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AGROBACTERIUM- MEDIATED TOBACCO TRANSFORMATION WITH WHEAT *DREB*2 GENE

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Plants encounter various biotic and abiotic stresses at different growth stages throughout their life. To grapple and adjust with persisting stress conditions, plants have developed certain mechanisms through the mean of transcription. Transcription factors manipulate the feat of multiple stress response in a contemporized way. Among them, drought resistant element binding proteins *DREB* may act as a defense impediment to minimize plant water loss. The *DREB2* gene (PTTa00075.1） amplified from wheat cv. Chinese spring was cloned in pCAMBIA 1304 under the control of CaMV35S promoter. To modify the genome and plant traits, *Agrobacterium*-mediated transformation may transmit the desired genes. The transgenic tobacco plants harboring the desired gene were developed through tissue culture technique. The GUS (*β*-glucuronidase) staining confirms the gene transformation. The putative transgenic plants were selected on selective antibiotic (hygromycin) media. The PCR analysis of T_1 generation confirmed the incorporation of the transgene into the tobacco genome for further evaluation.

Keywords: *Agrobacterium,* GUS-staining, *DREB*2, tobacco, wheat

INTRODUCTION

The crop improvements and their genetic modifications have been in practice since centuries to feed the globe through conventional breeding techniques. The genetic manipulations in these methods involved tightly linked genes which were undesirable to the breeders (Ye and Smith, 2008). To develop and improve an elite genotype, selection of superior traits for many generations is time consuming and laborious. The invention and advancement in biotechnology have opened new areas of research like transgenic plant technology to get the genetically modified plants. The gene transformation in plants was investigated by (Gelvin, 2003) to analyze the structure and function of gene. Recently, the purpose of these studies is to explore the biotic stresses (insects, fungus, nematodes, disease) and abiotic factors (salt, drought, heavy metals in the soil) to understand the pathways to achieve higher crop yield.

To transfer a gene into plants can be accomplished by direct methods like micro-projectile bombardment, microinjection, electroporation and protoplast fusion whereas viral vector and *Agrobacterium*-mediated gene transfer approaches used as indirect transformation techniques. Although the biolistic transformation techniques are relatively simple and no binary vector is required but the intracellular targets are random (cytoplasm, nucleus, vacuole, plastids, etc.,) and the transfer DNA (T-DNA) is not protected. Transformation with plant viruses is much easier but they do not settle into the host genome and not proceed to the next generation through meiosis. Therefore have little worth as transformation vectors (Twyman *et al*., 2002).

Agrobacteria are naturally occurring, gram-negative rod shaped, ubiquitous soil borne pathogens classified in genus Rhizobium and family Rhizobiaceae and induce crown gall in plants (DeCleene and DeLey, 1976). The T-DNA is transferred through chromosomal and vir-gene of the bacterial cells that produce hormones and opines. Hormones stimulate the transformed tissues to grow into calli while opine feed bacteria (Gelvin, 2005). The bacterium commonly used to genetically transform the majority of dicotyledonous plants (Dong and Qu, 2005). The desired results for monocotyledonous species were achieved with the development of binary vectors in barley, rice and wheat (Himmelbach *et al*., 2007). Albeit there is extensive use of transformation *via Agrobacterium* but the plant cell step of T-DNA transfer to the genome is poorly understood. Better recognizing about the transfer of T-DNA would be useful to develop the transformation technology.

Plants under prevailing ecological extremes acquire specific mechanism and structures to cope with harsh environments. Transcription factors control the activity of multiple stress response genes and depict as a targets for application in molecular plant breeding (Yamaguchi-Shinozaki and Shinozaki 2006). Several families of transcription factors such as AP2/ERF, MYB, MYC, NAC and bZIP ameliorate crop yield against different natural ambiances. These factors bind with specific *cis*-elements in the promoter region of genes. *DREB* factors of AP2/ERF family genes accelerate the genes to combat the water stress tolerance conditions.

The members of the family contain a single AP2 DNA-binding domain, with a conserved (TACCGACAT) box. *DREB* proteins were stably over-expressed under constitutive and/or drought inducible promoters in some dicotyledonous and monocotyledonous species (Rashid *et al*., 2012). The resulting transgenic plants frequently demonstrated increased resistance to drought and/or cold stress at the seedling stage in laboratory tests.

