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Full Length Article

Genetic Transformation of *Brassica napus* with the Antifungal *chitinase* Gene

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Abstract

Brassica napus is one of the most important sources of vegetable oil in the world. Efficient and reproducible protocols were developed for *Agrobacterium*-mediated transformation of *B. napus* in the current study. Synthetic *chitinase* (*NIC*) gene encoding antimicrobial protein in plasmid *pEKH* was transformed to Abasyn-95 cultivar of *B. napus* to produce transgenic plants. The role of a number of factors such as 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 *in vitro* grown seedlings were used as explants for transformation. Culturing of the explants on solidified MS plates supplemented with different hormone ratios and concentrations showed that the media supplemented with 2 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.5 mg L^{-1}benzylaminopurine (BAP) and 0.1 mg L^{-1}naphthaleneacetic acid (NAA) is the best combination among all tested conditions for callus induction. Maximum shooting was observed on MS medium containing 0.5 mg L^{-1} BAP and 2 mg L^{-1} 2, 4-D. The transgenic nature of the calli and regenerated shoots was confirmed by PCR analysis via*NIC* gene specific primers using their isolated genomic DNA as template. © 2013 Friends Science Publishers

Keywords: *Agrobacterium*; Transformation; Auxin; Cytokinins

Introduction

Brassica is an important genus belonging to the family *Brassicaceae*, also known as the mustard family or Cruciferae. In Pakistan, rapeseed and mustard are considered the most important source of oil after cotton (Abbas *et al*., 2009). The major constraints in growing mustard are non-availability of high yielding varieties suitable for high input conditions, fluctuations in weather conditions, aphid pests, frost injury and diseases (Kumar, 1999). Fungal diseases have served as a major cause of yield losses (Oerke *et al*., 1994).The Black leg disease caused by the fungus *Leptosphraeria maculans* is the most common disease of oilseed rape. Other fungal diseases like Club root (*Plasmodiophora brassicae*), and Alternaria leaf spot *(Alternaria brassicae)* can also cause serious yield losses. Depending upon the severity of the infection, the losses in productivity of rapeseed and mustard only due to *Alternaria* blight may range from 30-70% (Singh, 1990).

Brassica napus has high potential for improvement and is therefore the main target for crop improvement. To this end, researchers and plant breeders are working hard to develop new varieties with improved agronomic characteristics like disease resistance, herbicide resistance and increased tolerance to certain biotic and abiotic stresses (Malik, 1990). Since the agrochemicals and conventional breeding approaches often have certain limitations, the utilization of genetic engineering approaches therefore has emerged as a promising alternative.

Genetic modifications to introduce desirable traits in different crop plants have already made a significant impact on Agriculture worldwide. Genetic transformation has become an established technology for the improvement of many crop plants. The availability of an efficient *in vitro* system for regeneration amenable to genetic transformation is essential for developing transgenics. Various *Brassica* species have been shown to be highly susceptible to *Agrobacterium tumefaciens* and plants of *B. napus* have been successfully transformed in the past (Radke *et al*., 1988; De Block *et al*., 1989; Moloney *et al*., 1989; Wallbraun *et al*., 2009). Efforts to transform *B. napus* genotypes in Pakistan are generally hampered due to lack of prior characterization and recalcitrant nature of Pakistani commercial canola (*B. napus*) genotypes. Here we report successful transformation and regeneration of one of the major cultivated *B. nupus* cultivar i.e., Abasyn-95 of Khyber Pakhtunkhwa with a synthetic *chitinase* (*NIC*) gene that catalyzes the hydrolysis of Chitin which is a β-1, 4-linked homopolymer of N-acetylglucosamine (GlcNAc). Chitin is not present in plant cells, but is present as a major component in the cell wall of almost all true fungi. Chitinases play important role in plant defense against fungi by hydrolyzing the polymer of chitin found in fungal cell wall (Collinge *et al*., 2008). By using *Agrobacterium*-mediated gene transformation different plants like tobacco (Liu *et al*., 2004), potato, cucumber and tomato (Punja and

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Raharjo, 1996) have already been transformed with *chitinase*s.

