene recombination and crossing barriers. Advances in genetic engineering has overcome the species barrier and made it possible to introduce a gene of interest into the plant system from different sources.

With the rapid growth of recombinant DNA technology a number of genes have been identified, isolated and modified to study their regulation and function. Such modified genes are then introduced into the defined recipients for obtaining desired plants. As a result, development of these modern biotechnological approaches and exploitation of transgenic plants has become increasingly important for better and sustainable production of oilseed rape. Various *Brassica* species have been shown to be highly susceptible to *Agrobacterium tumefaciens* (Charest *et al.,* 1989) and plants of *B. oleracea* (David & Tempe, 1988), *B. napus* (Moloney *et al.,* 1989) and *B. juncea* (Mathews *et al.,* 1990) have been transformed through *Agrobacterium tumefaciens.*

Fertile transgenic plants of *Brassica* spp. carrying genes of interest have been produced by different methods like *Agrobacterium* (Narasimhutu, 1992), electroporation of protoplast (Bergman & Glimelius, 1993), and biolistic transformation (Chen & Beverdorf, 1994). *Agrobacterium tumefaciens*-mediated transformation is preferred than other methods of transformation for its simplicity and cost effectiveness (Barfield & Pua, 1991; Shen *et al.,* 2012; Pental *et al.,* 1993; Dutta *et al.,* 2005; Das *et al.,* 2006). This biotechnological approach have already been applied for the improvement of *B. juncea* in the area of phytoremediation (Zhu *et al.,* 1999), herbicide resistance (Mehra *et al.,* 2000), salt tolerance (Zhang *et al.,* 2001), hybrid seed production (Jagannath *et al.,* 2002), oil quality (Hong *et al.,* 2002; Das *et al.,* 2006) and aphid resistance (Kanrar *et al.,* 2002; Dutta *et al.,* 2005).

Defensins are a class of evolutionarily and structurally related small, highly basic, cysteine-rich peptides, possessing a broad-spectrum *In vitro* antifungal activity (Thomma *et al.,* 2002). They are low-molecular-weight (5 kDa) proteins found in roots, stems, leaves and seeds of a number of plant species that are toxic to bacteria, fungi and yeast *In vitro*. They cause permeabilization of fungal membranes, leading to the inhibition of fungal growth (Commue *et al.,* 1992; Broekaert *et al.,* 1995). Japanese horseradish (*Wasabia japonica* L.) is a potential source of antimicrobial proteins. Antimicrobial protein, *WjAMP-1* gene, isolated from leaves of *Wasabia japonica*, inhibited bacterial and fungal growth when expressed in *Nicotiana benthamiana* (Saitoh *et al.,* 2001; Kiba *et al.,* 2003). This

antimicrobial protein also called as *Wasabi defensin* has been successfully integrated in many crops like Petunia (Khan *et al.,* 2010), Water melon (Ntui *et al.,* 2010), Tobacco (Ntui *et al.,* 2010), Potato (Khan *et al.,* 2006), Rice (Kanzaki *et al.,* 2002) and Phalaenopsis (Sjahril *et al.,* 2006) for resistance against *Botrytis cinerea* (gray mold), *Alternaria* leaf spot and *fusarium* wilt, gray mold (*Botrytis cinerea*), blast fungus (*Magnaporthe grisea*) and *Erwinia carotovora,* respectively. The goal of the present study was to integrate *Wasabi defensin* gene via *Agrobacterium tumefaciens*-mediated transformation method into the genome of *Brassica juncea* for enhancing its resistance in the long run. To this end stable transformation of *Brassica juncea* with *Wasabi defensin* gene was carried out to produce disease resistant transgenic plants. *Brassica juncea* cultivar i.e. NIFA Raya was transformed with *Wasabi defensin* gene (*WjAMP-1*) under 35S promoter. Our aim in this study was to optimize and establish reproducible sterilization, regeneration and transformation protocols for future oilseed rape transformation programs.

Material and Methods

Transformation of *Brassica juncea* plants with *Wasabi defensin* gene was carried out with *Agrobacterium* strain *EHA 101*. The vector plasmid, *pEKH-WD* containing the *wasabi* gene, isolated from *Wasabia japonica* (Saitoh *et al.,* 2001; Kiba *et al.,* 2003*)*, kindly provided by Dr. Ikuo Nakamura, Graduate School of Horticulture, Chiba University, Japan. The construct, *pEKH-WD*, harboring the chimeric *Wasabi defensin* gene, encoding antimicrobial protein, the gene for *neomycin phosphotransferase II* (*nptII*), which confers kanamycin resistance (Bevan, 1984).