The development of genetic engineering and molecular breeding has opened a new era to ameliorate stress tolerance of crops (Fleury *et al*., 2010). Recently, successful gene transformation in crop plants for water and other stresses were accomplished (Bhattacharya *et al*., 2004; Chandra Babu *et al*., 2004). In this canvas the wheat *DREB2* gene was transferred in model system tobacco through *Agrobacterium*-mediated leaflets transformation and the inserted gene was confirmed through GUS-staining and *DREB2* transgene T_1 generation through PCR analysis.

MATERIALS AND METHODS

***Source of Agrobacterium strain and plasmid*:** *Agrobacterium* strain octopine type (LBA4404) was used which forms octopine in the plant and catabolism in the bacteria (Hellens *et al*., 2000). The plasmid pCAMBIA1304 (12361 bp) with GFP-GUS; green fluorescent protein and β-glucuronidase gene fusion, (*hph*); hygromycin phosphotransferase selectable marker under the control of CaMV35S promoter and nos-terminator was taken from the wheat lab at School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China as shown in Figure 1.

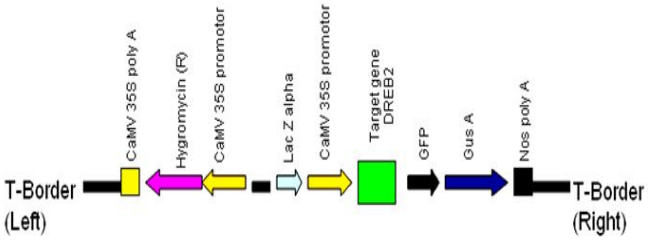

Figure 1. Sense vector pCAMBIA1304 containing *DREB2* gene, (CaMV35S) Cauliflower Mosaic Virus 35S RNA Promoter

***Plant transformation vector (pCAMIA1304+ DREB2)*:** For the construction of *DREB2* sense vector, the entire DNA coding sequence from wheat cDNA library (PTTa00075.1) was amplified through PCR. The sense primer 5' GGAAGATCTGCAGGGAGAAGAGCGAGG 3' contain--ing a *Bgl*II recognition site (underlined) and reverse primer 5' GGACTAGTGAGGCGTACTGATGTTGGGAG 3' with a *Spe*I recognition site (underlined) were used. After digesting the PCR product with *Bgl*II and *Spe*I, the resulting PCR product was inserted and ligated at the *Bgl*II and *Spe*I predigested modified expression vector pCAMBIA1304. The required product was confirmed by sequencing. Further confirmation was done by digestion with *Bgl*II and *Spe*I restriction enzymes (Fig. 2). The resulting vector harboring *DREB2* gene was under the control of the CaMV35S promoter with nopaline synthase terminator (Nos). The construct was then transferred into *Agrobacterium tumefaciens* (LBA4404) by freeze and thaw method (Burow *et al*., 1990).

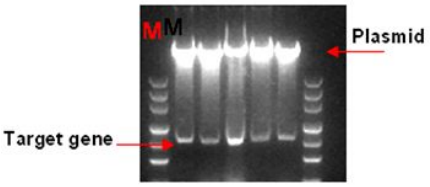

Figure 2. Confirmation of *DREB2* gene by digestion with *Bgl*II and *Spe*I restriction enzymes

The transformed *Agrobacterium* colonies were selected on solid LB medium supplemented with 50 mg/L kanamycin and 50 mg/L streptomycin after 2 days keeping at (28°C) in the darkness. These colonies were cultured at 28°C in 5 mL of liquid LB medium at 240 rpm. The presence of *DREB2* gene in *Agrobacterium* was confirmed through *Agrobacterium*-clone PCR as shown in Figure 3.

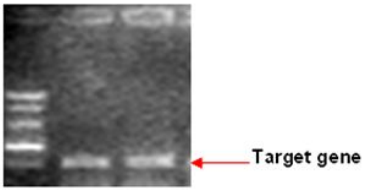

Figure 3. *Agrobacterium* clone PCR to confirm the *DREB*2 gene.

***Tobacco transformation*:** After growing the bacteria culture to OD_{600} to 0.4–0.6, the *DREB*2 gene was transferred into tobacco with the leaf disc method through with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA1304 +*DREB*2) (Gurel, 2001). The leaf discs infected with *Agrobacterium w*ere co-cultured on MS-medium containing 6-BA (2 mg/L) and NAA (0.2 mg/L) at (25°C) in the dark for 3 days. Further co-culturing on fresh solid MS-medium supplemented with (2 mg/L 6-BA, 0.2 mg/L NAA, 500 mg/L hygromycin and 500 mg/L cefotaxime) at (25°C) in the light for 25-30 days were carried out for callus and shoot regeneration. The shoots (2-3 cm tall) transferred to ½ MS-medium containing 250 mg/L cefotaxime and 500 mg/L Hyg+ for root regeneration.