In this study a synthetic *chitinase* gene named as *NIC* (Nakamura Ikuo Chitinase) of 1271-bp length has been used. It has been synthesized to encode for identical amino acid sequence as that of *chitinase 1* gene (*chi1*) in *Rhizopus oligosporus* except ^{25}Ser to ^{25}Arg (Yanai *et al.*, 1992). Although it shares 82%sequence similarity at the amino acid level to that of *chic1* but based on codon usage database (http://www.kazusa.or.jp/codon/), nucleotides at third position of about 18% codons in the *NIC* gene were substituted from fungal (AT-rich) type to plant (GC-rich) type (Khan *et al.*, 2012). The optimization of methodology for callus induction and regeneration will provide base line information for future *B. napus* transformation programs.

Materials and Methods

Plant Material

Brassica napus cultivar "Abasyn-95" was used for transformation. The seeds of this cultivar were kindly provided by the Nuclear Institute for Food and Agriculture (NIFA), Peshawar. Surface sterilized seeds were germinated for 8-10 days on half-strength MS basal medium under short day conditions (8 h light and 16 h dark) at 22±1°C.

Vector Construct and Inoculum Preparation

The construct for *NIC* gene (*pEKH-NIC*) as described in Khan *et al*. (2012) was kindly provided by Dr. Ikuo Nakamura, Graduate School of Horticulture, Chiba University, Japan. A single transformed colony of *Agrobacterium tumefaciens* strain EHA101 was picked from solidified Lysogeny Broth (LB) medium (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract,10 g L^{-1}tryptone, pH 7.2) and was cultured overnight on a rotary shaker at 28^0C in liquid LB medium in the presence of proper concentration of selective agents. The bacterial culture was centrifuged for 10 min at 3000 x g and after the removal of supernatant the pellet was diluted to OD_{600} = 0.5-0.9 in double distilled H_2O (ddH_2O) prior to inoculation.

Explants Pre-culturing

Cotyledons and hypocotyls excised from 10-14 days old seedlings were used as explants for pre-culturing under sterilized condition inside laminar flow hood. Plates containing pre-culture media supplemented with hormones (1-3 mg L^{-1}2, 4-D, 0.5 mgL^{-1}BAP and 0.1 mgL^{-1} NAA) were used. The plates were kept in growth chamber at 22±1^{o}C under short day conditions (8 h light and 16 h dark).

Co-cultivation and Selection

The preformed infection inoculum was used to infect the pre-cultured hypocotyls and cotyledons. One drop of silwet L-77 (surfactant) was added to infection solution. All the explants were dipped in infection inoculum solution for 8-10 min. The explants were then dried on sterilized filter paper and transferred to plates containing 30 mL MS medium supplemented with hormones (1-3 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA, 0.8% agar) for co-cultivation. The plates were then wrapped in aluminum foil and shifted to dark room maintained at 22±1ºC. After the co-cultivation period of 48 h all the infected and control explants were shifted to the selection MS media containing hormones (1-3 mg L^{-1}2,4-D, 0.5 mg L^{-1}BAP and 0.1 mg L^{-1} NAA), 100 mg L^{-1} kanamycin as a selective agent and 300 mg L^{-1} cefotaximeas bactericidal agent. The sub culturing to the fresh medium was performed after 8-10 days. After about 15-20 days callus was produced on the media. The calli were then transferred to shoot regeneration media which contained different concentration of 2,4-D and BAP.

Molecular Analysis for *NIC* gene in Transformed Callus and Shoots

The putative transgene in the plants was confirmed by polymerase chain reaction (PCR). Genomic DNA from callus was extracted by using 2X CTAB DNA extraction protocol (Rogers and Bendich, 1988) while, from transformed shoots, the extraction of DNA was performed by using Edwards (Edwards and Johnstone, 1991) DNA extraction protocol with some modifications. The following primers were used for amplification:

NIC forward 5′-GGTCGATGCCGTCCTCCTGTCCTT-3′
NIC reverse 5′-CGCCTTGGTGGTGGTCTTGATGGT-3′.

For PCR conditions, the DNA template was denatured at 94ºC for 1 min, amplified by 30 cycles (30 s at 94ºC, 30 sec at 59ºC and 1 min at 72ºC), and a final extension was performed at 72ºC for 5 min. The amplified products were analyzed by Gel electrophoresis on 1% Agarose gel using TBE buffer. The DNA was visualized using the gel documentation system.

