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Isolation and functional characterization of the promoter of a DEAD-box helicase *Psp68* using *Agrobacterium*-mediated transient assay

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Helicases are molecular motor proteins that perform a variety of cellular functions including transcription, translation, DNA replication and repair, RNA maturation, ribosome synthesis, nuclear export and splicing processes. The p68 is an evolutionarily conserved protein which plays pivotal roles in all aspect RNA metabolism processes. It is well established that helicases provides abiotic stress adaptation in plants but analysis of cis-regulatory elements present in the upstream regions is still infancy. Here we report isolation and functional characterization of the promoter of a DEAD-box helicase *Psp68* in response to abiotic stress and hormonal regulation. The promoter of *Psp68* was isolated by gene walking PCR from pea genomic DNA library constructed in BD genome walker kit. In silico analysis revealed that promoter of *Psp68* contained a TATA, a CAAT motif and also harbors some important stress and hormone associated cis regulatory elements, including E-box, AGAAA, GATA-box, ACGT, GAAAA and GTCTC. Functional analyses were performed by *Agrobacterium*-mediated transient assay in tobacco leaves. Very high level of GUS activity was observed in agroinfiltrated tobacco leaves by the construct carrying the *Psp68* promoter::GUS, subjected to abiotic stress and exogenous hormonal treatments. Stress-inducible nature of *Psp68* promoter opens possibility for the study of the gene regulation under stress condition. Therefore, may be useful in the field of agriculture and biotechnology.

Introduction

p68, a member of DEAD-box helicase family is highly conserved in eukaryotes and involved in almost all RNA metabolic processes. Recently, a number of studies showed that helicases are not only involved in many cellular processes including plant growth and development[1-7] but also provides stress tolerance in transgenic plants.[3,7-13] The appropriate regulation of gene expression is important for all cellular processes, in which transcriptional control is primarily concerned with improved survival. Mostly, genes are expressed in transgenic plants under the control of promoter, a DNA sequences required for appropriate spatial and temporal expression pattern. The most widely used promoter for expression of transgenes is CaMV 35S promoter (cauliflower mosaic virus) but sometime it may causes some undesirable effect in plants such as gene silencing, delayed growth, dwarfism and low yield.[14-18]

So, inducible and tissue-specific promoters are required to study the gene regulatory networks in plant.[19,20] Cis-acting regulatory elements present in promoter sequence may function as molecular switch by controlling transcriptional regulation of gene activities. Previously, it was reported that promoter of helicases contained stress responsive cis-elements[21-24] but the isolation of stress-inducible and tissue-specific promoters[25,26] is still interest in the fiend of molecular breeding, biotechnology and agriculture.

In this study, we have isolated and functionally characterized the promoter of *Psp68* in response to abiotic and hormonal treatment by A*grobacterium*-mediated transient assay. In silico analysis also identified that the promoter of *Psp68* harbored multiple stress responsive cis-acting elements. Transient assay showed that promoter of *Psp68* drives high levels of GUS expression under abiotic stress and hormonal treatment. Therefore, this promoter could be used for the study of the spatio-temporal expression pattern and development of stress tolerant transgenic crops in the future.

Results

Isolation of the promoter of *Psp68*

Pea genomic DNA library was prepared by digesting genomic DNA with different restriction enzymes (EcoRV, DraI, PvuII and SspI) in 4 separate tubes to generate blunt ends of genomic DNA. The digested genomic DNA was purified and further ligated into BD genome walker kit. The primary PCR was done by using AP1 as forward (5′-GTAATACGAC

