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OPTIMIZATION OF AN EFFICIENT NON-TISSUE CULTURE TRANSFORMATION METHOD FOR *BRASSICA JUNCEA*

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Abstract

The major hurdles in successful *in vitro* transformation of *Brassica juncea* through standard tissue culture (STC) method are: culture contamination, somaclonal variations, and lack of expertise. Moreover, the current STC method is time consuming and needs continuous electricity. In the present study, the *in planta* transformation method through floral dip with or without vacuum infiltration was optimized for successful transformation of *B. juncea*. The *B. juncea* CV "RAYA Anmol" was used for transformation through *Agrobacterium tumefaciens* strain *GV3101* harboring the binary vector plasmid *pBinGlyBar4-EADcT*. Based on the resistance reaction to the herbicide Basta, 20 and 40 resistant seedlings were obtained from ~2000 seed germinated from the plants transformed through floral dip and vacuum infiltration methods, respectively. The PCR analyses further confirmed the presence of transgene in 3 floral dipped plants without vacuum infiltration and 17 floral dipped plants with vacuum infiltration, giving the transformation frequencies of $1.5x10^{-3}$ and $8.5x10^{-3}$, respectively. This method, which avoids tissue culture, will reduce the somaclonal variation accompanying prolonged culture of cells in a dedifferentiated state, will facilitate functional genomics and improvement of *Brassica juncea* with novel desirable traits while reducing time and expense.

Key words: Transformation, *Brassica juncea*, Facile floral dip, Vacuum infiltration.

Introduction

The genus *Brassica* includes many important crop species, including *Brassica juncea*. *Brassica juncea* is an important oilseed crop globally. In Pakistan, rapeseed and mustard is the second most important source of oil after cotton (Abbas *et al*., 2009). However, the low oil production and poor seed biochemical characteristics, which are the major obstacles in increasing the area under rapeseed cultivation, need to be addressed. Quality traits like oil, protein and glucosinolate contents as well as fatty-acid can be modified by classical breeding (Nasim & Farhatullah, 2013; Sidra *et al*., 2014) and genetic approaches (Mahwish *et al*., 2014). Molecular breeding and transformation technology are the two main strategies for its improvement.

Standardizing plant regeneration, selection of an efficient transformation method and gene of interest are the main requirements for genetic modifications in many crop (Gul *et al*., 2015). May transformation methods have been employed for the production of fertile transgenic plants of *Brassica* species. These include *Agrobacterium*-mediated (Narasimhulu *et al*., 1992) electroporation of protoplast (Bergman & Glimelius, 1993) and biolistic transformation (Chen & Beversdorf, 1994). However, the A*grobacterium tumefaciens* mediated transformation is preferred over others because of its simplicity and cost effectiveness (Das *et al*., 2006). This method of transformation also has increased chances of single copy transgene integration to transgenic plants (Hiei *et al*., 1997) and has reduced multiple copy transfer which often leads to gene silencing through RNAi (Bhalla & Singh, 2008). Several agronomically important genes have been identified in the *Brassica* species and some from other organisms have also been transferred using these transformation methods.

Agrobacterium mediated genetic transformation is most widely used for *Brassica* and it is generally quite efficient and practical for most species in the genus. However, there are some problems such as the time consuming *In vitro* regeneration step, labor-intensive, genotype dependent response to tissue culture practices and the requirement of highly skilled personnel. Also the regenerated plants might exhibit some undesirable somaclonal variation along with reduced fertility (Curtis, 2003; Jain, 2001). These problems may complicate the function or regulation of the introduced genes (Chang *et al*., 1994). It is, therefore, highly desirable to develop a plant transformation procedure that eliminates the *In vitro* regeneration step. In the attempts, scientists developed a successful *Agrobacterium*-mediated gene transfer to Arabidopsis using vacuum infiltration (Bent, 2000). Many other plants such as canola (Wang *et al*., 2003) radish (Park *et al*., 2005), *Notocactus scopa* (Seol *et al*., 2008) and rice (Lin *et al*., 2009) were also successfully transformed using vacuum infiltration. This floral dip transformation approach has already been applied for the improvement of *Brassica 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 (Das *et al*., 2006; Hong *et al*., 2002) and aphid resistance (Kanrar *et al*., 2002, Dutta *et al*., 2005). However, due to the varietal differences that may exist, it is necessary to develop an optimized method for our local genotypes. For doing so, we aimed to optimize an easy non-tissue culture based transformation system for the genetic modification of local *Brassica juncea* genotype. This method of transformation can be used to modify plants in short time with less expertise.

