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AGROBACTERIUM MEDIATED TRANSFORMATION OF *BRASSICA JUNCEA* (L.) CZERN. WITH *CHITINASE* GENE CONFERRING RESISTANCE AGAINST FUNGAL INFECTIONS

BASHIR AHMAD[1*], AMBREEN[1], MUHAMMAD SAYYAR KHAN[2] ALI HAIDER[3] AND IBRAR KHAN[1]

[1]*Centre of Biotechnology and Microbiology, University of Peshawar, Khyber PakhtunKhwa, Pakistan*
[2]*Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar, Pakistan.*
[3]*PGMI Lady Reading Hospital Peshawar, Pakistan*
[*]*Corresponding author e-mail:* * bashirdr2001@yahoo.com

Abstract

Brassica juncea (Czern & Coss., L.) is an important oilseed crop. Since it is attacked by several bacterial and fungal diseases, therefore, we developed an easy and simple protocol for the regeneration and transformation of *B. juncea* variety RAYA ANMOL to give rise to transgenic plants conferring resistance against various fungal diseases. The transformation was carried out using *Agrobacterium* with *Chitinase* gene. This gene was isolated from *Streptomyces griseus* HUT6037. We used two types of explants for transformation i.e. hypocotyls and cotyledons. Only hypocotyls explants showed good results regarding callus initiation. Different hormonal concentrations were applied i.e. BAP 2, 4 and 6 mgL^{-1} and NAA 0.1, 0.2 and 0.3 mgL^{-1}. However, high transformation efficiency was observed by supplementing the medium with combination of 2 mgL^{-1} BAP and 0.2 mgL^{-1} for initiation of callus. Similarly 10 mgL^{-1} kanamycin and 200 mgL^{-1} cefotaxime also proved successful for the selection of transformed callus. In order to confirm the presence of transgenic callus Polymerase chain reaction was performed using specific primers for *Chitinase* gene.

Key words: *Agrobacterium*, *Brassica juncea*, *Chitinase* gene, Fungal infections.

Introduction

Brassica is considered as an imperative genus in *Brassicaceae* family. Its species have gained much importance primarily because of foremost improvements in the seed oil and quality of meal. In *Brassica*, a number of innovative phytochemicals are present some of which are useful against cancer (Steinmetz & Potter, 1996). *Brassica juncea* is an important species in the genus *Brassica* (Woods *et al*., 1991; Gentinet *et al*., 1996; Turi *et al*., 2012). It takes up small levels of heavy metals such as Nickel (Ni), Zinc (Zn), Cadmium (Cd), Lead (Pb), Copper (Cu), Sulphur (S), Boron (B) and Chromium (Cr) etc. therefore partially effective for the remediation of the soil that has been polluted with heavy metals (Ebbs & Kochian, 1997; Epstein *et al*., 1999; Jiang *et al*., 2004).

Due to scarcity of edible oil in Pakistan, large quantities of edible oil are imported to fulfill the domestic requirements (Ullah *et al*., 2014). A huge amount of Rs.168.36 billion was spent on the import of 2.49 million tons of edible oil and 0.638 million tons of oilseeds during 2008-09 (Anonymous, 2010). Rapeseed and mustard (belonging to *Brassica* genus) are traditional oilseed crops of Pakistan and contribute over 16 % to the domestic oilseed production, however, the yield per acre is very low due to many reasons but lack of high yielding varieties is the most important one (Khan *et al*., 2014). The national average yield of rapeseed and mustard is quite low (812 kg/ha) and the average in Khyber Pakhtunkhwa (KP) is the lowest (418 kg/ha) of the four provinces (Anonymous, 2010**)**. Thus, development of disease resistant and high yielding varieties of oilseed *Brassica* is required to increase the local production of oilseed in the country.

