Characterisation of abiotic stress inducible plant promoters and bacterial genes for osmotolerance using transgenic approach

ABSTRACT SUBMITTED TO

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ABSTRACT

Abiotic stress is the primary cause for the loss of crop and reduces average yield for most major crop plants by more than 50%. Salt in soil water inhibits plant growth by osmotic/water deficit stress and/or ion-toxicity effects. Among the abiotic factors that contribute to plant evolution, water availability is the most important. Drought, salt and low temperature stresses together are the major problematic cause for agriculture as these adverse environmental factors prevents plants from realizing their full genetic potential. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affects plant growth and productivity. Plants respond and adapt to these stresses at the molecular and cellular levels to survive under stress conditions.

In a different approach, several genes encoding for osmoprotectants have been isolated from different species and have been used for transformation of crop plants to improve tolerance to salt and drought stress. Compatible solutes are small organic metabolites that are highly soluble in water and non-toxic at high concentrations, and act as osmoprotectants. Glycine betaine is one of the osmoprotectants. Most commonly known biosynthetic pathways for glycine betaine include the two-step oxidation of choline. In addition, a novel pathway for glycine betaine biosynthesis has been reported in *Ectothiorhodospira halochloris* and *Aphanothece halophytica*. In this pathway, glycine betaine is synthesized from glycine by a three-step methylation reaction that is catalyzed by two N-methyltransferases. *GSMT* gene encodes glycine sarcosine methyltransferase that catalizes first two methylation reactions that convert glycine to sarcosine and subsequently to dimethylglycine. *DMT* encodes dimethylglycine methyltransferase that is responsible for the specific methylation of

dimethylglycine to generate glycine betaine. *ApGSMT* and *ApDMT* has been reported to improve growth of higher plants under salt stress.

In the present study, the promoter activity of different abiotic stress-inducible promoters isolated from different plant sources has been investigated. The 5' flanking sequences of the identified genes were used to drive the *GUS* reporter gene. This study has twin interests of gaining a basic understanding of the nature of the promoter elements that drive abiotic stress-inducible expression of these genes and the potential application of the identified promoters in plant genetic engineering. Simultaneously, the role of glycine betaine in stress tolerance was examined by genetic engineering of *Arabidopsis* with stress-inducible expression of *ApGSMT* and *ApDMT* cDNA from *Aphanothece halophytica*. Thus in the present study was carried out with the following objectives:

- **To study the stress responsiveness, tissue-specificity and strength of three stress-inducible promoters namely Arabidopsis RD29A (responsive to dehydration), Arabidopsis LEA4LEA4 (Late embryogenesis abundance) and HMGR (hydroxyl methyl glutaryl CoA reductase) by transgenic approach using GUS as a reporter gene in model plant Arabidopsis thaliana.**
- **To study the role and mechanism of action of ApGSMT and ApDMT genes driven by stress-inducible promoters in osmotolerance of a model plant Arabidopsis thaliana.**

Cloning and characterization of different abiotic stress inducible plant promoters

In the present study the abiotic stress-responsive promoters were cloned and their activity was assessed in transgenic *Arabidopsis* by using promoter::*GUS* reporter gene system. CaMV35S promoter-driven *GUS* was used as control.

The *cis*-acting regulatory elements are short consensus DNA sequence present in a eukaryotic promoter, which serves as the binding sites for transcription factors. The specific and combinational interaction initiates transcription and regulates the gene expression. Identification of these regulatory elements is crucial in understanding the nature of promoter function. *In silico* based tools were employed in the present study for identification of the potential *cis*-acting regulatory elements in the 5' upstream sequence of the genes *AtRD29A*, *BcLEA4*, *BnLEA4*, *BjLEA4* and *SmHMGR* using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) lead to the identification of important consensus sequences.

These six confirmed recombinant pCAMBIA1200 derived clones were transformated in *A. tumefaciens* strain LBA4404 using freez thaw method of transformation and further transformed in *Arabidopsis* by floral dip method.

The hygromycin resistant putative transgenic plants were further confirmed for the presence of the transgene by *GUS* staining analysis. The transgenics were grown and maintained in the National Phytotron Facility, IARI under controlled conditions (20-24^{o}C day/night temperature, 60-75% relative humidity, 16/8 hr day/night photoperiod and 125µmol m^{-2} s^{-1} light intensity.

Three PCR positive T_3 transformants *Arabidopsis* plants from each of the six constructs were used for further molecular, histochemical, fluorometric and physiological analysis. Plant genomic DNA from wild type and T_3 transformed

Arabidopsis plants was PCR amplified using *GUS* transgene gene specific forward and reverse primers. An amplicon of 785 bp corresponding to the predicted size confirmed the transgene integration in the plant genome. No amplification was observed in the non-transformed wild type plants. The pCAMBIA derived promoter construct was used as positive control.

