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UNDERSTANDING THE GAMMA-VACUOLAR PROCESSING ENZYME GENE REGULATION BY PROMOTER-GUS FUSION APPROACH

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Abstract

Vacuolar processing enzymes (VPEs) are vacuole localizing cysteine proteinase, responsible for the maturation of seed storage proteins in plants and are also known to be involved in programmed cell death. In the present study, an attempt has made to understand the regulatory mechanism of *AtγVPE* gene expression by studying promoter region of *AtγVPE*. We isolated approximately 1.2 kb fragment upstream of the initiation codon of *γVPE* gene sequence and deletion mutations were made from 5' end of promoter region, fused with GUS reporter gene to analyze the transient GUS activities in *Nicotiana benthamiana* leaves. The full-length promoter (1282 bp) was found to show lower GUS activity in comparison to the deletion fragments. However, P6 (-349 bp) promoter had the maximum activity and on further deletion the activity reduced, with little or no activity in P8 (-176 bp), signifying P6 having the maximal promoter activity. *In-silico* analysis revealed the presence of several putative *cis*-regulatory elements in the promoter region, including hormone-responsive elements for ABA, GA, SA and ethylene, elements involved in abiotic stress, development, and several repressor elements. Functional validation of putative *γVPE* promoter in tobacco leaves also proved the promoter activity was induced by abiotic stress and phytohormones.

Key words : Programmed cell death, abiotic stress, phytohormones, *Nicotiana benthamiana*, transcription factors, *cis*-regulatory elements.

Introduction

Gama vacuolar processing enzymes (VPEs) were first reported in maturing pumpkin seeds and categorized as cysteine proteinase responsible for the maturation of seed storage proteins in the protein-storage vacuoles of maturing seeds and thus named it as vacuolar processing enzyme (VPE) (Hara-Nishimura *et al*., 1991). In *Arabidopsis thaliana*, four isoforms of VPE have been reported, α, β, γ and δ, of which β and *δVPE* show abundant expression in seeds whereas α and *γVPE* are expressed mainly in the vegetative tissues (Hara-Nishimura *et al*., 1998). However, Shimada *et al*. (2003) showed that α and *γVPE* along with β *VPE* also have a role in processing and maturation of storage proteins in *Arabidopsis* seeds (Shimada *et al*., 2003; Gruis *et al*., 2004). The vegetative *VPE* (*γVPE*) up-regulates in the lytic vacuoles during senescence and stress associated PCD, showing its involvement in environmentally induced PCD (Kinoshita *et al*., 1999). The *γVPE* is inhibited not only by the VPE inhibitor ESEN-CHO but also by caspase-1 inhibitors and in *VPE*-silenced *Nicotiana benthamiana* plants, the VPE activity was correlated to the reduction of caspase-1-like activity, showing that γVPE have caspase-like activity (Hatsugai *et al*., 2004). Furthermore, there is a similarity in the substrate specificity between VPE and caspase-1 which is constant with several structural resemblances between the two enzymes, such as similar substrate pockets and similar active sites (Hara-Nishimura *et al*., 2005; Hatsugai *et al*., 2006). *γVPE* by inducing PCD plays numerous roles in development as well as in biotic and abiotic stress response. VPE expressed in petals and leaves during senescence and in tuber apical bud meristems to start PCD and the release of apical dominance in potato (Kinoshita *et al*., 1999; van Doorn and Woltering, 2008; Muller *et al*., 2010). Promoter-GUS analyses have shown enhanced *αVPE* expression in dying cortical cells positioned subsequent to the developing lateral roots and

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in senescent leaves. However, *γVPE* expression was found at an early stage of senescence and advanced stage of pollen development (Kinoshita *et al*., 1999; Hatsugai *et al*., 2015). Hybrid lethality is another condition where zygotes, after formation, is unable to survive due to vacuolar collapse and cell death (Mino *et al*., 2007). VPEs are induced by fungal toxin in plants and is upregulated in response to immune responses, including the hypersensitive response (HR) resulting in localized PCD and limiting the spread of the pathogen. *γVPE* activity has also been shown to increase during fungal infection (*Hyaloperonospora arabidopsidis*, *Fusarium moniliforme*, *Botrytis cinerea*), which otherwise is less in the γ*vpe*-null mutant (Wang *et al*., 1991; Rojo *et al*., 2004). It is a well-known fact that under stress MAPK6 activity increases and MAPK6 has been shown to activate *γVPE* leading to the subsequent execution of heat stress-induced PCD (Li *et al*., 2012). The *γVPE* has also been reported to express strongly in guard cells in response to water stress, maneuvering the closing and opening of stomata (Bates *et al*., 2012; Albertini *et al*., 2014). Salt, drought and O^3 stress leadfing to accumulation of ROS, like H_2O_2 in plants also triggers activation of γVPE mediated PCD (Deng *et al*., 2011; Kim *et al*., 2014; Kadono *et al*., 2010). The γVPE has also been associated to involve in UV and heavy metal stress-induced caspase-1 or caspase-3 activity, vacuolar collapse and PCD (Danon *et al*., 2004; Sanitadi and Gabbrielli, 1999).

