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Designing of a *35S::DREB1A* molecular construct to produce environmental stress tolerant plants

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Abstract

Environmental stresses affect plant growth and cause losses worth hundreds of million dollars to agricultural industry each year. Many genes are induced in response to environmental stresses. The *DREB1A* gene is a stress-inducible transcription factor which its ectopic over-expression improves plant tolerance to environmental stresses. To produce environmental stress tolerant plants carrying the *DREB1A* gene, the full length cDNA of the *DREB1A* gene was amplified from *Arabidopsis thaliana* Col-0 plants by gene specific primers and cloned into pGEMT-Easy vector, and transformed into *E.coli*. Presence of the *DREB1A* gene was confirmed by restriction analysis as well as DNA sequencing. A 668-bp *Xba*I/*Bam*HI digested fragment of *DREB1A* gene from the *pGEMT::DREB1A* construct was sub-cloned into the pBI121 binary vector. The recombinant plasmids were transferred into *Agrobacterium tumefaciens* cells (strain LBA4404) and screened on LB medium supplied with kanamycin/rifampicin (50 mg/l). Positive bacterial colonies were selected based on colony-PCR analysis and saved for further application in plant materials.

***Keywords*:** abiotic stress, *DREB1A* gene, transgenic plants, *Agrobacterium tumefaciens*

Introduction

The world population is increasing with an alarming rate, while food products are decreasing due to the adverse effects of various abiotic stresses. For the most nations of the world it is a major area of concern to cope with increasing food requirements by minimizing the effects of harsh environmental conditions. Drought, high salinity, heat and freezing temperatures are main stresses which reduce growth and production of crop plants (Mahajan and Tuteja, 2005). Heat and low temperatures induce mechanical damages on the cell, while drought and salinity disturb the water balance and ion homeostasis (Serrano and Navarro, 2001). Plant tolerance to these stresses, is well known to be a multigenic trait resulted from expressions of many genes under stress conditions. Identification of genes related to the Ca^{2+} signaling pathway and environmental stress response showed interaction and correlation between different stress response pathways (Farooq et al., 2009). Although the roles of Ca^{2+} signaling pathway and Abscisic Acid (ABA) are not avoidable in stress response, transcription factors (TF) play central role in plant responses to biotic and abiotic stresses (Chaves and Oliveira, 2004).

The CBF/DREB TFs family, bind to the CRT/DRE elements, have been shown to be induced by drought, salinity, heat and low temperature stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). Most of the stress responsive genes contain the DRE or DRE-related core motifs in their promoter regions. Several reports indicate that over-expression of the DREB TFs leads to an increase in the expression of stress responsive genes which in turn result in plant tolerance to the environmental stresses (Jaglo et al., 1998; Kasuga et al., 1999; Hsieh et al., 2002).

The *DREB1A* gene is a member of the CBF/DREB TFs family, expressed in most plant tissues and developmental stages. The expression of *DREB1A* gene, induced by low temperature, strongly up-regulates more than 50 downstream stress responsive genes (Ito et al., 2006; Hong et al., 2009). This would result in adaptation of plants to the stress conditions and exercise specific tolerance mechanisms. Over-expression of the *DREB1A* gene in crop plants can also result in high tolerance to the abiotic stresses, thereby increasing the efficiency of plant production (Zhao et al., 2007; Hong et al., 2009; Li et al., 2011). Interestingly, the *DREB1A* over-expressing wheat plants consistently had a higher total number of heads and better head development (Pellegrineschi et al., 2004).

We chose *DREB1A* gene to design a molecular construct useful for production of environmental stress tolerant plants. The *DREB1A* gene has many advantages such as central role in stress response signaling pathway, comprehensive effect on the environmental stress tolerance, short length and low

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number of exons, expression in all plant tissues and developmental steps, conservation in most of plant families, involvement in regulation of many downstream stress responsive genes and overcoming of silencing problem in host plants. Here, we report isolation of the *DREB1A* gene from *Arabidopsis thaliana* leaves followed by its cloning into the pGEMT-Easy vector and pBI121 binary vector, and subsequent transformation of *E.coli* and Agrobacterium cells.

Materials and Methods

Materials and growth conditions

The *Arabidopsis thaliana* wild type (WT) plants accession Columbia (Col-0) was used in this study. Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 30 sec followed by soaking in 20% mercuric chloride for 10 min, and five times rinses in sterile distilled water. Sterilized seeds were placed on Murashige and Skoog (MS) medium solidified with 0.8% (w/v) agar. Seeds were stratified in darkness at 4°C for 4 days before transferring to growth chamber. After this period, they were grown for 4 weeks at 22-24°C under a 16/8-h light/dark photoperiod.

The *E.coli* (strain DH5α) cells were cultured on solid or liquid LB medium and incubated at 37°C for 16 h. The *Agrobacterium tumefaciens* (strain LBA4404) cells were also cultured on LB medium supplemented with 50 mg/l rifampicin and incubated at 28°C for 48 h. The bacterial cells containing pGEMT-Easy vector were selected on LB medium supplemented with 100 mg/l ampicillin and the pBI121 plasmid containing cells were screened on LB medium supplied with 50 mg/l kanamycin.