Cloning and stress inducible expression of *ApGSMT* and *ApDMT* genes encodes for glycine betaine

In the present study genetic engineering of glycine betaine overproduction in *Arabidopsis* is achieved by stress inducible expression of *ApGSMT* and *ApDMT* cDNA from *Aphanothece halophytica*. Transgenic plants co-expressing *ApGSMT* and *ApDMT* under the control of *AtLEA4* and *AtRD29A* promoter revealed high levels of tolerance to salt, drought and low temperature stresses as compared with non transformed and constitutively co-expressing *ApGSMT* and *ApDMT* transgenic *Arabidopsis* plants. These three confirmed recombinant pCAMBIA1200 derived clones were used for transformation of *Agrobacterium tumefaciens* strain LBA4404 using freez thaw method of transformation and further transformed in *Arabidopsis* plants.

The hygromycin resistant putative transgenic plants were confirmed for the presence of the transgene by molecular analysis such as PCR and RT-PCR. Transgenic plants were grown and maintained in the National Phytotron facility, IARI under controlled conditions (20-24^{o}C day/night temperature, 60-75% relative humidity, 16/8 hr day/night photoperiod and 125µmol m^{-2} s^{-1} light intensity.

The transgenic approach allows scientists to study the mechanisms governing stress tolerance by either over expression or antisense suppression of the transgene into the model plant species and to monitor the phenotypical and biochemical changes

before and after a specific abiotic stress treatment. For achieving the desired level of expression of the transgene, it needs to be accurately regulated. Strong abiotic stress-inducible promoters are required for transgene expression at different stages of growth. Selection of abiotic stress-inducible promoter has become increasingly important for successful gene transfer and expression of transgenes in plants. The availability of a broad spectrum of stress-inducible promoters that differ in their ability to regulate the expression patterns of the transgene can dramatically increase the successful application of transgenic technology for improved tolerance to abiotic stress in crop plants. In general, selection of a suitable promoter is critical for devising intelligent strategies for genetic engineering.

In summary, we conclude that two genes *ApDMT*/*ApGSMT* under the control of stress- inducible *AtRD*29A and *AtLEA*4 promoters were transferred to *Arabidopsis*. The present results indicated that the successful integration of *ApDMT*/*ApGSMT*, which catalyzes the glycine-methylation biosynthetic pathway of glycine betaine driven by *AtLEA*4 promoter, subsequently enhanced the tolerance of transgenic plants against a variety of abiotic stresses including salt, drought, cold and heavy metal in comparison to driven by CaMV35S and *AtRD*29A promoters. This tolerance was assessed by the reduction in the inhibitory effects of salt against chlorophyll content, cell membrane damage and the maintenance of higher water retention capacity under salinity conditions. In this study, promoters of this gene, *AtRD29A*, *BnLEA4*, *BcLEA4*, *BjLEA4* and *SmHMGR* were selected for characterization.

The important summary of the present study are:-

1. *GUS* histochemical analysis revealed that PCaMV35S driven *GUS* expression was constitutive in all the tissues including roots, as expected.

2. *AtRD29A* promoter-driven *GUS* expression under non-stress and abiotic stress conditions were found strongly in all parts of the plants.
3. The *BcLEA4* and *BnLEA4* promoter-driven GUS expression was regulated in transgenic *Arabidopsis* treated with abiotic stresses (high salt, cold and high temperature) with the highest up-regulation under salt stress treatment showed constitutive basal activity under control (no stress) conditions.
4. Transgenic plants expressing GUS under the transcriptional control of *BjLEA4* promoter showed weak level of expression under control condition and salt induction of *BjLEA4* was also significantly higher as compared to high and low temperature treatments.
5. *SmHMGR* promoter driven expression of *GUS* in transgenic *Arabidopsis* was weak and down-regulated during cold and high temperature treatments and salt induction of *SmHMGR* promoter was comparatively high.
6. Co-expressing *ApGSMT* and *ApDMT* driven by *AtLea*4 promoter in transgenic *Arabidopsis* showed better seeds germination and relatively healthier growth pattern of seedling under salinity, heavy metal and drought stresses.
7. Under saline stress, decrease in total chlorophyll was less in the inducible *AtLEA*4 promoter co-expressing *ApGSMT/ApDMT* transgenic than in the *RD*29A promoter, constitutively (CaMV35S) co-expressing *ApGSMT/ApDMT* and wild type *Arabidopsis* plant.
8. Transgenic plant co-expressing *ApGSMT* and *ApDMT* driven by *AtLea*4 promoter had a slower decrease in WRA than in the *RD*29A, constitutively (CaMV35S) co-expressing *ApGSMT/ApDMT* and wild type *Arabidopsis* lines.

9. The plants co-expressing *ApGSMT/ApDMT* driven by *AtLEA*4 and *AtRD*29A promoters showed significantly less ion leakages than constitutively co-expressing *ApGSMT*/*ApDMT* transgenics and wild type plants.

10. Transgenic plants co-expressing *ApDMT/ApGSMT* driven by *AtLEA*4 comparatively also evidenced higher induction of salt responsive up-regulation of α-Linolenic acid metabolism than in the *RD*29A promoter, constitutively (CaMV35S) co-expressing *ApGSMT/ApDMT*.