Results

Agrobacterium-mediated Genetic Transformation

Agrobacterium-mediated genetic transformation was employed to transform the explants (hypocotyls and cotyledons) with the transformation vector (Khan *et al.*, 2012) in which the expression of *chitinase* gene (*NIC*) is driven by 35S promoter (Fig. 1). Since the choice of explant is an important determinant for successful transformation and tissue culturing, we therefore used hypocotyls and cotyledons as explants

Transformation of the explants was carried out by dipping the explants in the transformation solution for 8-10 min. Silwet L-77 was added at a concentration of 300 µL L^{-1}

Table 1: Concentration of 2,4-D, BAP and NAA for callus formation

Plant growth regulators concentration	Response of callus formation
1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	No response
1.5 mg L^{-1} 2 , 4-D + BAP 0.5 mg L^{-1} + NAA 0.1 mg L^{-1}	Poor callus
2 mg L^{-1} 2 , 4-D + BAP 0.5 mg L^{-1} + NAA 0.1 mg L^{-1}	Good callus

Table 2: Combination and Concentration of Plant growth regulators for shooting

Plant growth regulators concentration	Response of shoot Regeneration
2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	No shooting
1 mg L^{-1} 2, 4-D + 0.2 mg L^{-1} BAP	Poor shooting
2 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BAP	Good shooting

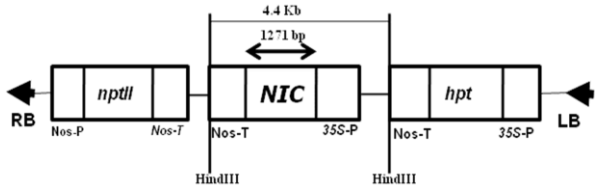

Fig. 1: T-DNA region of the binary vector *pEKH* used for *B. napus* transformation. The expression of *NIC* gene is driven by 35S promoter (35S-P). RB: Right border sequence of the T-DNA, LB: Left border sequences of the T-DNA

Fig. 2: Callus produced after 15-20 days on selection medium hormones (2 mg L^{-1} 2, 4-D, 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) and antibiotics kanamycin 100 mg L^{-1} and 300 mg L^{-1}cefotaxime

to the infection solution having an OD_{600} = 0.5-0.9. Silwet L-77is a subclass organo silicone copolymer surfactant that has the ability to reduce the surface tension of water and enhances the susceptibility of explants to infection. This allows the uniform spreading of water droplets over the leaf surface to penetrate the stomatal opening. The explants were then dried on sterilized filter papers and transferred to sterilized petri plates containing solidified MS medium inside Laminar flow hood. Co-cultivation was carried out by transferring the petri plates to the dark at 22±1ºC for about two days. After co-cultivation the development of callus started at the cut surface of the explants after 15-20 days (Fig. 2).

Optimization of Concentrations for Plant Growth Regulators

To determine the optimal concentrations of plant growth regulators, different concentrations and combinations of plant growth regulators (Table 1) ranging from 1-3 mg L^{-1} 2, 4-D, 0.1-0.5 mg L^{-1}BAP, 0.1-0.5 mg L^{-1}NAA were used for callus induction. We found out that 2 mg L^{-1} 2, 4-D, 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA is the best combination of plant growth regulators for callus induction. After callogenesis the calli were shifted to regeneration medium containing antibiotics 100 mg L^{-1} kanamycin, 300 mg L^{-1} cefotaxime and different concentrations and combinations of plant growth regulators (Table 2) ranging from 1-3 mg L^{-1} 2, 4-D, 0.1-0.5 mg L^{-1} BAP in the presence of 20 µM $AgNO_3$.The calli showed good response to shoot regeneration when 2,4-D was used at a concentration of 2 mgL^{-1}in combination with BAP at a concentration of 0.5 mgL^{-1}. The shoots started to develop after 6-7 weeks (Fig. 3).Apart from the above mentioned growth regulators, the shooting medium also contained 100 mgL^{-1} Kanamycin and 300 mgL^{-1}cefotaxime. After the formation of shoot, rooting was achieved by shifting the transformed calli to rooting medium (2 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} NAA) to regenerate whole plants (Fig. 5). The regenerated whole plants were then shifted to soil for acclimatization (Fig. 6).