DNA extraction and PCR analysis

Leaves were frozen in liquid nitrogen and DNA was extracted according to Dellaporta et al. (1983). The following PCR primers were used to amplify the *DREB1A* full length cDNA: 5′-AGCTCTAGAATGAACTCATTTTCTGCTTTTT CTG- 3′ (forward) and 5′-AGCGGATCCTTAATAACTCCATAACGATAC GTCG- 3′ (reverse). The restriction sites for *Xba*I and *Bam*HI were also included, respectively. The amplification reaction contained 1xPCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 µl TaqDNA polymerase (1 u/µl). The final concentration of each primer was 0.4 µM. The amplification reaction protocol included an initial denaturation at 95°C for 3 min, followed by 35 amplification cycles of 95°C for 30 sec, 64°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 5 min. The PCR amplification products were separated on 1% (w/v) agarose gels using TBE buffer.

Fragment cloning into the pGEMT-Easy vector

The *pGEMT-Easy* vector has single 3′-T overhangs at the insertion site which greatly improve the efficiency of ligation of a PCR product into the plasmids (Promega, USA). Hence, after purification of the PCR products by purification kit (BioNEER, Korea) they were ligated into the pGEMT-Easy vector. For this, 2 µl of the purified PCR product was mixed with 1 µl of 10x ligation buffers, 1 µl of T_4DNA ligase and 1 µl of pGEMT-Easy vector in final volume of 10 µl. Reaction tubes were incubated overnight at 4°C. *E.coli* competent cells were prepared by ice-cold 50 mM $CaCl_2$ solution (Sambrook and Russel, 2006). 5 µl of the overnight ligation mixture was added to 100 µl fresh competent cells and left on ice for 30 min. Transformation was performed by the heat shock method by 45 sec incubation at 42°C, followed by a 2 min cooling period on ice. An aliquot (900 µl) of SOC solution was added and cells were incubated at 37°C for 3 h. The cells were transferred onto LB-Ampicillin-IPTG-XGal (LAIX) medium for blue/white colonies screening and incubated overnight at 37°C. The transferred plasmids were purified by plasmid isolation kit (BioNEER, Korea) according to the supplied protocol.

Restriction enzyme and sequence analysis

Restriction analysis was performed at 37°C for 3 h. The 20 µl restriction reaction contained 2 µl of 10x *Bam*HI or Tango buffer, 0.5 to 2 µl of *Xba*I with or without *Bam*HI (10 u/µl), 1 to 2 µl (0.05 ng) of plasmids which were extracted from blue/white colonies. At the end of the reaction, the restriction enzymes were inactivated at 80°C for 20 min. The digested products were separated on 1% (w/v) agarose gels. *Xba*I/*Bam*HI digested fragment was cleaned using a DNA purification kit (BioNEER, Korea), and then used for ligation into the pBI121 binary vector.

For confirmation of the cloned fragment, recombinant plasmids were sequenced with forward and reverse primers of *DREB1A* gene. The identity of the sequenced fragment was checked by BLAST (Basic Local Alignment Search Tool) searches available in TAIR (http//www.arabidopsis.org/Blast/).

Gene over-expression construct

To make over-expression construct, pBI121 binary vector was used. The *DREB1A* gene was

recovered as *Xba*I/*Bam*HI fragment from the pGEMT-Easy vector and subcloned into the same sites of pBI121. The 10 µl ligation reaction contained 1 µl of 10X ligation Buffer, 1 µl of T_4DNA ligase, 2 µl of *Xba*I/*Bam*HI digested pBI121 vector, and 2, 4 or 6 µl of gene digested fragment. Reaction tubes were incubated at 4°C for 16 h. By this strategy the *DREB1A* gene was placed under the control of CaMV 35S promoter and nopaline synthase (nos) terminator. The *pBI121::DREB1A* was introduced into *Agrobacterium tumefaciens* LBA4404 cells by freeze-thaw (5 min in liquid nitrogen and then 5 min at 37°C) transformation procedure (Sambrook and Russel, 2001). One ml fresh liquid LB was added and the cells were incubated at 28°C for 2 h. The cells were transferred onto the LB medium, supplied with kanamycin/rifampicin (50 mg/l) and incubated at 28°C for 48 h. Recombinant bacteria were confirmed by PCR analysis and saved for further application in plant materials.

Results

Here, we designed the *pBI121::DREB1A* molecular construct *in-silico*, by using the clone manager software. The sequences of *DREB1A* gene, pGEMT-Easy vector and pBI121 binary vector were scanned to select suitable no-cutter restriction enzymes and *Xba*I and *Bam*HI were selected. These sites were added to the 5′ ends of the primers. Presence of these two different sites has several advantages in next steps of cloning.