Plants experiences different environmental stresses during their lifecycle. These stresses include temperature, water, salinity, ultraviolet rays, metal toxicity stress and diseases etc. (Shinwari *et al*., 1998). Depending on the level of these stresses, the growth and yield of the plant is severely affected. Therefore plants have modified themselves in such a way to level down the degree of these stresses (Nakashima *et al*., 2000). Therefore different approaches have been applied to control different diseases caused by the fungal pathogens for example (1) Traditional breeding is used to create *Brassica* varieties which may possibly provide resistance against fungal infections but there is no such variety that confers resistance against these fungal infections at a satisfactory degree, mainly because of the lack of suitable resistant donors (Pradhan *et al*., 1993; Khan *et al*., 2012), (2) Chemical method is used to control spreading of the disease but it is very costly and eco-unfriendly. It is also not enough to fight against the new emerging pathophytes. Similarly use of pesticides is useful but its severe use causes health problems and environmental pollution (Singh, 2008) and (3) Genetic Engineering techniques are the use of plant biotechnology methods to overcome the limitations caused by conventional breeding (Narusaka *et al*., 2003). Through the use of different biotechnology techniques, crop yield and nutrient values can be improved. Crops can be genetically engineered to produce such plants that help in resistance against abiotic stresses, various diseases, and insect pests. As a result of improvement in biotechnology in the past ten years, various genes have been isolated, identified and modified in order to study the structure and function of genes (Kidokoro *et al*., 2009). Such tailored genes are inserted into specific recipients to get the desired plants. Hence different plants were transformed using different transformation methods. These are protoplast electroporation (Bergman & Glimeius, 1993), *Agrobacterium* mediated (Narasimhulu *et al*., 1992) and biolistic transformation (Chen & Beverdorf, 1994). However, the most commonly used method for the transformation of dicotyledonous plants is *Agrobacterium* mediated DNA transfer. *Brassica juncea* is also manipulable to recombinant DNA technology (Grover &

Pental, 2003). *Agrobacterium tumefaciens*-mediated transformation is best because of its cost, ease and efficacy (Dutta *et al*., 2005, Barfield & Pua, 1991; Pental *et al*., 1993; Das *et al*., 2006). This biotechnological technique has been practiced for the development of *B. juncea* in the fields such as fight against herbicides, to improve oil quality (Das *et al*., 2006; Hong *et al*., 2002; Sivaraman *et al*., 2004; Zada *et al*., 2013, 2013a) development of cross breed seed (Jagannath *et al*., 2002), fight against insect pest (Kanrar *et al*., 2002), phytoremediation (Zhu *et al*., 1999) and salt tolerance (Zhang *et al*., 2001).

Chitinase is an enzyme which is a pathogenesis-related protein catalyzing the degradation of chitin **(**Boller, 1985**)**. Chitinases, a glycosyl hydrolase, have two domains i.e. chitin-binding domain and catalytic domain (Collinge *et al*., 1993). Chitinase C *(ChiC)* was first discovered in *Streptomyces griseus* HUT6037 (Itoh *et al*., 2006). This gene was expressed in different transgenic *Brassica* providing resistance against fungal infections (Broglie *et al*., 1991; Chatterjee & Mondal, 2000; Nishizawa *et al*., 1999). This research was intended to check whether the introduction of such antifungal proteins would provide any physiological advantage in terms of enhance resistance against selected fungal pathogens. *Brassica juncea* was transformed with *chitinase* gene through *Agrobacterium* and confirmation was carried out by PCR using *ChiC* gene specific primers.

Materials and Methods

Seeds of *Brassica juncea* variety RAYA ANMOL were provided by Nuclear Institute for Food and Agriculture (NIFA) Peshawar, Pakistan. The complete experiments were carried out *In vitro*. Seeds were first surface sterilized with tap water 3-4 times and then with distilled water. The seeds were then soaked in ethanol (70%) for 30 sec. They were surface sterilized with 1-2% Na-hypochlorite solution containing 2-3 drops TWEEN-20 for 10 minutes. At last they were washed with double distilled water inside Laminar Flow Unit (LFU).

After sterilization the seeds were cultured on half strength Murashige & Skoog (MS) media. Each flask (100ml) contained 20-25 seeds. All the flasks were covered and kept in growth room for germination. The temperature of the growth room was maintained at 23 ± 1°C.

The hypocotyls and cotyledons of 5 to 7 days old were used as explants for pre-culturing. The media used for pre-culturing was MS media supplemented with different concentrations of 6-Benzylaminopurine (BAP) i.e., 2, 4 and 6 mgL^{-1} and Naphthalene acetic acid (NAA) i.e., 0.1, 0.2 and 0.3 mgL^{-1}. Thirty plates were used for pre-culturing and 10-15 explants were pre-cultured on each plate. The total numbers of explants on all plates were 450.

The *Agrobacterium tumefaciens* EHA101 harboring the binary vector *pEKB/ChiC* was used for inoculum preparation. This strain was then used to infect *Brassica juncea* explants. Plant materials used for infection were (1) Cotyledons and (2) Hypocotyls. The precultured explants were dipped in transformation solution for 10 minutes for infection.

The explants were placed on sterilized filter paper for drying. After drying they were moved to MS medium containing 30g sucrose, 2 mgL^{-1}, 4 mgl^{-1} and 6 mgL^{-1} BAP and 0.1 mgL^{-1}, 0.2 mgL^{-1}, 0.3 mgL^{-1} NAA and 8 gL^{-1} agar. Controls were also cultured on MS medium but they were not infected with *Agrobacterium*. All the co-cultivated and control explants were sealed with aluminium foil and positioned in dark for 2-3 days in culture room at 23 ± 1°C.

