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GENETIC IMPROVEMENT OF CANOLA AGAINST ABIOTIC STRESS THROUGH INCORPORATION OF DREB GENE

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ABSTRACT

Salinity, sodicity, drought and water logging are serious problems of agriculture in Pakistan that add to poverty in the country. Pakistan, in spite of being a large agricultural country, imports huge amounts of edible oil. *Brassica* species in general, have good agronomic characteristics and under favorable conditions can give reasonable yields; however, under stress conditions their productivity is severely compromised. The main focus of this study is to develop salt and drought tolerant germplasm of *Brassica napus* var. wester by incorporating DREB gene that induces expression of stress tolerance genes which ultimately result in salt and drought tolerance of the variety. The paper describes the successful incorporation of DREB factor into *Brassica napus* var. wester genome that does seem to have regulated the expression of stress inducible genes as evidenced by increased content of proline in transformed plants when subjected to high salt level.
Keywords: *Brassica napus*, Transformation, *Agrobacterium tumefaciens*, DREB, Transcription factor

INTRODUCTION

Salinity, water scarcity and extreme temperature are drastic environmental stresses that adversely affect plant growth and decrease crop productivity. The detrimental effects of salinity are estimated to desolate about 20% of the irrigated land in the world (Yeo, 1999). This land mainly included arid and semi-arid areas which comprise 25% of the total land of our globe (Yamaguchi and Blumwald, 2005). Plants respond and acclimatize to these stresses at the cellular and molecular as well as biochemical and physiological levels.

At molecular level transcription factors play essential role in the gene regulation in plants, they bind to multiple target sequences in the genome and regulate multiple genes expression in a complex manner. Different genes such as radical-induced cell death1 (*rcd1*) binds with these transcription factors to induce the activity of abiotic stress tolerance genes (Anjum et al., 2015). Several abiotic stresses can induce the DREB gene to transcribe these factors which then bind to the DRE promoter element of stress related genes, and trigger their expression (Garg et al., 2008). The core sequence (TACCGACAT) of Dehydration Response Element (DRE) is recognized by the DREB subfamily proteins (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). The DRE related signature sequences have been found in the promoter region of wheat and *Brassica napus* (Canola) genes (Jiang et al., 1996; Ouellet et al., 1998; Qamarunnisa et al., 2012). The DREB proteins endure water balance in plant cells to make it abiotic stress tolerance by inducting a set of abiotic stress responsive genes (Haake et al., 2002; Kizis et al., 2002; Dubouzet et al., 2003).

Brassica napus is one of the most economically important crop species in the world. In addition to classical methods, genetic engineering techniques have made it possible to produce new varieties of canola with desired and improved characters. The worldwide demand for canola oil is encouraging more research in canola breeding for producing higher yielding varieties; conventional breeding techniques are time consuming and are not as specific as genetic engineering that also offers an alternate method for trait improvement (Cardoza and Stewart, 2004). Moreover, successful tissue culture is necessary for genetically modified cells to grow. Since techniques for tissue culture of canola have already been evolved, this species is an attractive candidate for use in transformation studies. Transformation of canola has been performed using various explants, however, hypocotyls remain the most attractive explants for transformation (Radke et al., 1988; De Block et al., 1989; Cardoza and Stewart, 2003).

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Canola transformation has been done using various techniques such as electroporation, biolistic gene gun and *Agrobacterium tumefaciens*-mediated transformation, the last considered the easier and cost effective. (Cardoza and Stewart, 2004).

In the present study, attempt was made to successfully transformed DREB factor into canola genome with high transformation efficiency and this makes possible the development of a larger number of salt/drought tolerant canola plants.

MATERIALS AND METHODS

Plant Material and Germination

Seeds of canola provided by Nuclear Institute of Agriculture (NIA) Tandojam were sterilized using 2.5% sodium hypochlorite followed by washing with sterile distilled water, germinated on MS medium and allowed to grow for 6 days.

Inoculation, Co-cultivation and Selection

The *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector of DREB gene with inducible *rd29* promoter, *nptII* and *uidA* gene was inoculated in Luria-Bertani medium in the presence of 100mg/l streptomycin and 50mg/l kanamycin at 28 ^{o}C in the dark until OD_{600} reached 0.5-0.8.