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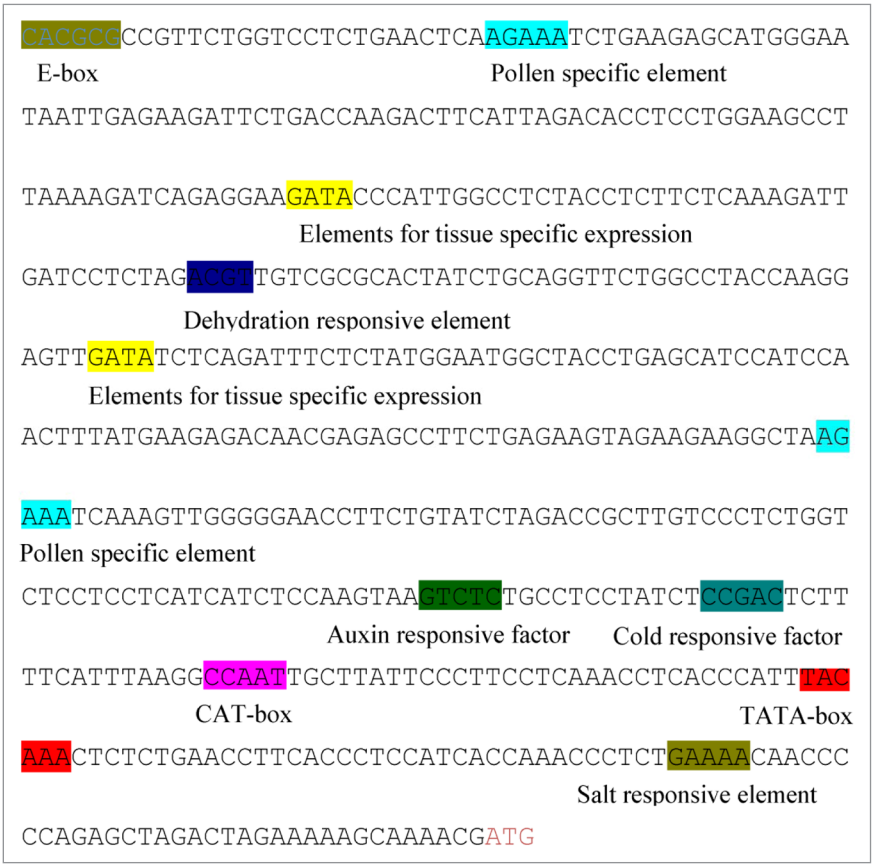

Figure 1. *Psp68* promoter sequence. A schematic representation showing various cis-elements present in the upstream region of *Psp68* gene as determined by PLACE program. TATA-box and CAAT sequences and various cis-acting elements are shown in different color.

TCACTATAGG GC-3′) primer and gene specific reverse primer R3 (5′-CCTCGCATTC TCTTCCTCGT A-3′). Four DNA genomic libraries were used as a template for the first PCR. The PCR products were resolved on a 1% agarose gel and a smear was observed in all the 4 libraries. Secondary PCR was done using primary PCR product as template (1: 10 dilution) with AP2 (5′-ACTATAGGGC ACGCGTGGT-3′) as forward and R2 (5′-AGAAGAGTTG GAGTGA-GGGTACG-3′) as a reverse primer. The PCR products were resolved on 1% agarose gel but only library 4 gave a band size of 750 bp. After the nested PCR, the specific band was purified and cloned into pGEM-T vector (data not shown) and sequenced. After sequencing, 531bp upstream regions was successfully isolated and verified by finding the overlapping regions at the 3′ end of *Psp68* gene.