Subsequent co-cultivation of 2-3 days all the infected and control explants were shifted to selection medium provided with different concentrations of BAP (2, 4 and 6) mgL^{-1} and NAA (0.1, 0.2 and 0.3) mgL^{-1} and 200 mgL^{-1} cefotaxime as a bactericide and 10 mgL^{-1} kanamycin as a selective chemical. These explants were left at culture room condition for 2 weeks with biweekly subculturing to fresh medium.

After two weeks, initiation of callus induction was observed on selection medium. A small piece from this callus was taken and subjected to PCR for inspection whereas the left over part of the callus was cultured on shooting medium for shoot regeneration. It was also observed that the control explants died on selection media after few weeks.

Polymerase chain reaction (PCR) was used to select transformants for *chitinase* gene (*ChiC*). Cetyl trimethylammonium bromide (CTAB) method was used for the extraction the genomic DNA from kanamycin-resistant as well as from the control *Brassica juncea* (Rogers & Bendich 1988). In order to perform PCR analysis genomic DNA and *ChiC* primers were used. The sequences of primers are as follows.

Forward ChiC1 5′-CGGGATCCGTCATGAGTCTGCTGGTCGC-3′
Reverse ChiC2 5′-ACGCGTCGACATCAGCAGCTCAGGTTCGGAC-3

Results and Discussion

In the present work an attempt was made to develop a protocol for the transformation of *Brassica juncea* variety RAYA ANMOL with *chitinase* gene using *Agrobacterium* mediated transformation. Successful transformation not only requires well established tissue culture system but also a stable and effective resistance of the engineered plants across the environment. For this reason, different conditions were optimized for the transformation of *Brassica juncea*.

For expression of *ChiC* in *B. juncea,* explants were transformed through *Agrobacterium* using transformation vector in which *chitinase* gene expression was driven by cauliflower mosaic virus 35S promoter as shown in Fig. 1.

In order to increase transformation efficiency, preculturing of explants played a fundamental role. A higher number of transgenic callus were obtained when explants precultured for 24-48 hours (Fig. 2). However explants conditioned for 72 hours decreased the efficiency. Preculturing for 2 days have also been reported (Babic *et al*., 1998; Wang *et al*., 2005). In contrast preculturing for 72 hrs has also been reported (Valvekens *et al*., 1988). Similarly co-infection was also considered important. Higher transformation efficiency was observed when co-infection time was 10 min. On the other hand when co-infection time was increased from 10 min, transformation efficiency was decreased.

Efficiency of transformation was also co related with co-cultivation. Co-cultivation period of 3-5 days was applied and it was observed that co-cultivation time of 2 days was the best. Increasing co-cultivation time greatly affected tissues of explants. Excessive growth of *Agrobacterium* was observed when co-cultivation was increased from more than two days. When explants were co-cultivated for two days, less growth of *Agrobacterium* was seen. Many reports were received on co-cultivation period of various transgenic plants. Co-cultivation of 2 days has been reported (Wenck *et al*., 1999; Xiang *et al*., 2000). On the contrary co-cultivation for 3 days has been used (Miguel & Oliviera 1999). Likewise explants were co-cultivated for 3-5 days. But explants co-cultivated for 2 days were found to be sufficient for transformation of *Brassica juncea*. Longer co-cultivation period can result in necrosis of explants due to excessive growth of bacteria (Khan *et al*., 2003).

After co-cultivation, the explants were shifted to selection medium and checked for callus induction. Following three weeks callus was induced at the cut plane of the explants as shown in the Fig. 3. However control explants on selection medium did not show any callus initiation.

Cotyledons and hypocotyls were provided with different concentrations of BAP i.e., 2 mgL^{-1}, 4 mgL^{-1} and 6 mgL^{-1} and NAA i.e. 0.1 mgL^{-1}, 0.2 mgL^{-1} and 0.3 mgL^{-1}. However, callus was only induced when MS medium was provided with a mixture of 2 mgL^{-1} BAP and 0.2 mgL^{-1} NAA. Thus this proved to be the best hormonal combination for callus initiation in *Brassica juncea*. Parallel consequences have been revealed in other reports (Muhammad *et al*., 2002).

Callus was induced from the cut end of the explants and ultimately extended all over the explants. It was also observed that hypocotyl explants showed rapid callus initiation as compared to cotyledon explants. Similarly white, thick and hairy adventitious roots also developed from the explants.