Hypocotyl explants (300) of canola var. wester were pre-cultured on callus induction medium including MS (Murashige and Skoog) medium added with 1mg/l 2,4-D for 3 days in dark. Another batch of 300 explants was also raised as control plants in which no transformation was carried out. After 3 days, one batch of 300 explants were inoculated with *Agrobacterium* suspension for five minutes, blotted dry on sterile filter paper and transferred on fresh callus induction medium for co-cultivation at 25 ^{o}C in dark for 2 days. After 2 days the hypocotyls were transferred onto selection medium containing callus induction medium supplemented with 50mg/l kanamycin and 500mg/l timentin. After 15 days, they were transferred to shoot induction MS medium with 0.5mg/l IAA, 2mg/l BAP, 500mg/l timentin and 50mg/l kanamycin. After 2 weeks, they were transferred to shoot elongation MS medium with 0.05mg/l BAP, 50mg/l kanamycin and 500mg/l timentin. Regenerated shoots were rooted on half strength MS medium containing 0.5mg/l IBA, 50mg/l kanamycin and 500mg/l timentin. Regeneration percentage and transformation efficiency was calculated.

Histochemical Assay

Transient expression of *gus* gene was assayed using Jefferson et al. (1987) method. Leaf segments from transformed plants were dipped in GUS staining solution and incubated at 37 ^{o}C for 24 hours and then bleached with ethanol. Beta-glucuronidase activity in transformed plants was analyzed.

Confirmation of Transformation

Transformation of DREB gene was confirmed by PCR analysis of DREB and *nptII* gene. Total genomic DNA was isolated from fresh leaves of transformed and control canola plants using standard CTAB method (Murray and Thompson, 1980). PCR was performed using specific primers of DREB gene and *nptII* gene in ABI 3100 thermal cycler.

Proline Determination

Proline content in control and transformed plants subjected to salt stress at 0mM, 75mM, 125mM, 175mM and 225mM were analyzed by Bates et al. (1973) method.

Table 1: Regeneration percent of non-transformed and transformed plants and transformation frequency

Genotype	No. of hypocotyls cultured	Callus inducing explants	Callus induction %	No. of shoot initiation	No. of regenerated plants	Regeneration %	No. of PCR positive plants	Transformation frequency
Wester Non-transformed	300	270	90	211	186	88	0	
Wester Transformed *(on selection media)	300	132	44	84	52	62	39	13

*Selection media include the basic media supplemented with 50 mg/l kanamycin

Figure 01: Callus induction from cut end of Brassica hypocotyls after 10 days on MS medium supplemented with 1 mg/l 2,4-D

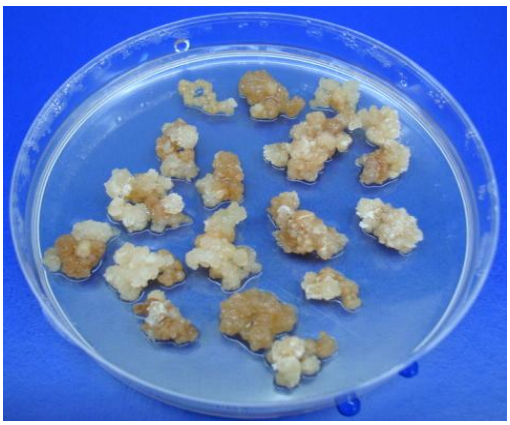

Figure 02: Plant cells received the *nptII* (kanamycin resistance gene) grew normally under selective pressure and cells continue to propagate while non-transformed cells became necrotic and dead.

Figure 03: Multiple shoots emergence on shoot induction medium under selection of kanamycin.

Figure 04: Shooted plants rooting on root induction medium

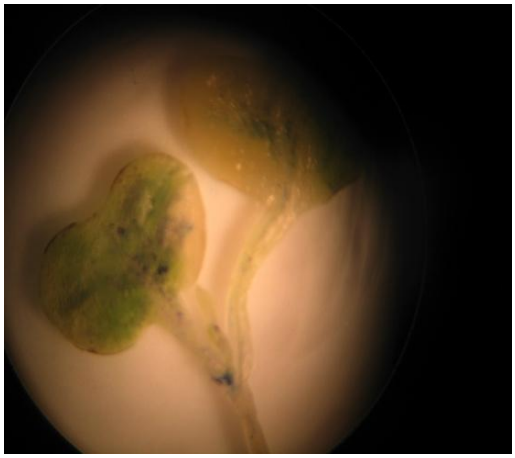

Figure 05: Histochemical assay of transformed plants. Blue spots showing transient expression of *gus* gene.