***Histochemical GUS assay*:** The GUS-gene was used as a selective marker and was under the control of CamV35S

promoter, Histochemical GUS-staining was performed (Jefferson, 1987) in order to observe the transgene expression. The transformed explants were incubated overnight at 37°C in 1.0 mM chromogenic substrate X-GlcA (5-bromo-4-chloro-3indolyl-β-D-glucuronic acid) with GUS substrate solution. The explants were dipped in fixation solution for minimum 4 hours. After fixation the explants were transferred into 75% ethyl alcohol for overnight for decolorization.

***Selection of transgenic plants and PCR analysis*:** The T_0 generation seeds of putative transgenic plants were germinated on ½ MS-medium in Petri dishes supplemented with hygromycin (50 mg/L). The selected transgenic tobacco lines on the hygromycin media confirmed the gene transformation. Total genomic DNA of control and hygromycin-resistant tobacco leaves from T_1 generation was extracted by CTAB method (Richards *et al*., 1994). The *DREB2* transgene was amplified by using the Sense primer 5' GGA AGATCTGCAGGGAGAAGAGCGAGG 3') and antisense primer (5' GGACTAGTGAGGCGTACTGATGT TGGGAG 3'). The underlines areas mentioned the enzyme sites.

The *hygromycin* gene was amplified by using forward Hyg-F (5' TGCGCCCAAGCTGCATCA T 3') and reverse Hyg-R (5' TGAACTCACCGCGACGTCTGT 3') primer pair. The PCR was carried out in a 20 µL reaction volume comprised of 30 ng DNA template per 20 uL reaction volume, 10 pMol each of primers, 10 mMol dNTPs, 1X PCR buffer, 1X $MgCl_2$ and 0.5 U Taq polymerase (Takara, China). The PCR reaction was run at (94°C) for 3 min followed by 32 cycles of amplification (1 min at (94°C), 1 min at (58°C) and 1 min at (72°C) with a final extension at (72°C) for 5 min.

RESULTS

***Vector construction*:** The complete DNA coding sequence of *DREB2*-gene (PTTa00075.1) was placed in sense orientation of pCAMBIA 1304 under the control of CaMV35S promoter as shown in (Fig. 1). The construct transferred to *Agrobacterium* was reconfirmed through digestion with *Bgl*II and *Spe*I restriction enzymes and clone PCR analysis as shown in Figure 2 and 3.

***Histochemical GUS staining*:** GUS-gene expression was evaluated to confirm reporter gene for plant transformation. GUS expression in callus and leaf tissues reflects the transformation of *DREB*2 gene. The blue color in transgeics was found observed as compared to the control (Fig. 8).

***Shoot regeneration*:** The putative transformed leaf discs on hygromycin selected media initiated callus development whereas the untransformed leaf discs turn yellow after 3-5 days. The putative discs formed bright green callus and the shoots that were emerged 3 to 5 leaflets in 25-30 days (Fig. 4).

***Root regeneration*:** *In vitro* shoots with 3-5 leaflets started root development in 20-25 days (Fig. 5). The putative transgenic plants have well developed roots whereas untransformed plants did not develop roots (data not showed), which kept on growing. Hygromycin resistant *DREB2* transgenic tobacco lines were generated which exhibited normal phenotypes. The transgenic plants maintained *in vitro* conditions were transferred to soil pots in growth chambers for seed set.

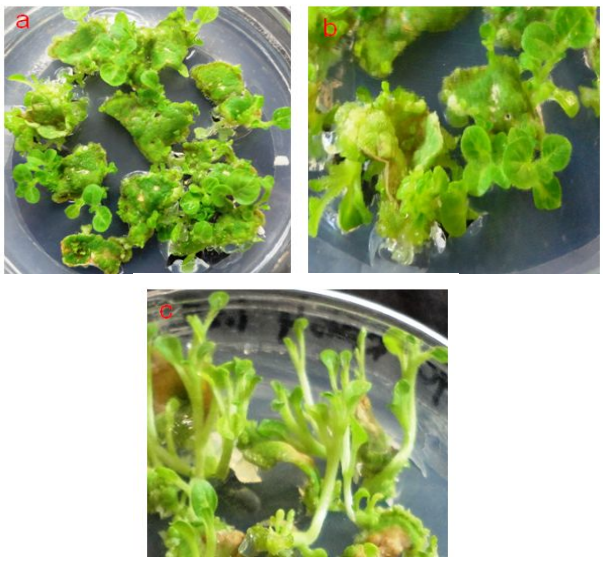

Figure 4. Transgenic tobacco shoots/plantlets developed from callus induced on selective MS-medium