In silico analysis of *Psp68* promoter

To identify the transcription start site, putative TATA box and CCAAT box, the promoter sequence of P*sp68* were analyzed by using Plant Prom Database. The promoter region has a TATA (TACAAA, consensus TATAAA) and CCAAT box at position −81 and −117bp respectively (**Fig. 1**). To identify the cis-regulatory elements, present in the P*sp68* promoter, the sequence was analyzed using PLANTCARE and PLACE databases (**Fig. 1**). Various cis-acting elements including E-box, AGAAA, GATA-box, dehydration and salt responsive elements (ACGT and GAAAA) and auxin response factor (GTCTC) were identified in the promoter sequence (**Fig. 1**). The additional cis-acting elements presents among others are 8 transcriptional activators elements (NGATT, GANTTNC, MACCWAMC and CTGACY), 4 mesophyll-specific gene expression elements (YACT), 6 pollen specific activator elements (GTGA and AGAAA), 10 (AAAGAT, CTCTT, AAAGAT and CTCTT) nodule specificity regulatory elements, one light-activated (ACTTTG), and one WRKY transcription factors (TGAC) element (**Table 1**). A complete list of all predicted cis-elements present in the *Psp68* promoter was shown in **Table 1**.

Cloning of *Psp68* promoter in binary vector and *Agrobacterium* transformation

The PCR amplified *Psp68* promoter fragment was first cloned into pGEMT easy vector. It was then released by BamHI and HindIII restriction digestion and further cloned in pCAMBIA-1391Z (promoter less vector) binary vector in the same restriction site. Cloning of the *Psp68* promoter was confirmed by colony PCR and restriction analysis (data not shown). The fusion construct containing *Psp68* promoter-GUS (β-glucuronidase) in pCAMBIA-1391Z was further transformed *Agrobacterium tumefaciens* strain (LBA4404) and verified by colony PCR using promoter specific primers.

Table 1. Prediction of cis-regulatory elements of *PsP68* promoters using PLACE database

Element name and number	Sequence	Function
ARR1AT (5) EECCRCAH1 (1) MYBPLANT (1) BOXNTCHN48 (1)	NGATT GANTTNC MACCWAMC CTGACY	Transcriptional activators
ACGTATERD1 (2)	ACGT	Responsive to dehydration
ARFAT (1) SURECOREATSULTR11 (3)	TGTCTC GAGAC	Auxin response factor
CAATBOX1 (3) GATABOX (5) NTBBF1ARROLB1 (1)	CAAT GATA ACTTTA	Responsible for the tissue specific promoter activity. Tissue-specific and auxin-regulated expression
CACTFTPPCA1 (4)	YACT	Elements for mesophyll-specific gene expression
CAREOSREP1 (1)	CAACTC	Gibberellin-upregulated proteinase expression
CCAATBOX1 (1)	CCAAT	Enhanced expression of chimaeric heat shock
DOFCOREZM (5)	AAAG	Transcription factors
GTGANTG10 (3) POLLEN1LELAT52 (3)	GTGA AGAAA	Late pollen gene Responsible for pollen specific activation
INTRONLOWER (1)	TGCAGG	Catalog of splice junction
MYBCOREATCYCB1 (1)	AACGG	Activator of reporter gene
MYBPZM (1)	CCWACC	Controls phlobaphene pigmentation
NODCON1GM (2) NODCON2GM (3) OSE1ROOTNODULE (2) OSE2ROOTNODULE (3)	AAAGAT CTCTT AAAGAT CTCTT	Nodule specificity of cis-acting regulatory elements. Activated in the infected cells of root nodules
SEBFCONSSTPR10A (1)	YTGTCWC	Potato silencing element binding factor
SORLIP2AT (1)	GGGCC	Involved in the network of phytochrome A-regulated gene expression
TAAAGSTKST1 (1)	TAAAG	Transcription factors in guard cell-specific gene expression
TBOXATGAPB (1)	ACTTTG	Light-activated gene transcription
WBOXNTERF3 (1)	TGACY	Rapid and transient activation of transcription of the ERF3 gene by wounding in tobacco leaves
WRKY71OS (1)	TGAC	Early nuclear events in plant defense signaling: rapid gene activation by WRKY transcription factors

Regulation of *Psp68* promoter activities

To compare the regulation of *Psp68* promoter activity, we used transient expression by agro infiltration in the tobacco leaves.[27] This method was selected to avoid long-time regeneration protocol. The *Psp68* promoter was fused with GUS reporter gene in pCAMBIA-1391Z vector and infected into the leaves of tobacco by *Agrobacterium* infiltration. The CaMV35S promoter fused with GUS and WT tobacco plants used was as positive and negative control respectively, in order to determine *Psp68* promoter activity.