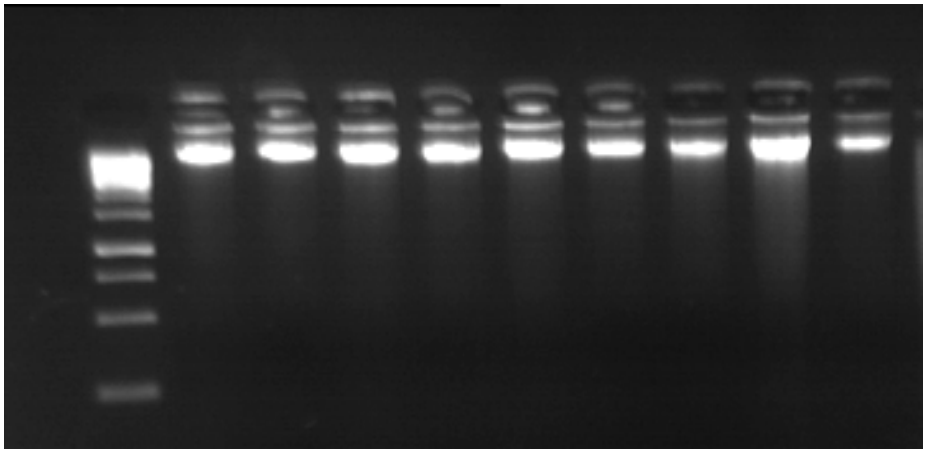

Figure 06: Gel electrophoresis of canola genomic DNA extracted by CTAB method along with 1 kb DNA marker in first lane

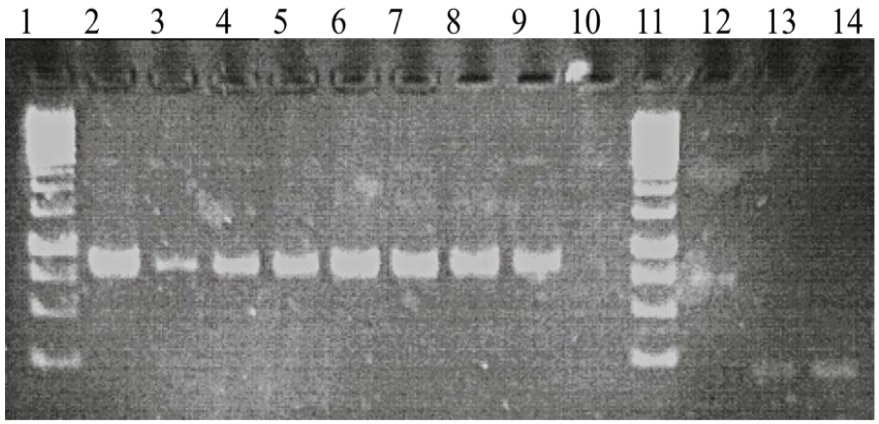

Figure 07a: PCR analysis of DREB gene in transformed and non-transformed plants. Lane 1: 1 kb DNA marker, Lane 2-9: transformed plant DNA showing expected fragment of DREB gene (800 bp), Lane 10: negative control, Lane 11: 1 kb DNA marker, Lane 12-14: non transformed plants DNA.