Genomic DNA was isolated from control and transgenic callus obtained on selective medium by using CTAB method of DNA extraction. PCR was performed in order to confirm the presence of chitinase gen integration in transgenic callus. For negative control the DNA from transgenic and control was used whereas for positive control the plasmid DNA from *Agrobacterium* was used. Successful transformation was achieved in callus as shown by the presence of the PCR product in the transformed calli (Fig. 5).

After three weeks control explants died on the selection medium as shown in Fig. 4C. Explants on medium having BAP 4 mgL^{-1} and 6 mgL^{-1} with NAA 0.1 and 0.3 mgL^{-1} showed not as much of callus initiation. However explants supplemented with concentration of BAP 2 $mgL^{-1}$and NAA 0.2 mgL^{-1} showed fast callus initiation and maximum callus development as shown in Fig. 4 A& B.

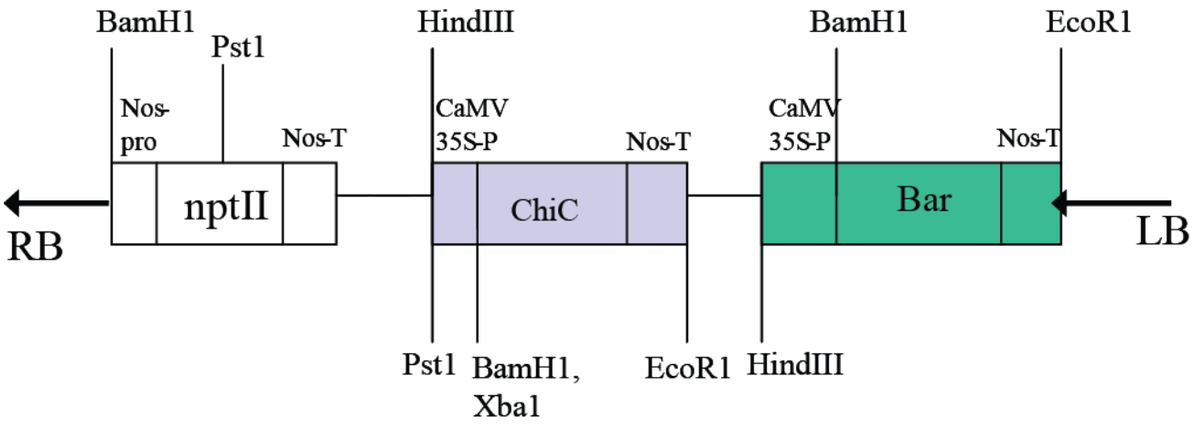

Fig. 1. Construct of *Chitinase* gene, *Chitinase* and *bar* genes are in the T-DNA region of pEKH vector. Neomycin phosphotransferase (*nptII*) gene is driven by nopaline synthase promoter (*nos-p*) whereas the *bar* gene is driven by 35 S promoter of cauliflower mosaic virus.

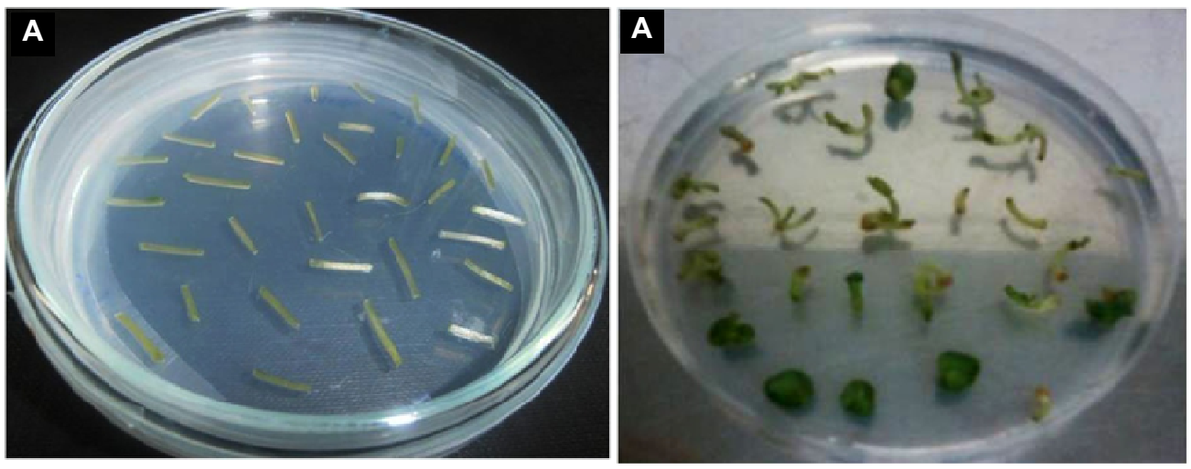

Fig. 2. Preculturing medium containing cotyledons and hypocotyls.