Seeds of *Brassica juncea* variety NIFA RAYA were used for transformation which were kindly provided by Mr. Iftekhar, senior scientist at Nuclear Institute for Food and Agriculture (NIFA). For surface sterilization, *Brassica* seeds were washed with tap water and dipped in 70% ethanol for 30 seconds. Then the seeds were treated with 1-2% sodium hypochlorite solution (1% active chlorine) plus 1-2 drops of TWEEN-20 for 15-20 min followed by rinsing 3-4 times with sterilized water in a sterile beaker inside the Laminar Flow Hood. After sterilization, seeds were cultured for germination on half strength MS medium.

The surface sterilized seeds were cultured on half MS medium (in half MS medium each component MS medium is taken half except sucrose) in six 100 ml flasks, each containing 20-25 seeds. The flasks were then placed in the controlled environment of growth room maintained at 23 ± 1°C to regenerate *In vitro* plants.

Cotyledons and hypocotyls excised from 4-7 days old plants in the microbe free environment of Laminar Flow Hood were used as explants for pre-culturing. A total of 20 plates each containing 30 ml MS medium with hormones (1-6 mgL^{-1} BAP and 0.1-0.6 mgL^{-1} NAA) were used for pre-culturing. The number of explants pre-cultured in each plate was 20. So a total of 400 explants were pre-cultured in the controlled environment of growth room maintained at 23 ± 1°C.

Three types of plant material were used for infection:

1. Hypocotyls from *In vitro* growing plants
2. Cotyledons from *In vitro* growing plants
3. Leaves from green house growing plants

The infection inoculums prepared from *Agrobacterium tumefaciens* strain *EHA* 101 harboring the binary vector plasmid *pEKH-WD* were used to infect both types of plant material in separate flasks. Some of the hypocotyls and cotyledons were pre-cultured for 2 days before infection while leaves from green house growing plants and some hypocotyls and cotyledons were used directly for infection. All these explants were left in infection inoculum for 10-15 minutes.

After infection all the plant material were dried on sterilized tissue paper and then co cultivated on different plates. The infected explants were co cultivated on plates containing 30ml MS medium with hormones (1-6 mgL^{-1} BAP and 0.1-0.6 mgL^{-1} NAA). Controls were treated in the same way but without *Agrobacterium* infection. All co cultivated and controls explants were then rapped completely in aluminium foil and placed in dark room maintained at 23 ± 1°C.

After co-cultivation, for two days the treated and controls both types of explants were transferred to selection MS media with hormones (1-6 mgL^{-1} BAP and 0.1-0.6 mgL^{-1} NAA) containing 10-100 mg l^{-1} kanamycin with 300 mg l^{-1} cefotaxime. These explants were transferred to fresh medium after an interval of 7-10 days. After 2-3 weeks we got callus on selection medium from all explants which were then subjected to PCR analysis, for which DNA was extracted from a small parts of callus. And from the remaining part of callus we developed shoots and only cotyledon and hypocotyls explants produced shoots and green house leaf explants produced only callus not shoots which was also confirmed by PCR.

Polymerase chain reaction (PCR) was employed to screen transformants for *wasabi defensin* genes integration. Genomic DNA from kanamycin-resistant and control *Brassica juncea* explants calli and shoots was extracted as described by Sharma *et al.,* (2003) with some modification. PCR was performed using genomic DNA as a target and the following oligonucleotide primers of *Wasabi defensin* gene (Khan *et al.,* 2006).

Forward: 5′-TTTGCTTCTATCATCGCTCTTC-3′
Reverse: 5′-TTATTAGTACAACAAACCAACA-3′

Results and Discussion

Agrobacterium mediated transformation was employed to transform the explants from all three categories by using a transformation vector in which the expression of *Wasabi defensin* gene is driven by 35S promoter (Fig. 1). The choice of explant is an important determinant for successful transformation and tissue culturing. To find out the role of explants in transformation and on callus and shoot induction, different types of explants were used in this study. In most of our experiments hypocotyls and cotyledons from 4-7 days old seedlings have been used as explants either directly or after pre-culturing on pre-culturing media (Fig. 2). Alternatively leaves from the green house growing 40-50 days old plants were also used as explants directly after cutting and sterilization in some experiments.