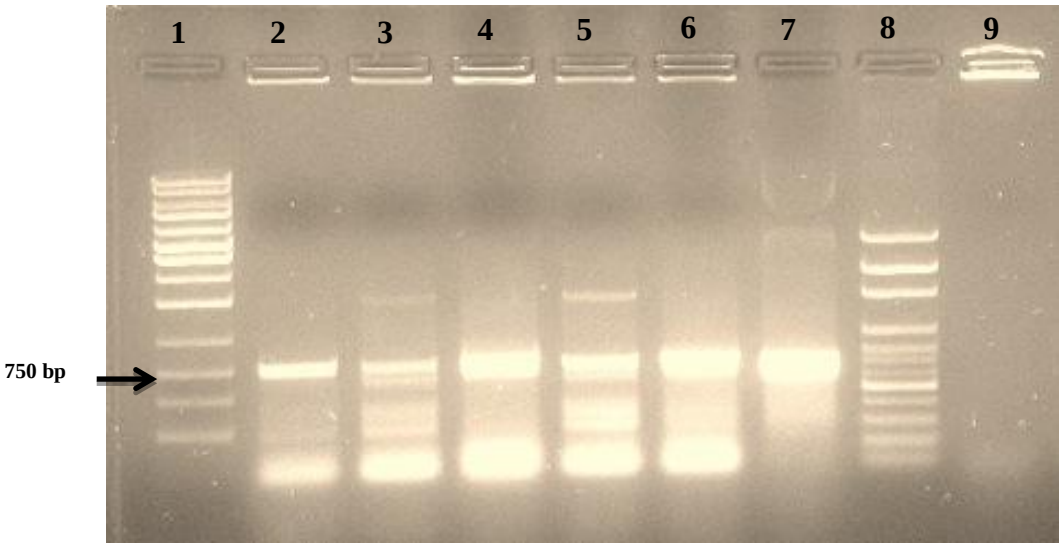

Figure 07b: PCR analysis of *nptII* gene in transformed and non-transformed plants. Lane 1 = 1kb DNA molecular weight marker, Lane 2 – 6 = transformed plants DNA showing expected band of 750 bp, Lane 7 = positive plasmid, Lane 8 = 100 bp DNA molecular weight marker and Lane 9 = non-transformed plant DNA

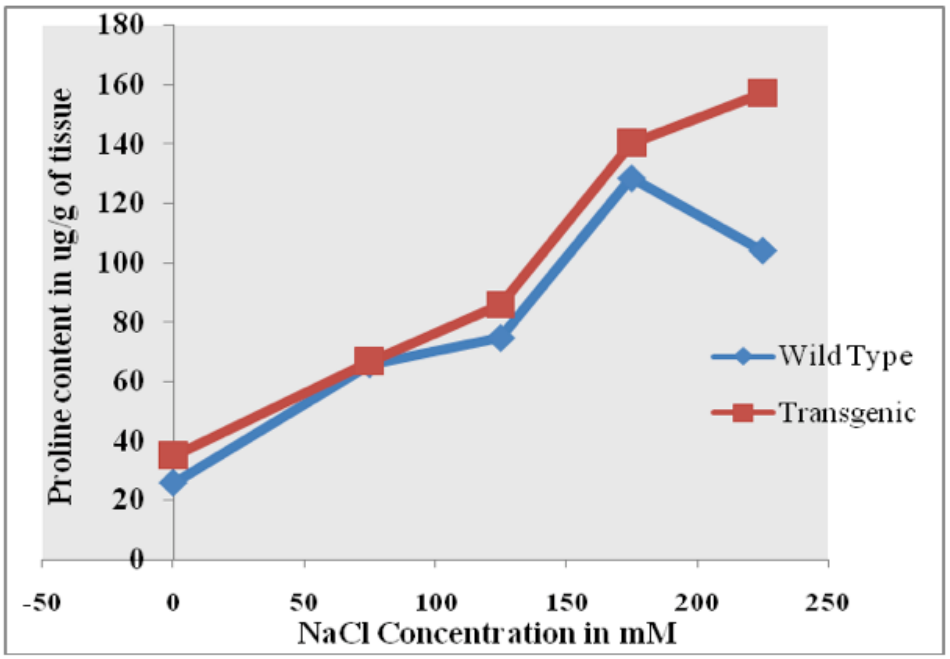

Figure 08: Proline content determination in transformed and non-transformed plants. Proline content was higher in transformed plants than non-transformed plants (wild type)

RESULTS

MS medium proved to be good for germination of canola seeds of var. wester and healthy hypocotyls were produced within 6 days. *Agrobacterium* strain LBA4404 with binary vector of DREB gene grew successfully under selective antibiotic pressure showed that the vector was present along with antibiotic marker. The callus induction medium used in this study was found to be efficient in inducing callus from cut end of hypocotyls within 10 days (Figure 01). After the calli were transferred to the shoot induction medium, the transformed callus was green and healthy, while the non-transformed was necrotic and brown (Figure 02). Timentin successfully controlled the growth of *Agrobacterium* and no more visible growth was observed in the presence of timentin. Multiple shoots

regenerated from the transformed callus within 2 weeks (Figure 03). Preconditioning of explants played a very critical role in increasing the transformation efficiency. Regenerated transformed shoots successfully rooted on root induction medium. All the shoots exhibited root within a month (Figure 04). Histochemical assay confirmed the expression of *gus* gene in transformed plants showing blue spots (Figure 05). Genomic DNA of canola was extracted using CTAB method and the gel showed heavy molecular weight, intact band of canola genome (Figure 06). PCR analysis of putative transformed plants showed the expected band size of 600 bp for DREB gene and 750 bp for *nptII* gene while in control plants no such amplification occurred (Figure 07a and 07b). The average shoot regeneration percentage in control and transformed explants was calculated and transformation efficiency was also calculated (Table I). Out of 300 explants 186 plants regenerated from non-transformed explants and 52 plants from transformed sectors. The regeneration percentage in non-transformed explants was found to be 88% while in transformed ones it was 62%. Transformation frequency was calculated to be 13%. Proline content in transformed plants was respectively higher when compared with non-transformed plants (Figure 08).