Activities of *Psp68* promoter in tobacco leaves

To check whether the isolated 5′-flanking region of *Psp68* genes have promoter activity, the constructs containing promoter of *Psp68*::GUS and CaMV35S::GUS were agroinfiltrated into the leaves tobacco. Both the constructs drove strong levels of GUS expression but maximum GUS gene expression was driven by the CaMV35S promoter (**Fig. 2**). No GUS expression was observed for the negative control. These results indicated that the promoter sequence isolated from the upstream of the *Psp68* gene was functional in tobacco leaves.

Abiotic stress-induced activities of *Psp68* promoter

The impact of abiotic stress (salt, PEG and cold) on the activities of *Psp68* promoter was verified by transient assay in the leaves tobacco (**Fig. 3**). Abiotic stress treatment applied on agroinfiltrated leaves increases the expression of GUS activity (**Fig. 3A**). The effect of abiotic stress was varied for the *Psp68* promoter; GUS activity increased ~15-folds in response to salt stress but upon PEG and cold treatment the expression was increased ~20 and ~11-folds respectively (**Fig. 3B**). The CaMV 35S promoter also displayed high GUS activity levels. These results indicated that the promoter of *Psp68* is a stress inducible promoter.

Hormone-induced GUS Activity

In the agroinfiltrated leaves of the tobacco, the *Psp68* promoter construct showed GUS positive expression in response to hormonal (Auxin, ABA and MeJA) treatments (**Fig. 4**). High

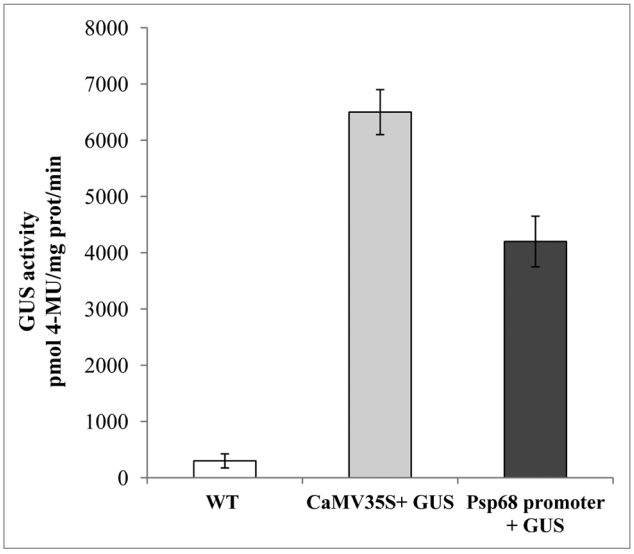

Figure 2. Transient expression of *Psp68* promoters in agroinfiltrated tobacco leaves. GUS activity was determined 48h after infiltration of tobacco leaves with Agrobacterium ($OD_{0.7}$) containing promoter::GUS and CaMV35S::GUS constructs, or WT (negative control). Data represent the mean and SD of 4 independent experiments.

GUS expression was observed in response to Auxin and MeJA followed by ABA treatment (**Fig. 4A**). To quantify the GUS expression, equal amounts of protein was isolated from agro-infiltrated leaves and assayed for fluorescence. GUS activity increased ~11, ~8 and ~10-folds respectively by application of Auxin, ABA and MeJA treatments (**Fig. 4B**). The variation in the activity may be due to present of hormone-induced cis-acting elements in different position of *Psp68* promoter.