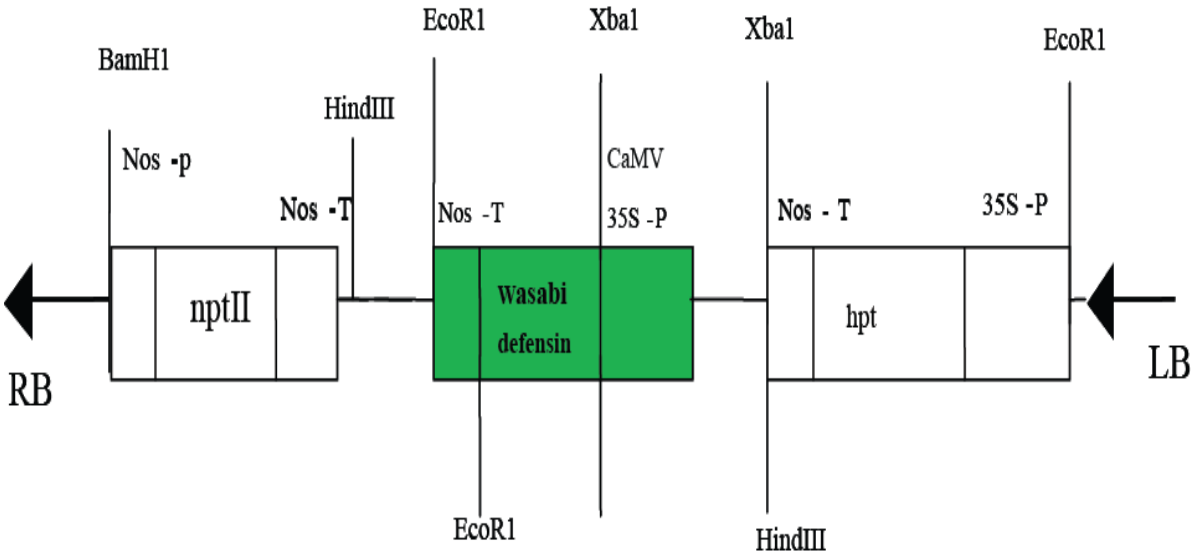

Fig. 1. Over expression construct of *Wasabi defensin* gene used for transformation.
T-DNA region of the binary vector pEKH-WD harboring wasabi and hpt genes. The genes for *Wasabi* and *hpt* are driven by CaMV 35S promoter, and the gene for *neomycin phosphotransferase* (*nptII*) by *nopaline synthase* promoter (*nos-p*). LB and RB, Right and Left border sequences of the T-DNA region, respectively. CaMV 35S-P, cauliflower mosaic virus *35S* promoter, nos-T, terminator of the nopaline synthase gene. Recognition sites of restriction enzymes are also indicated.

Fig. 2. Cotyledons and hypocotyls explants on pre-culturing medium (Color print needed)
Cotyledons and hypocotyls explants on pre-culturing medium containing BAP 2 mgL^{-1}, NAA 0.2 mgL^{-1}.

Transformation of the explants was carried out by dipping the explants in the transformation solution for 10-15 minutes. The explants were dried on sterilized filter paper and transferred to petri plates containing MS medium inside Laminar Flow Hood. Afterwards these petri plates were covered with aluminium foil and kept in the dark at 23 ± 1°C for 2 days. After two days of co-cultivation, the explants were shifted to long day (16 h light/8 h dark) condition at 23± 1°C and observed regularly for callus initiation and/or shoot formation