DISCUSSION

Environmental stresses derive in many forms but all of them do affect plant water status. Salt, drought and low temperature stress decrease the water content causing injury to the plant and under severe conditions, these abiotic stresses can result in plant death (Qiang et al., 2000). There is, indeed, a need to produce plants that are resistant to these environmental stresses. DREB transcription factors play a significant role in abiotic stress management by regulating the expression of many stress tolerance genes. Although a considerable amount of research has been conducted on cis-acting element DRE (Yamaguchi-Shinozaki and Shinozaki, 1994), however there is still a gap in detailed mechanisms by which the DREB1/CBF family is regulated. Different studies proposed that the stimulation of these genes is regulated at the transcriptional level, and it is possible that they are not subject to autoregulation (Gilmour et al., 1998: Medina et al., 1999).

In our study, callus from *Brassica* hypocotyls was produced within 10 days on callus induction medium. A similar result was also obtained by Muhammad et al. (2002a). These finding are also supported by the study of Turget et al. (1998). Shoot regeneration from callus became higher in the presence of higher amount of BAP (Narasimhulu and Chopra, 1998). Muhammad et al. (2002a) reported that maximum shoot regeneration from callus was achieved on the medium with 2.0mg/l BAP and 0.5mg/l IAA suggesting that exogenous BAP effectively promoted shoot induction in canola var. wester.

The most common and successful method for the transformation of *Brassica napus* is via *Agrobacterium* because brassica is a dicot plant and *Agrobacterium* hosts in nature are mostly dicot plants. There are some crucial factors that affect the generation of transgenic plants, such as pre-cultivation of explants, co-cultivation time, explant condition, inoculation time and OD of *Agrobacterium*. Improvement of transformation efficiency upon preconditioning of the explants has been observed in *Arabidopsis thaliana* (Schmidt and Willmitzer, 1985; Sangwan et al., 1992) and several other plants. The preconditioning of the explant has also been reported in *Brassica napus* (Ovesná et al., 1993). Our results also showed that preconditioning of hypocotyls enhance the transformation efficiency. Application of 2,4-D pretreatment before co-cultivation has been reported to increase callus growth (Janssens et al., 1995). The regeneration protocol of the present study was efficiently used to transform canola plant with DREB and *nptII* gene. However gene transformation frequencies cannot be compared with those reported by different researchers due to different genotype and explants used (Mashayekhi et al., 2008). Hypocotyl explant compared to cotyledon, is suggested to be more suitable for tissue culture and transformation because it has more potential of regeneration.

Multiple tools have been used to identify transgenic plants, including PCR, southern hybridization and many others. Among all methods PCR is the fastest and more sensitive method useful for preliminary analysis. It has been observed that insertion of single gene in the plant has advantage over multiple genes insertion, because the insertion of multiple

copies of T-DNA tends to reduce the expression of exogenous gene in host plant genome (Takasaki et al., 1997). The current study confirmed the presence of inserted genes into *Brassica napus* genome, which ultimately reported the successful transformation of DREB factor into *Brassica napus*.

Among various amino acids, the accumulation of proline is reported in many plants under stress conditions (Hare and Cress, 1997). However, the role of proline in stress tolerance accumulation is still subject of further research. Whether it acts as an osmo-protector (Csonka, 1989), osmo-regulator (Delauney and Verma, 1993) or a regulator of redox potential of cells (Bellinger and Larher, 1987) has not been decided. The elevated level of proline in transformed plants compared to control indicates the stimulation of abiotic stress tolerance genes in transformed *Brassica napus*. Therefore, the current study confirmed the successful incorporation of DREB gene into canola genome making it promising to be used at larger scale on salt/drought land.

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ABBREVIATIONS

DREB: Dehydration responsive element binding protein, PCR: Polymerase chain reaction, CTAB: Cetyltrimethylammonium Bromide, GUS: beta-glucuronidase, MS: Murashige and Skoog, IBA: Indol butyric acid, BAP: Benzylamino purine, IAA: Indol Acetic Acid, LB: Luria Broth

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