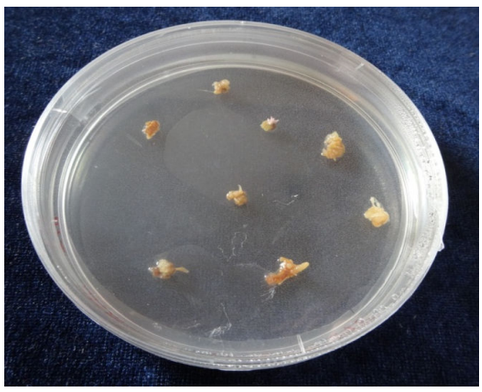

Fig. 3. Callus initiation after co-cultivation.

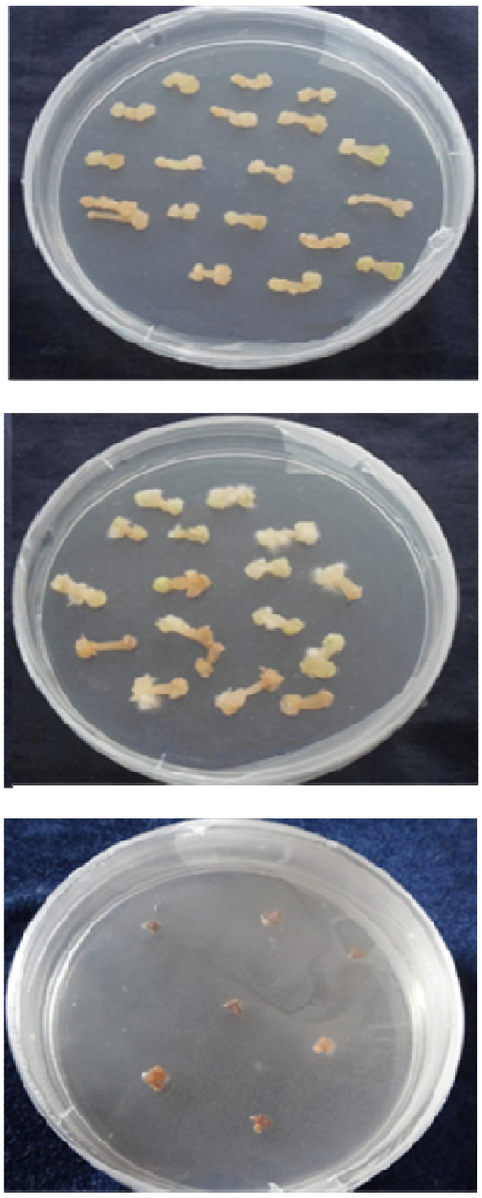

Fig. 4. Callus initiation on selection media. A and B are transformed callus and C is control callus.

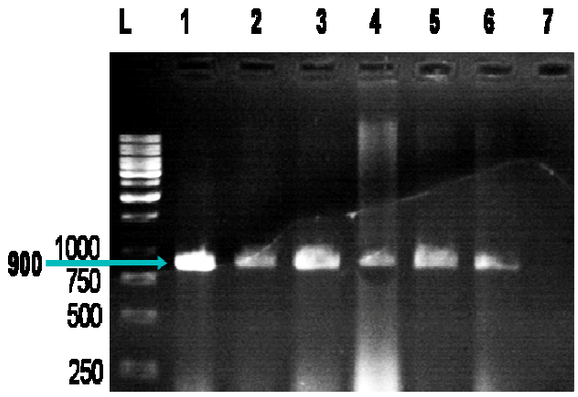

Fig. 5. PCR analysis of transformed calli.
L is 1 kb ladder. Lane 1 is positive control, i.e., Plasmid DNA from transformed *Agrobacterium* and 2-6 is amplification of *ChiC* gene (0.90 kb) in transgenic callus. Lane 7 is negative control (DNA from non-transformed callus)

Conclusion

We conclude from our experiments that hypocotyls explants are more responsive in terms of callus initiation and transformation. Our results indicated that among the different hormonal concentrations and combinations test during this study, 2 mgL^{-1} BAP and 0.2 mgL^{-1} was the best concentration and combination of hormones initiation of callus in transformed explants. PCR analyses revealed that *chic* gene has been successfully integrated into the genome of the transformed calli as shown by the presence of transgene specific PCR product in the transformed calli.

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