Material and Method

Plant material: A *Brassica Juncea* CV genotype "RAYA Anmol" was used for all experiments. For transformation, seeds were directly germinated in 9 cm pots filled with the mixture of soil, vermiculite and horticulture (Clinton, OK, USA) (4:2:1) along with ½ spoon osmocot in the greenhouse. The growth conditions in the greenhouse were 21/16^{o}C (day/night) with natural lighting. The relatively humidity was maintained at 60%.

***Agrobacterium tumefaciens* strain and binary vector:** The *Agrobacterium tumefaciens* strain *GV3101* harboring the binary vector plasmid *pBinGlyBar4-EADcT* was used for the transformation of *Brassica* plants. The plasmid, *pBinGlyBar4-EADcT*, harboring the chimeric *EaDAcT* gene isolated from *Euonymus alatus* (burning bush) plant (Durrett *et al*., 2010), encoding diacylglycerol acetyltransferase with kanamycin and Basta resistance genes for selection, was constructed and transferred into *Agrobacterium tumefacien*s strain *GV3101*.

***Agrobacterium* transformation:** For transformation of Agrobacterium, frozen competent cells of *Agrobacterium tumefacients* were thawed on ice. About 100µL competent cells with 100ng of plasmid DNA were taken in an electroporation cuvette and an electric pulse of 2.4 kV was given for a brief period of 5 milliseconds. After electroporation, the transformed *Agrobacterium* cells were removed and mixed with ~1ml YEP. The YEP added Transformed cells were then grown by shaking at 28^{o}C for about 3 hours. Then serial dilutions of the transformed cells were plated on YEP medium with triple antibiotics selection having kanamycin sulphate (50mgL^{-1}), Gentamycin (50mgL^{-1}) and Rifampicin (25mgL^{-1}). The antibiotics resistant bacterial colonies were confirmed through PCR using *EaDaCT*gene specific primers.

***Agrobacterium* inoculum preparation and vacuum infiltration transformation:** A single transformed Agrobacteriumcolony was inoculated in a culture tube containing 5 ml YEP medium (10 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, pH6.8), supplemented with 50 mg/ml kanamycin. From overnight culture, about 1.6ml was transferred into a 2l flask containing 160 ml of the same medium and incubated overnight at 28^{o}C. Bacterial cells were harvested by centrifugation at 6,000 rpm for 20 min and the pellet was suspended in the in-filtration medium, consisting of half- strength MS salts, 50 g/l sucrose and 0.05% (v/v) Silwet L77 (Lehle Seeds, Round Rock, TX, USA). The pots with plants at the flowering stage were placed inside a 310 mm- height vacuum desiccator (Bel-Art, Pequannock, NJ, USA) and the inflorescences were immersed into the inoculum contained in a beaker. The vacuum was applied and held for 5 min at a pressure of 85 kPa (Fig. 1). The treated plants were covered by plastic bags and placed in dark for 24 h before returning to normal growth in greenhouse until maturity.

Floral dip transformation: In this method the brassica inflorescences were directly dipped in infiltration medium without using vacuum desiccator. *Brassica juncea* plants at the early flowering stages (Fig. 2) were used for transformation. The flower buds were submerged for 1–3 min in Agrobacterium-containing solutions without using vacuum desiccator.

Fig. 1 Infected *Brassica juncea* plants in desiccator.