In line with the previous reports (Muhammad *et al.,* 2002; Moghaieb *et al.,* 2006; Bano *et al.,* 2010), more than 90% of the hypocotyls and cotyledons explants responded to the pre-cultured conditions by expanding in size within 48 hour. Callus started developing from the co-cultivated explants in the second week after co-cultivation (Fig. 3A & B) on selection medium containing antibiotics and hormones. Although callus also developed from the leaves derived explants, however their response was much slower than those observed for the most commonly used hypocotyl explants used in *Agrobacterium*-mediated *B. juncea* transformation experiments (Barfield & Pua, 1991; Pental *et al.,* 1993; Dutta *et al.,* 2005; Das *et al.,* 2006) and cotyledons. Moreover, we found out that shoots regeneration occurred only from the callus developed from the hypocotyls and cotyledons derived explants. Pre-cultured control explants died on the selection medium after three weeks of their transfer to the selection medium (Fig. 3C). Explants on medium containing BAP 1,4,5 and 6 mgL^{-1} with NAA 0.1,0.4,0.5 and 0.6 mgL^{-1} showed very less callus initiation and formation while BAP 2 mgL^{-1}, NAA 0.2 mgL^{-1} showed fast callus initiation and BAP 3 mgL^{-1}, NAA 0.3 mgL^{-1} showed maximum callus formation for all the 3 types of explants.

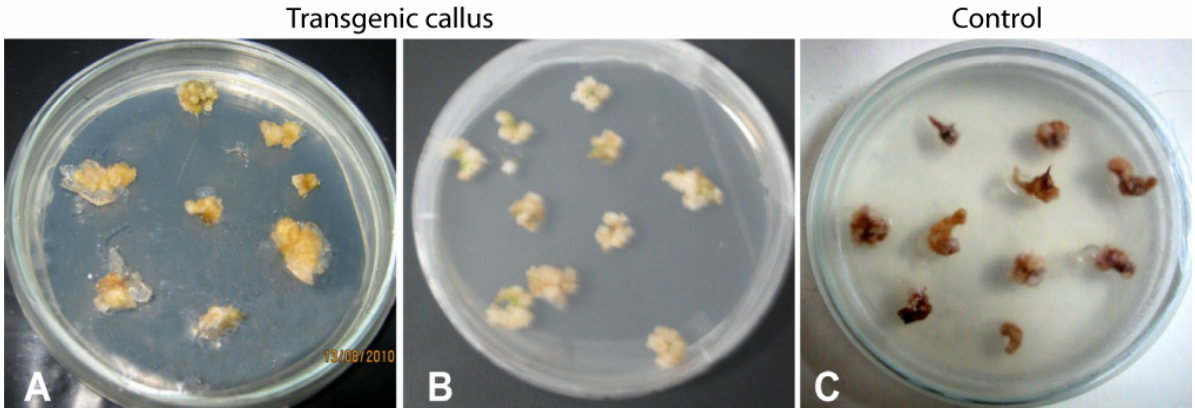

Fig. 3. Transgenic and control callus (Color print needed)
A and B, transformed callus., C, control callus on selection medium (Ms medium containing BAP 3 mgL^{-1} and NAA 0.3 mgL^{-1}, Kanamycin 30 mgL^{-1} and cefotoxime 300 mgL^{-1}).

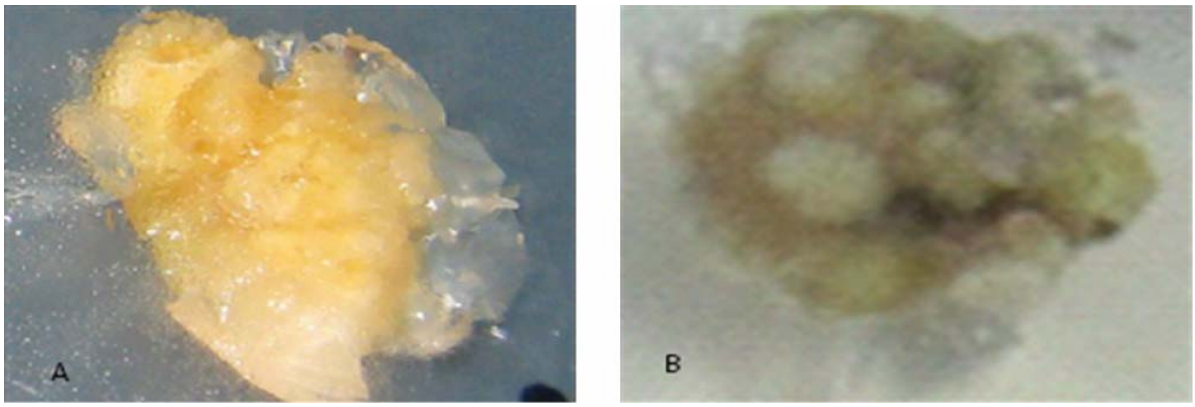

Fig. 4. The effect of $AgNO_3$ on callus formation and shooting (Color print needed)
Transformed callus on shooting medium containinng BAP 3 mgL^{-1}, NAA 0.5 mgL^{-1}, Kanamycin 50 mgL^{-1} and 300 mgL^{-1} cefotaxime. A, without silver nitrate B, with 20 µM silver nitrate.