Discussion

Cis- regulatory elements present in the promoters of stress-responsive gene controlled many essential biological processes including abiotic stress responses, hormone responses and developmental processes. In plants, a number of cis-regulatory elements have shown to be essential for the transcription of stress-responsive genes.[28,29] A recent chromatin immunoprecipitation study identified that ETHYLENE RESPONSE FACTOR1 bind with stress-specific GCC or DRE/CRT elements and upregulates specific suites of genes in response to abiotic stresses.[30] Drought and salt stress lead to increase ABA accumulation which may triggers adaptive responses.[31] The presence of either a single ABRE or multiple ABREs is sufficient to confer ABA-mediated osmotic stress.[32] The significance of a few cis- regulatory elements (G-box and ABREs) combinations have also been showed that stress-responsive genes are regulated by multiple transcription factors. [33,34] Therefore, to understand the regulatory gene networks in stress-responsive cascades, functional analyses of cis-acting elements is desirable.

The promoter of *Psp68* contain canonical E-box element which is critical for p68 promoter activity. [35] E-box motifs can be recognized by Myc-Max heterodimers that are known to

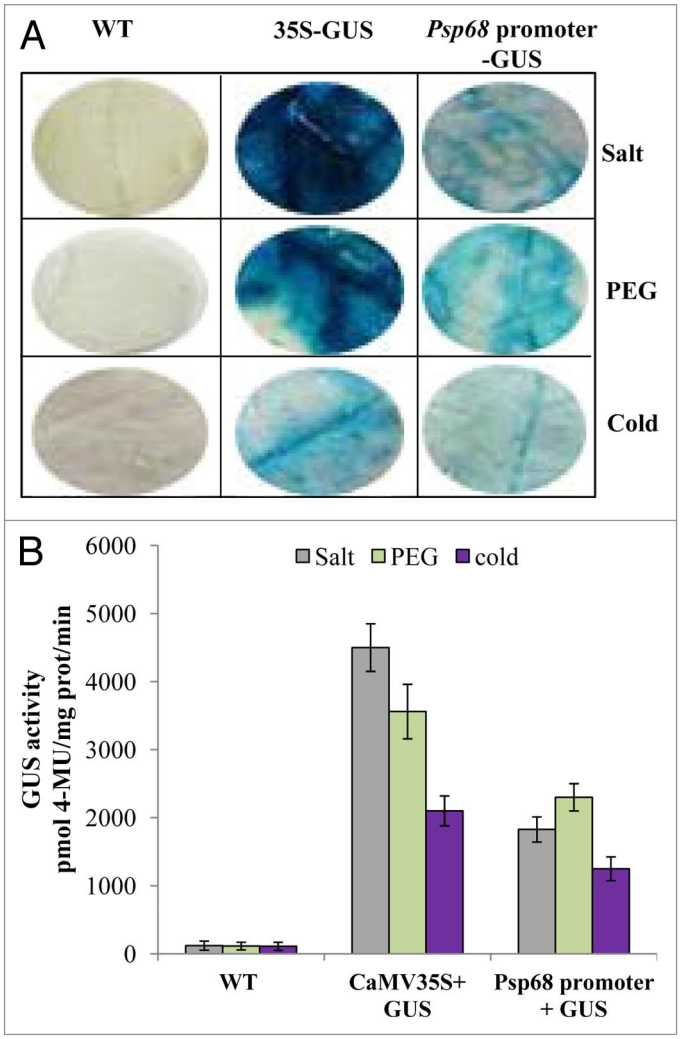

Figure 3. *Psp68* promoter-GUS analysis in response to abiotic stress. (**A**) GUS activity in agroinfiltrated leaves. GUS was detected in X-Gluc solution followed by stress treatment. (**B**) Effect of abiotic stress on the transient expression of *Psp68* promoter in agroinfiltrated tobacco leaves. Two days after infiltration with *A. tumefaciens* ($OD_{0.7}$) containing either *Psp68* promoter::GUS or CaMV35S::GUS constructs, tobacco leaves were sprayed with 200 mM NaCl, 20% PEG. For cold treatment infiltrated leaves were kept on 4 °C. After treatment, all the samples were used for quantification assay. Infiltrated leaves of WT without treatment (water) used as negative control. Data represent the mean and SD of 4 independent experiments.