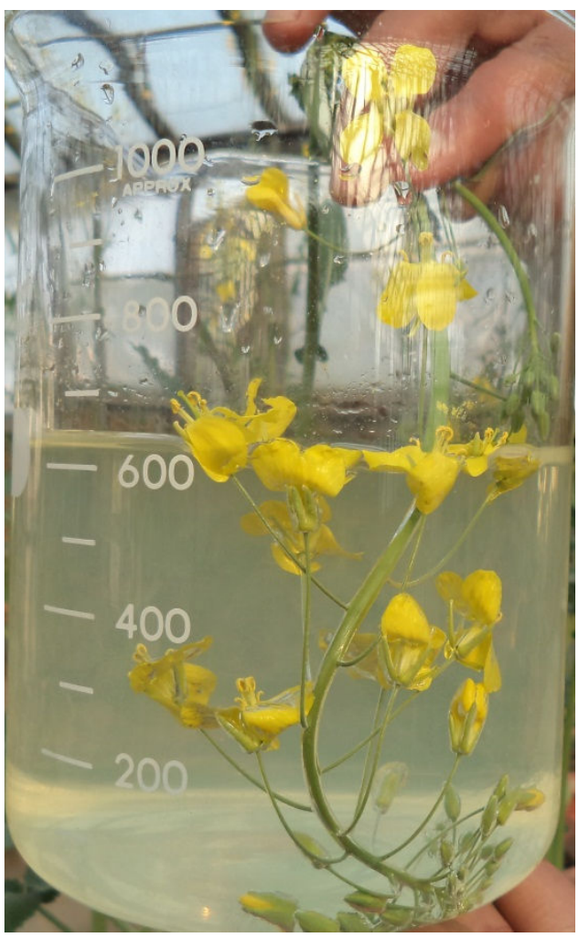

Fig. 2. Transformation using floral dip without desiccator.

Basta screening: Initially the efficiency of basta herbicide was checked by spraying three different basta concentrations, i.e. 0.1%, 0.2% and 0.3% on wild plants. All three were found to kill the wild type plants in different time spans. However, the 0.2% basta spray was the best for killing wild plants.

For screening, the *Brassica juncea* seeds of transgenic plants were germinated in soil and the plants were sprayed with 0.2% basta herbicide outside the growth chamber (Fig. 3B). Spraying was preformed after five days of germination and repeated five times at two days interval. The transgenic plants continued to grow while non-transgenic plants became white, remained small and died after 15 days of selection (Fig. 3C).

Molecular analysis of T_0 plants: Polymerase Chain Reaction (PCR) was employed to confirm the putative transformed plants. Genomic DNA from basta resistant and control *Brassica juncea* plants was extracted using cetyl-trimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1985). The PCR reaction was carried out in a mixture containing 50ng genomic DNA, 200µM dNTPs, 0.4 µM of each *EaDACT* specific primers (Table 1), 2.5mM $MgCl_2$,2µl taq polymerase buffer (10X) and 2.5 units Taq polymerase enzyme (Thermoscientific®, USA) with volume adjusted to 20µl using PCR grade water. The mixture was firstly heated at 95°C for the complete denaturation of genomic DNA and then amplified in a 30 cycled reaction with the thermal profile of 94°C for 30 sec, 57°C for 30sec and 72°C for 1min. A final extension at 72°C was given for 5min at the end of reaction.

Fig. 3. Floral dip transformation of *Brassica juncea*. **A.** Plants grown in green house for floral dip transformation. **B**. T_1 Plants grown from the seeds obtained. **C.** Application of basta spray on the grown T_1 plants. **D.** Showing the mature T_1 plants confirmed through PCR analysis.

Table 1. Primers used for the PCR amplification of target *EaDACT* gene in the putative transformed *Brassica* plants.

Name	Sequence	GC Contents	Tm (°C)	Expected size
EaDACT-F	ACCCAATTGATGATGGATGCTCATCAAGAG	44%	58.9	0.9KB
EaDACT-R	AGACCTGCAGGTTAAGCGTAATCTGGAACATC	47%	68.9	

Table 2. Summary of the *In planta* transformation using *Agrobacterium strain GV3101* harboring the binary vector pBinGlyBar4-EADcT.

Method	No of plants infected	No of seeds recovered	Germination frequency	No of plants resistant to basta	No of PCR confirmed plants	Transformation frequency
Floral dip	30	~2000	99%	25	3	$1.5x10^{-3}$
Vacuum infiltration	30	~2000	99%	40	17	$8.5x10^{-3}$

Results and Discussion

The vacuum infiltration method of *Agrobacterium* mediated transformation has successfully been used for genetic modifications in different model plants like Arabidopsis (Bechtold *et al*., 1993) and *B. napus* (Wang *et al*., 2003) bypassing tissue culture and regeneration of target plants. The method used for transformation of these species utilized vacuum infiltration of whole flowering plants with *Agrobacterium*, followed by collection of seeds and screening of transformed progeny by selection on media containing antibiotic or herbicide (Bechtold *et al*., 1993).

Initially for the *In planta* transformation of crops such as *B. napus*, plants were uprooted and then immersed in a container filled with an *Agrobacterium* suspension prior to vacuum infiltration (Wang *et al*., 2003). However, in our transformation method, we immersed the florescence of *Brassica juncea* in a suspension of *Agrobacterium GV3101* harboring the binary vector *pBinGlyBar4-EADcT* without uprooting. Transformation frequencies of the optimized methods ($1.5x10^{-3}$ and $8.5x10^{-3}$) were found higher than the transformation frequency in the harvested seeds over several years (1x10-4 to 3 x10 -4) (Xu *et al*., 2008).