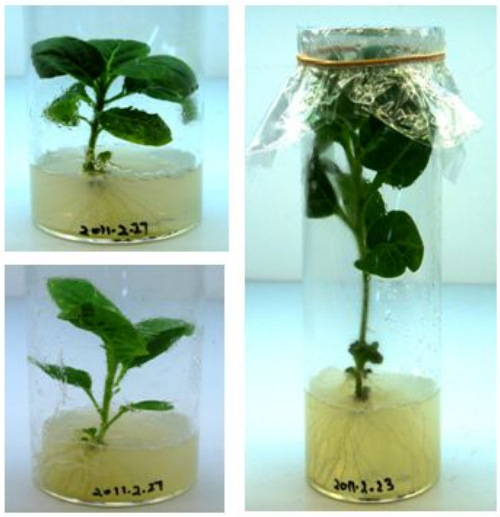

Figure 5. Root development of transgenic tobacco plantlets on MS-medium supplemented with hygromycin.

***Selection of transgenic plants and PCR analysis*:** The

plants of T_1 generation were transferred from the ½ MS-medium to selectable hygromycin supplemented MS-media. The plantlets which remained green and developed roots on hygromycin were found resistant to selective antibiotic (Fig. 6). The similar findings were studied for transgenic tobacco plants (Pathi *et al*., 2013).

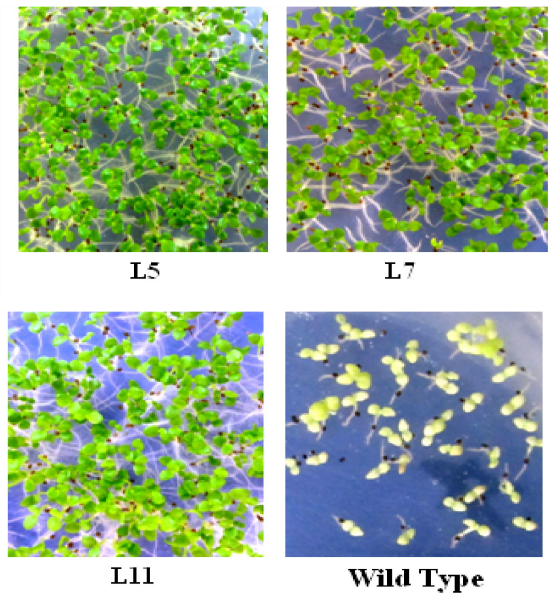

Figure 6. Selection of T_1 generation on MS-medium supplemented with hygromycin. The green plantlets with elongated roots are transgenic while the yellowish plantlets with little roots are wild type and are sensitivity to hygromycin.

Hygromycin resistant T_1 lines were generated from independent transgenic plants. PCR analysis of transgenic lines strengthened that the transformed tobacco genome comprised of both, the *DREB2* and plant selectable *hygromycin* marker gene while the PCR results of the control plants exhibited negative behavior for *DREB2* as well as for hygromycin (Fig. 7).

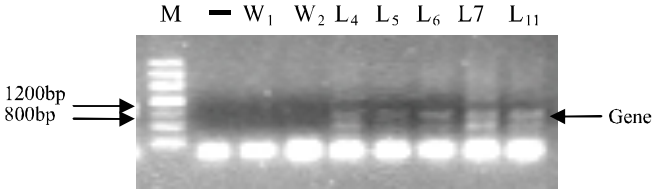

Figure 7. PCR analysis of *DREB2* transformed plants along with hygromycin selective marker resistance gene in tobacco T_1 generation. W_1 and W_2 non-transgenic wild type, L_4, L_5, L_6, L_7 and L_{11} the transgenic tobacco lines.

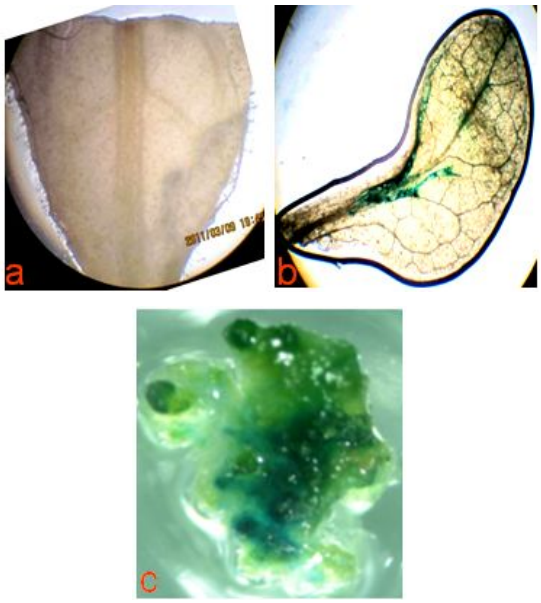

Figure 8. Histochemical GUS-assay of transformed and untransformed plant. a) Control (Left), b) Transformed leaf (Mid) and c) Callus.