function in the regulation of many growth regulating genes. E-box motif is found in other DEAD-box proteins in human Ddx5, mouse MrDb (Myc-regulated DEAD-box protein) and *Drosophila*.[36,37] The p68 function as transcription coactivators by binding with CBP, the CREB-binding protein.[38] The CBP bridges the CRE/CREB complex to components of the basal transcription apparatus and it is possible that p68 directly influence its own transcription. Furthermore, the promoter of *Psp68* contains overrepresentation of different transcriptional activators elements. These findings indicated that transcription of *Psp68* might be highly complex and developmentally regulated. Several putative cis-regulatory elements associated with

tissue-specific expression (GATA and CAAT motifs), pollen specific activator elements (GTGA and AGAAA motifs), mesophyll (YACT motif) and guard cell-specific (TAAAG motif) gene expression elements and nodule specific regulatory elements (AAAGAT, CTCTT, AAAGAT and CTCTT motifs) were identified in *Psp68* promoter sequences. Apparent enrichment of these tissue-specific expression regulatory elements indicates the involvement of *Psp68* gene in wide range of cellular process but need to validate.

The expression of *Psp68* was induced by abiotic stress. Salt (GAAAAA) and dehydration (ACGT-box) responsive cis-acting elements were identified by computational analysis. The promoter of *Psp68* is able to drive GUS expression in agroinfiltrated leaves of tobacco challenged with NaCl, PEG and cold stress. The presence of the GT-1 like element (5′-GAAAAA-3′) in the upstream region of *Psp68* gene might be responsible salinity specific expression. Earlier report showed that in response to salinity stress, the GA sequence in GT-1 cis regulatory element bind to nuclear factor(s)[39] resulting salinity stress tolerance. In this study, GUS activity was also observed in response to PEG and ABA treatments. The existence of ACGT-box and ABRE elements might support the above statement. In response to dehydration, ABA levels increased. It has been reported that most dehydration-inducible genes are also induced by ABA[40,41] and ABA is known be involved in dehydration-inducible gene expression in land plants. [40-42] The promoter sequence of *Psp68* also contain cold responsive element like CCGAC (DRE). Earlier, the DRE/CRT and ABRE elements found together in the promoters of many well-studied cold-regulated genes in *Arabidopsis*[43, 44] which is consistent with a role for the ABA regulation of cold-induced genes.

Auxin is a major plant hormone, required for many developmental processes in plant[45] including root formation,[46] apical dominance[47] and growth-related tropisms.[48] The transcriptional response to auxin is mediated by the auxin responsive cis-regulatory elements present in the upstream region of auxin responsive genes.[49] We have identified 5 auxin responsive cis-regulatory elements in *Psp68* promoter sequence. Furthermore, high GUS expression was observed in the agroinfiltrated tobacco leaves upon treatment with auxin. This indicated that *Psp68* gene may play an important role in auxin-mediating signal transduction pathways. Although conserved similar sequence were observed in the promoter of many auxin responsive genes, [50,51] it remains need to be tested the functional significance of these conserved sequences. Jasmonates are another growth regulators[52] important for plant biotic and abiotic stress responses. [53-55] Either GCC or G-box elements are required for MeJA-inducible expression of different genes. A number of studies have been identified these elements in a variety of plant gene promoters and their role in response to light, anaerobiosis, and various phytohormones.[56,57] We found 7 GCC motifs in the promoter of *Psp68* and promoter:: GUS analysis also detected very high level of GUS expression under MeJA treatment, indicating a positive regulatory role of *Psp68* gene toward abiotic stress tolerance.