All three types of explants, when cultured on regeneration medium containing appropriate concentration of growth hormones produced trasformed callus. However, regenation of the shoots was not observed for all types of explants in the absence of $AgNO_3$. In line with the finding of the previous reports where shoots induction from explants of *Brassica* was highly increased with ethylene inhibitors like silver nitrate or aminoethoxyvinylglycine (Chi *et al.,* 1990; Pua & Chi, 1993; Burnett *et al.,* 1994; Zhang *et al.,* 1998; Dutta *et al.,* 2008), addition of 20 µM silver nitrate in the shooting medium in our case had a positive effect on regeneration of *Brassica juncea* as evident from the greenish color of the callus (Fig. 4B) compared to the callus developed in the absence of silver nitrate (Fig. 4A). Subsequently shoot regeneration only occured when 20 µM silver nitrate was present in the shooting medium (Fig. 5). The mode of action of silver nitrate in plant tissue culture is assumed to be associated with the physiological effect of ethylene with silver ions. $AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to supress shoot morphonegensis *In vitro* (Zhang *et al.,* 2001). It is however, important to mention that the calli developed from the leaves of approximately 3 months old plants did not produce shoots even in the presence of $AgNO_3$. The failure of these calli to regenerate shoots seems to be correlated with the age of the explants, which is supported by the previous reports (Tang *et al.,* 2003) who have found out that shoots regeneration frequency greatly decreases in Oilseed *Brassica spp.* with the increase in the age of the explant. The transgenic shoots developed roots when transferred to MS medium without plant growth hormones.

PCR analysis of the genomic DNA extracted from the control and transgenic calli using *Wasabi defensin* gene specific primers were carried out to confirm the transgenic nature of the transformed calli (Fig. 6). The presence of a ~ 450 bp band in lane 2 (poitive control), lane 3-6 (transgenic calli) and its absence in lane 7 (negative control) confirmed the transgenic nature of the transformed calli (Fig. 6).

Our studies suggest that hypocotyls and cotyledons from 4-7 days old seedlings are the most suitable explants when used for transformation. They were found to be far better than leaves taken from older plants in terms producing transgenic calli and shoots. Our studies further suggest that medium containing 2 mgL^{-1} BAP and 0.2 mgL^{-1} NAA is the best for callus initiation whereas 3mgL^{-1} BAP and 0.3 mgL^{-1} NAA is the best hormone combination for callus formation. The addition of silver nitrate has a very positive effect on shoot regenration as indicated by the fact that shoot regeneration occurred in our case when media containing 3 mgL^{-1} BAP and 0.5 mgL^{-1} NAA was suplemented with 20 µM $AgNO_3$.

Fig. 5. Transgenic shoots of *Brassica juncea* (Color print needed)
Transformed shoots on MS medium containing 100 mgL^{-1} kanamycin and 300 mgL^{-1} cefotaxime for rooting.

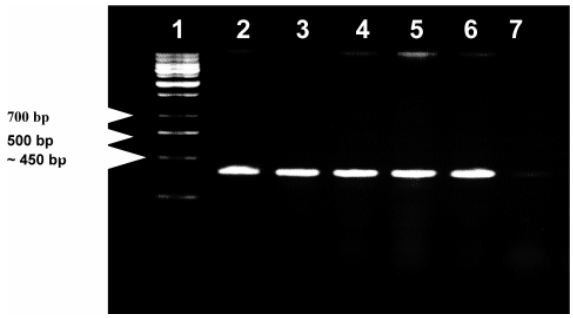

Fig. 6. PCR amplification *Wasabi defensin* gene of genomic DNA from calli
PCR analysis of DNA extracted from calli after infection. Lane 1, 1 Kb ladder, lane 2, plasmid DNA from transformed *Agrobacterium tumafaciens*, as a positive control, lane 3-6, four independent calli samples in which *wasabi* gene was amplified, lane 7, DNA from control non-transformed calli as a negative control.

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