DISCUSSION

Abiotic stresses are the most limiting factors of agricultural productivity all over the world. Classical plant breeding methods are restricted due to complexity of stress tolerance traits, low genetic variance and heritability of yield components under stress condition. Contrary, the advancement in understanding of the molecular biology of stress responses in plants and use of genetic engineering tools seem to have a potential in deriving relatively quicker solution for improving stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000). Engineering stress proteins or the enzymes of the biosynthetic pathways associated with stress responses has been evolving as an encouraging method for improving stress tolerance (Lata *et al*., 2011). Recently, high throughput gene discovery platforms have elucidated a number of candidate genes that might be useful in this endeavor. The explicit use of *Agrobacterium* in plant molecular biology research for genetic improvements is due to small copy number of T-DNA and durable expression of transferred genes (Murai, 2013). Plants respond to abiotic stress by modulating gene expression, which eventually leads to the restoration of cellular homeostasis and recovery of growth (Arie, 2002; Mizoi and Yamaguchi-Shinozaki, 2013) through transcription factors, which are also called 'gene switches'. Transcriptional regulation is the potential area for coordinated regulation of genes relevant to stress tolerance (Julieta, 2014). Isolation and cloning of genes coding for transcription factors, which normally express at

low levels, has been a major area of research in the present-day plant stress molecular biology (Shinozaki and Yamaguchi-Shinozaki, 2000).

Stress-related genes are found in all plant species. In the present investigation an important transcription factor *DREB2* from wheat (Chinese spring) cDNA was cloned and transferred in to tobacco through *Agrobacterium*-gene mediated transformation to alter the essential drought resistance barrier to adjust stress resistance in tobacco plants (Ho *et al*., 2013).

A recently available *DREB*2 sequence (PTTa00075.1) from wheat cDNA library was used to amplify gene from genomic DNA. Gene specific primers were designed with flanking restriction sites. An amplicon obtained from wheat genomic DNA was eluted and cloned into plant expression vector under CaMV35S promoter and then mobilized into *Agrobacterium tumefaciens* LBA4404. The tobacco leaf discs were infected with *Agrobacterium* to get the transgenic tobacco plants to impart desired traits (Maneesha *et al*., 2008; Teixeira da Silva, 2003; Zupan *et al*., 2000). The transgene expression in tobacco callus and leaf was determined through GUS-gene selective marker which was under the control of CamV35S promoter. Similar findings about the GUS-reporter gene have been canvased in tobacco plant (Nakashima *et al*., 2014).

Enhanced growth of transgenic tobacco seedlings in hygromycin medium and *in-vitro* culture alongwith dried control tobacco seedlings confirmed the transfer of *DREB*2 gene in the tobacco. Balance of selective agents along with *Agrobacterium* might be in dire need to raise transgenic plants (Silva and Fukai, 2001). Micropropagation confirm the gene transformation and to alter the growth characteristics of the regenerated plants ((Teixeira da Silva, 2003). *DREB*2 serves as genetic switches and activate the promoter of stress inducible genes in up and down regulated response to environmental stress (Shinozaki *et al*., 2003)

The transgenic tobacco was tested for the confirmation of *DREB*2 gene using gene specific primers through PCR. The 800 bp amplicon equal to the cDNA sequence of *DREB2* was observed in all the transgenic lines along with hygromycin gene. The wild type did not have the target gene presence. Thus, the *DREB*2 gene under 35S promoter is constitutively expressing. To develop transgenic plants, the hygromycin phosphotransferase (*hpt*) gene has been used as a selectable marker in different studies (Lim *et al*., 2012; Namuddu *et al*., 2013).

It is concluded that the *DREB*s can be used to produce transgenics with higher tolerance to drought, high salt and/or cold stress in combination with different promoters. In addition, the epigenomics of the genes and their signaling pathways in gene expression may provide help to trace the molecular responses to drought (Cao *et al*., 2010; Hanafy *et al*., 2013; Janani *et al*., 2013; Movahedi *et al*., 2012).

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