PCR Analysis

Genomic DNA was extracted from independently obtained kanamycin resistant callusing and subjected to PCR with oligonucleotide specific primers for *NIC* gene. The result of PCR using kanamycin resistant calli gave the expected product of the right size for *NIC* gene (Fig. 4). The same was applied for the genomic DNA extracted from the regenerated transgenic shoots. The transgenic nature of the independently regenerated shoots was confirmed by the amplified DNA product of the expected size for the *NIC* gene using *NIC* gene specific primers (Fig. 7).

Discussion

In vitro morphogenic responses of explants cultured on media are affected by different combinations and concentrations of plant growth regulators. Plant growth regulators are organic substances formed in different parts of the plant and transported to other sites where they stimulate specific biochemical, physiological and morphological responses. Among the various combinations and concentrations of plant growth regulators, used for

Table 3: Standardized conditions for PCR

Stages	Initial denaturation temperature	Denaturation temperature	Annealing temperature	Extension temperature	Final extension	Cycle	Total cycles
Stage1	94°C, 1min					1	30
Stage2		94°C, 30 sec	59°C, 30 sec	72°C, 1 min			
Stage3					72°C, 5 min	1	

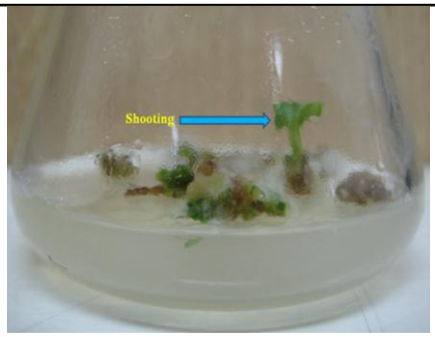

Fig. 3: Shoot regeneration from transformed callus on shooting medium (2 mg L^{-1} 2, 4-D, 0.5 mgvL^{-1} BAP)

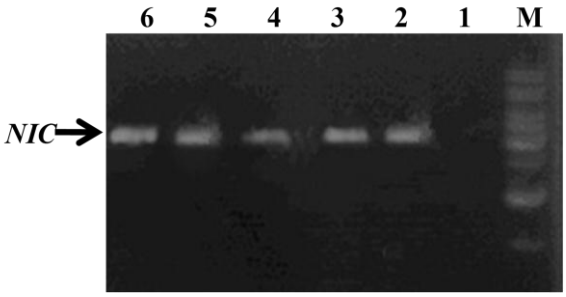

Fig. 4: PCR analysis of the transformed and non-transformed calli. Lanes M: I Kb Ladder, 1: non-transformed, 2-6: independent transgenic calli

Fig. 5: Regeneration of whole plants from transgenic calli on regeneration medium

Fig. 6: Transgenic plants in soil for acclimatization. Plants were shifted to soil and initially covered with plastic bags for acclimatization

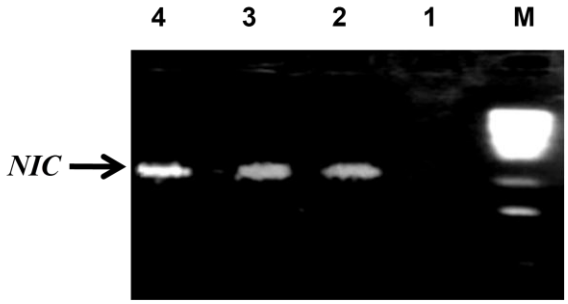

Fig. 7:PCR analysis of the transformed and non-transformed shoots. Lanes M: I Kb Ladder, 1: non-transformed, 2-4: independent transgenic plants

callus induction in *B. napus* cultivar Abasyn-95, 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA were found to be the best among all tested combinations and concentrations for callus induction. Similar concentrations of 2, 4-D has been reported for callus induction in *B. napus* (Al-Naggar *et al*., 2010; Hongbo *et al*., 2010) while some others have reported BAP and NAA for callus induction in *B. napus*. Since *in vitro* tissue culture responses are highly genotype dependent (Sparrow *et al*., 2004), it is therefore conceivable that hormonal requirements are different for different genotypes. In contrast to these, Bano *et al*. (2010) and Khan *et al*. (2010) obtained callus by using BAP and NAA. In this particular study shoots were regenerated from transgenic calli by using 2 mg L^{-1} 2, 4-D and 0.5 mg L^{-1}

BAP. Similar results were obtained by Ali *et al*. (2007), while some others have reported 4 mg L^{-1} BA and 0.04 mg L^{-1}NAA for regeneration in *B. napus* (Kong *et al*., 2010), presumably due to different genotypes of *B. napus* used in these experiments.