The promoter of *Psp68*, drive the expression reporter gene in response to abiotic stress and hormonal treatments. The *Psp68* promoter contains dehydration, salt, cold, auxin ABA and MeJA related cis-elements, which may regulate the expression of this gene. Therefore, *Psp68* promoter could be used as a new and powerful candidate for the study of tissue specific and stress responsive transgene expression in crop plants.

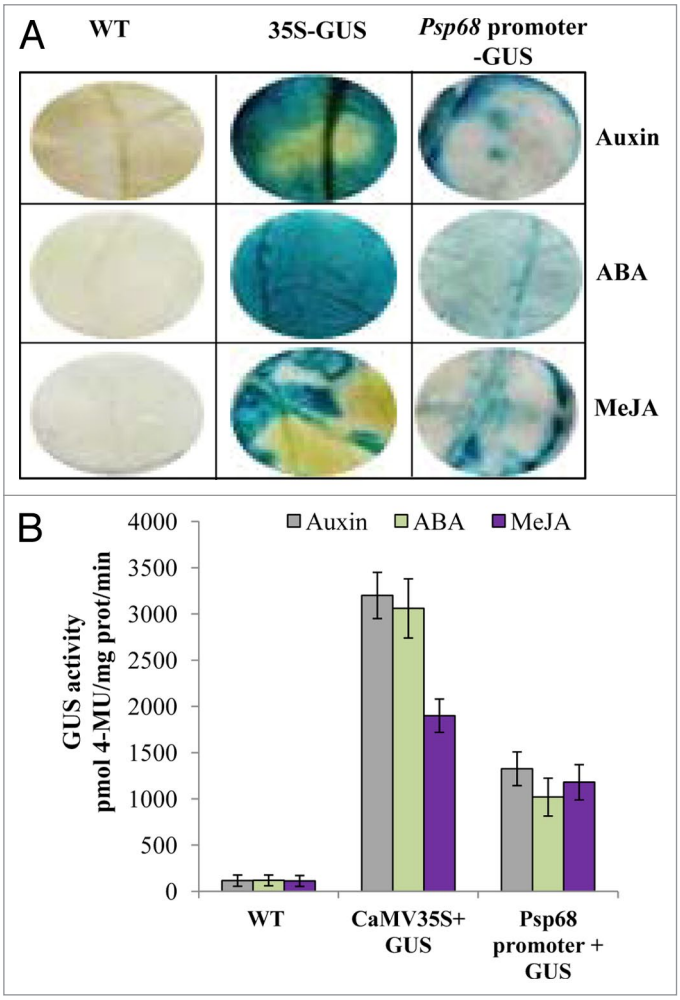

Figure 4. *Psp68* promoter-GUS analysis in response to hormonal treatment. (**A**) GUS activity in agroinfiltrated leaves. GUS was detected in X-Gluc solution followed by hormone treatment. (**B**) Effect of abiotic stress on the transient expression of *Psp68* promoter in agroinfiltrated tobacco leaves. Two days after infiltration with *A. tumefaciens* ($OD_{0.7}$) containing either *Psp68* promoter::GUS or CaMV35S::GUS constructs, tobacco leaves were sprayed with 100 µM Auxin, 100 µM ABA and 10 µM MeJA and used for quantification assay. Water treated infiltrated leaves of WT used as negative control. Data represent the mean and SD of 4 independent experiments.

Materials and Methods

Isolation of *Psp68* promoter: gene walking by PCR

Pea genomic DNA was isolated by a previously described method[58] and ~5 µg genomic DNA was digested overnight at 37 °C with 4 blunt end cutting restriction enzymes: DraI, EcoRI, PvuII and StuI/SmaI independently. Following digestion, the genomic DNA was purified by phenol-chloroform precipitation

and each pool of DNA fragments was ligated to the BD Genome Walker Adaptor as per the manufacturer's instruction. The primer AP1corresponding to gene-specific primer R3 was used for primary PCR reactions. The 50 µl reactions mixture contain 0.4 µl stock diluted DNA, 0.2 µM of each primer and 0.5 µl Advantage® 2 Polymerase mix (Clontech, USA) with the following conditions: 35 cycles, 94 °C 45s, 64 °C 30s, 72 °C 1 min. The primary PCR were then diluted in 50 fold for prior to the nested-PCR reaction using the primer AP2 in combination with R2 in the same cycle conditions. The bands of interest were separated by electrophoresis, purified and cloned into pGEMT vector and sequenced.