DNA extraction was performed from 2 to 3 weeks old *Arabidopsis thaliana* plants and the *DREB1A* fragment was amplified accordingly. At the end of the PCR reaction, a 700 bp DNA fragment was produced with the same size as with the *DREB1A* gene. The PCR products were cloned into the pGEMT-Easy vector after purification successfully. This experiment was further confirmed either by blue/white selection method or restriction digestion of the purified plasmids (figure 1). The plasmids were cut with both *Xba*I and *Bam*HI and a band of around 700 bp, corresponding to the *DREB1A* gene, was obtained (figure 1, lane 3). Finally, sequencing analysis and Blast program confirmed that the *DREB1A* gene was successfully amplified and inserted into the vector without any mutation.

We chose pBI121 binary vector for next step of cloning. *XbaI/BamHI* digested fragment, corresponding to the *DREB1A* gene, was purified from the gel and directly ligated into the cut pBI121 plasmid using *XbaI/BamHI* restriction sites. The pBI121 vector is a low copy number plasmid and it is very difficult to isolate enough amounts of pBI121 plasmid for restriction analysis. Therefore, we used colony-PCR to screen the growing colonies on the selective medium. Production of a band for DREB1A fragment would indicate that the *pBI121::DREB1A* construct had been made successfully.

Colony-PCR products were separated on the gel (figure 2A). There is a band corresponding to DREB1A fragment on lane 3. This colony was used again in another colony-PCR test with positive and negative controls (figure 2B), to ensure that PCR is free from any possible contamination. The sub-cultured colonies were shown to be all transfermants of the *pBI121::DREB1A* construct (figure 2C). We used two different restriction enzymes to clone the DREB1A fragment into the pBI121 vector. Recombinant Agrobacterium cells containing *pBI121::DREB1A* construct are used for production of environmental stress tolerant plants.

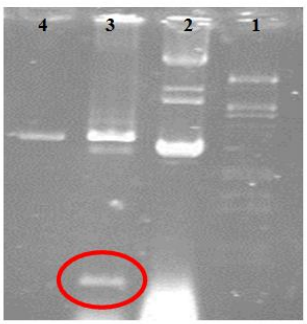

Figure 1. Restriction analysis of blue/white colonies isolated plasmids: lane 1 *Eco*RI/*Hind*III lambda DNA marker, lane 2 non-digested plasmid from white colonies, lane 3 *Xba*I/*Bam*HI digested plasmid isolated from white colonies which contain *DREB1A* fragment (circle), lane 4 none digested plasmid from blue colonies.

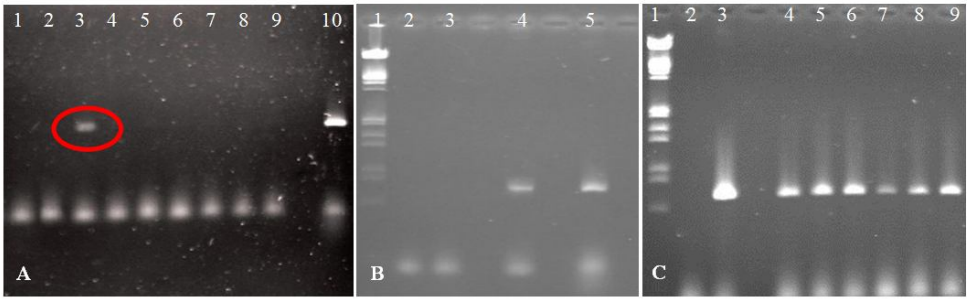

Figure 2. Agarose gel electrophoresis representing PCR products for confirmation of success rate of *DREB1A* gene cloning. A, lane 1 to 9 show colony-PCR products separation, Lane 3 has a band corresponding to DREB1A fragment, lane 10 show positive control with genomic DNA of *Arabidopsis thaliana*. B, lane 1 lambda DNA marker, lane 2 negative control with ddH_2O, lane 3 negative control with empty pBI121 vector, lane 4 colony-PCR containing *pBI121::DREB1A*, lane 5 positive control with genomic DNA of *A.thaliana.* C, lane 1 marker, lane 2 negative control with ddH_2O, lane 3 positive control with genomic DNA of *A. thaliana,* lane 4 to 9 colony-PCR from sub-cultured recombinant colonies.

Discussion

Environmental stresses have adverse effects on plant growth and seed production. We can overcome this problem with production of transgenic plant, over-expressing stress tolerance genes. It is very important to select a gene such as *DREB1A* which can confer high tolerance to various abiotic stresses. In this study we provided *pBI121::DREB1A* construct which could be applied for Agrobacterium transformation and consequently crop plant improvement. Transgenic plants would be more tolerant to the abiotic stresses such as drought, salinity, low and high temperatures (Vadez et al. 2007; Maruyama et al., 2009; Hong et al. 2009). It is also possible that over-expression of the *DREB1A* gene confer direct or indirect properties to plants to cope against heavy metal toxicity and harmful rays or mechanical injuries (Kohan and Bagherieh, 2011). These plants will have more stability and show more growth and productivity under stress conditions compared to the wild type plants.

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