Since different explants have different potential for callogenesis or organogenesis, transformation and regeneration therefore, requires complete assessment of media, growth regulators and culture conditions. Different kinds of explants such as hypocotyls, cotyledons, peduncle, pedicel, leaf, stem sections and inflorescence have been used by different researchers for organogenesis and callogenesis. Based on our previous experience with *Brassica species* (Attache and Khan, 2011; Bangash and Khan, 2011) transformation and other reports (Babic *et al*., 1998; Cardoza and Stewart, 2004; Jonoubi *et al*., 2005; Ali *et al*., 2007),hypocotyls and cotyledons were used as explants since they gave better tissue culture responses. The use of leaves as explants in *B. campestris* transformation in the past has ended up with no shoot regeneration (Attache and Khan, 2011). Similarly the age of explants is also an important determinant in plant tissue culturing. Young tissues consisting of rapidly dividing meristematic cells are potentially the best explants in most of the cases. Hypocotyls and cotyledons from10-14 day old seedlings in this study gave far better responses in terms of shoots regeneration compared to our previous results where leaves from 3 months old *B. campestris* plant genotypes were used (Attache and Khan, 2011). In this context our results are in line with many of the previous reports who have used explants of approximately the same age (Damgaard *et al*., 1997; Cardoza and Stewart, 2003; Al-Naggar *et al*., 2010; Hongbo *et al*., 2010; Kong *et al*., 2010; Tang *et al*., 2011).

As compared to direct use of explants for transformation, the explants pre-cultured for 48-72 h before co-cultivation produced high number of calli in our study. During this study explants were pre-cultured for 48 h before co-cultivation. Pre culturing for 48 h have also been reported by Cardoza and Stewart (2003), Jonoubi *et al*. (2005) and Khan *et al*. (2010). In contrast to this, Tang *et al*. (2011) pre-conditioned the explants for 3-7 days. The dissimilarity in pre-culturing period may be due to the difference in the age of the explants used and also different explants used. Co-infection time is a key factor in transformation. The explants co-infected for 8-10 min showed high transformation efficiency in our study. This result is in agreement with Tang *et al*. (2011) and Khan *et al*. (2010). In contrast to this, Mashayekhi *et al*. (2008) gave 5 min for co-infection.

In our study the transformed callus did not produce any shoots on regeneration media without silver nitrate ($AgNO_3$). With the addition of 20 µM $AgNO_3$ in to regeneration media, the shoots were produced successfully. The positive influence of $AgNO_3$on shoot regeneration of different *Brassica* sp. have been reported (Chi *et al*., 1990;Pua and Chi, 1993; Burnett *et al*., 1994; Zhang *et al*., 1998).The mode of action of $AgNO_3$in plant tissue culture is considered to be related with the physiological effects of ethylene with silver ions. $AgNO_3$ is a potent inhibitor of ethylene action and ethylene is believed to suppress shoot morphogenesis *in vitro* (Zhang *et al*., 2001).

In conclusion, hypocotyls and cotyledons taken as explants from young seedlings are the most suitable explants in terms of producing transgenic calli and shoots. Our studies further suggest that medium containing 2 mg L^{-1} 2,4-D, 0.5 mg L^{-1}BAP and 0.1 mg L^{-1} NAA is the best combination of plant growth regulators for callus induction. Similarly, 2 mg L^{-1} 2,4-D in combination with BAP at a concentration of 0.5 mg L^{-1} supplemented with 20 µM $AgNO_3$ was found to be the most suitable combination for shoot regeneration in Abasyn-95. The above mentioned concentration and combinations of hormones therefore can be used as base line information for future transformation programs of *B. napus* in general and for Abasyn-95 and closely related genotypes in particular.

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