PCR confirmation of plants: A total of 20 basta resistant plants were confirmed to have the introduced gene using PCR. Results of the PCR analysis of simple floral dip and vacuum infiltration are shown in Figs 4 and 5 respectively.

Transformation efficiency: The transformation efficiencies of both the simple floral dip and the vacuum infiltration were calculated. The vacuum infiltration was found to be more efficient as it gave more transgenic plants as compared to the normal floral dip (Table 2).

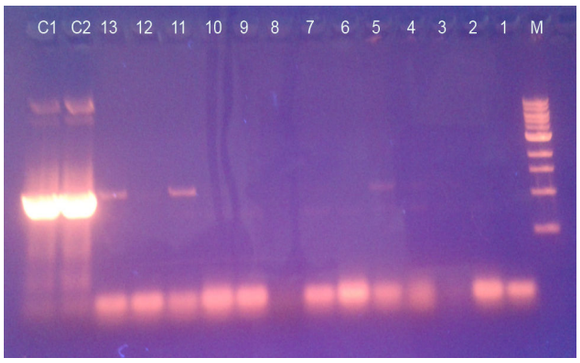

Fig. 4. Representing the PCR confirmation of the plants that were transformed using floral dip method of Agrobacterium mediated transformation. Lane M; 1kb DNA ladder, 1-13; plant samples; C1 and C2; plasmid controls.

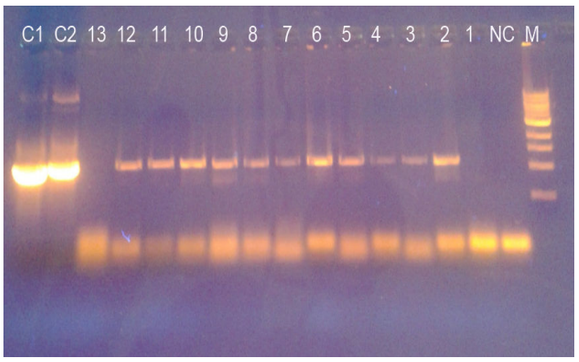

Fig. 5. Representing the PCR confirmation of plants that were transformed using vacuum infiltration method of Agrobacterium mediated transformation. Lane M; 1kb DNA ladder, NC; negative control, 1-13; plant samples, C1 and C2; plasmid controls.

The transformation frequency of both normal floral dip ($1.5x10^{-3}$) and vacuum infiltration method ($8.5x10^{-3}$) was found higher than many other studies reported like in a study on *Brassica rapa* that reported a transformation frequency in the range of $1x10^{-4}$ to 3 $x10^{-4}$in the harvested seeds (Xu *et al*., 2008).

Shoot regeneration is highly genotype dependent and the explants are sensitive to Agrobacterium (Pandian *et al*., 2006). Moreover, about 7–8 months is required for the production of transgenic *Brassica juncea* seeds using these tissue culture based methods with high expertise.

In the present study, we have successfully generated *Brassica juncea* transformants within 3 to 4 months with transformation frequencies of $1.5x10^{-3}$ and $8.5x10^{-3}$are almost as high as with other transformation methods (Clough and Bent, 1998, Curtis & Nam, 2001). Our new method, which avoids tissue culture, should reduce the somaclonal variation accompanying prolonged culture of cells in a dedifferentiated state and facilitate the functional genomics and improvement of Brassica with novel desirable traits while reducing time and expense.

Floral dip method by using vacuum infiltration optimized in this study has thus yielded higher transformation frequency as compared to that obtained by without vacuum infiltration method in *B. napus*. Mendelian segregation analysis revealed that many of the T_1 plants were single copy transgene. It is important to note that even though vacuum infiltration method of Arabidopsis transformation was developed in 1993(Bechtold *et al*., 1993), development of the floral dip method of Arabidopsis transformation by Clough & Bent (1998) has revolutionized the Arabidopsis transformation. Floral dip transformation being easy to perform requires less time, labor and cost and most importantly overcomes somaclonal variation related problems.

Conclusion

Here we have shown for the first time that the floral dip method can be successfully used for transformation by using vacuum infiltration in *Brassica juncea*. The facile floral dip transformation will facilitate transformation of oil seed *Brassica* species for functional genomics and crop improvement.

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