In silico analyses of promoter sequence

Homologies to sequence were searched in Basic local alignment search tools (BLASTN and BLASTX) and was aligned using the ClustalW program. The prediction of transcriptional start site, TATA-box and CAAT-box were done using Plant Prom Database.[59] Putative cis-acting elements were identified by using Plant CARE Database (http://bioinformatics.psb.-ugent.be/webtools-/plantcare/html/).

Plasmid constructions

The *Psp68* promoter was amplified from *Psp68* promoter-pGEMT clone, using the primers by introducing BamHI and HindIII restriction sites. The amplified bands were run on 1% agarose gel, cut, eluted and again ligated into pGEMT-easy cloning vector. The clone was verified by colony PCR and restriction digestion analysis with BamHI and HindIII enzymes. *The Psp68* promoter was further cloned in pCAMBIA-1391Z in the same restriction site. The colonies were checked by PCR, followed by restriction analysis with BamHI and HindIII restriction enzymes. The *Psp6*8 promoter cloned in pCAMBIA-1391Z vector was again transformed in *Agrobacterium tumefaciens* (LBA4404) and confirmed by colony PCR using *Psp6*8 promoter specific primers.

***Agrobacterium*-mediated transient assays**

Agroinfiltration assays were performed by a previously described method earlier.[27] The *Psp68* promoter, transform in *Agrobacterium tumefaciens* (LBA4404) were grown in LB medium containing 50 µg/ml rifampicin, 50 µg/ml kanamycin and incubated overnight at 28 °C. The cells were harvested by centrifugation at 3,0009 g for 15 min and further resuspended in infiltration media (10 mM MgSO4, 200 µM acetosyringone, 20 mM MES pH 5.6). Fully expanded leaves of tobacco (*Nicotiana tobaccum* cv USA) plants grown in greenhouse at 22 °C were agroinfiltrated by using a 1-ml syringe. After 48h, infiltrated leaf discs were collected.

Stress treatments

For salinity and drought stress, tobacco leaves were agroinfiltration with 200 mM NaCl and 20% PEG solution or water as a control and then collected after 24h. For cold treatment, infiltrated leaf discs kept on 4 °C and collected after 24h. For hormonal stress, tobacco leaves were agroinfiltration with 10 µM naphthalene acetic acid (auxin), 100 µM ABA and 10 µM MeJA respectively or water as a control and then collected after 24h.

GUS activity detection

The leaf discs were incubated overnight at 37 °C in GUS assay solution containing 1 mg/ml X-Gluc, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2% Triton X-100 in 100 mM sodium phosphate buffer (pH 7.4) followed by washing with 70% ethanol solution till the chlorophyll cleared.

GUS activity quantification

β-Glucuronidase activity was quantified by fluorometric GUS assays. Agroinfiltrated leaves were homogenized in 1 ml extraction buffer containing 10 mM EDTA, 50 mM NaH_2PO_4 pH 7, 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol and 0.1% Triton X-100. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C and finally supernatant was collected. The concentration of protein was measured by Bradford method[60] by using bovine serum albumin (BSA) as a standard. GUS activity was performed by earlier described method[61] and expressed as picomoles of 4-MU (methylumbelliferone) per minute per milligram of protein.

Contributions

MSAB performed the research, analyzed data and written the manuscript, KMKH performed the experiments and helped in writing the manuscript, NT designed research, analyzed the data and written the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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