Gama-vacuolar processing enzyme as evident from the literature has temporal and spatial expression patterns and plays a critical role in development as well as in stress response. In the present study, we have tried to understand the regulatory mechanism of temporal expression of *γVPE* by promoter-reporter fusion approach. We isolated approximately 1.2 kb stretch upstream of translation start codon, predicted to be the putative promoter, fused with GUS reporter gene, and transient GUS expression was assayed in *Nicotiana benthamiana*. To understand the temporal expression pattern of *γVPE*, we assayed the gamma-vacuolar processing enzyme putative promoter-GUS activity under various stresses. Further, the *in silico* analysis revealed various *cis*-regulatory elements and their association with GUS expression pattern with deletion mutations was also attributed.

Materials and Methods

Isolation and construction of *AtγVPE* promoter-GUS chimeric plasmids

Genomic DNA of *A. thaliana* (Col-0) was isolated, and ~1282 bp upstream sequences of *AtγVPE* gene (AT4G32940) was selected as promoter sequence and amplified by PCR using sequence-specific primers. The amplified PCR products were cloned in binary vector pCAMBIA 1304 at *Xba* I and *Bgl* II restriction sites and named as P chimera plasmid which was further used for generating deletion fragments. Eight subsequent *AtγVPE* promoter fragments were generated from -1096, -832, -666, -587, -446, -349, -253 and -176 bp relative to initiation codon by using a common reverse primer and position specific forward primers (supplementary table 1 and supplementary fig. 1), cloned into pCAMBIA 1304 and named as P1 (1096 bp), P2 (832 bp), P3 (666 bp), P4 (587 bp), P5 (446 bp), P6 (349 bp), P7 (253 bp) and P8 (176 bp) chimera plasmids.

In silico analysis of promoter sequence

The promoter region was analyzed using MatInspector programme, Matrix Family Library version 10.0 (http://www.genomatix.de/) with plant filter (*A. thaliana*) to search putative binding sites of transcription factors (TFs) (Cartharius *et al*., 2005).

Agrobacterium-mediated GUS transient expression

The full length and all promoter deletion plasmids were mobilized into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium* containing individual constructs were grown in LB media supplemented with kanamycin (50μg/ml) and rifampicin (40μg/ml). Overnight grew *Agrobacterium* cells were harvested by centrifugation and suspended to an OD_{600} of 0.6 in suspension solution [10mM MES buffer (pH5.6), 10mM $MgCl_2$ and 200mM acetosyringone) and incubated at room temperature for 2 hours. The *Agrobacterium* cells were infiltrated into the intracellular spaces on the abaxial side of fully expanded leaves of *N. benthamiana*. After 72 hours the infiltrated leaves were analyzed for GUS expression.

Plant growth condition and stress treatment

N. benthamiana plants were grown at 22±2p C with photoperiod 16 hours light/8 hours dark in the greenhouse. One-month-old plants were used for the experiment. For stress treatment, plants were infiltrated with *Agrobacterium* cells of an OD_{600} of 0.4 (Srivastava *et al*., 2014). After 48 hours of infiltration, the leaves were cut into disc with the help of disc-borer and floated in Petri plates containing 200 mM NaCl, 10% PEG-6000, 10 μM MeJA, 10 μM ACC, 500 μM Salicylic acid, 10 μM ABA, 15 mM $CdCl_2$ and 1 mM H_2O_2 as well as in water, that served as control, for 24 hour at 22±2^{0}C with photoperiod 16 hours light/8 hours dark. For cold

treatment, leaf discs were floated in the water and incubated at 4^0C for 24 hours.

GUS activity and quantification

For visual detection of GUS activity, leaf discs were vacuum infiltrated with a GUS solution [2mM 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-Gluc), 50mM sodium phosphate buffer (pH 7.2) and 0.2% Triton X] and incubated at 37p C overnight (Rustagi *et al*., 2015). The reaction was stopped by adding 80% ethanol and washed with the same to remove the chlorophyll. The fluorometric analysis of GUS activity was performed using 4-methylumbelliferyl-β-glucuronide (4-MUG, Sigma) (Jefferson *et al*., 1987). Stress treated and control leaf disc were crushed in liquid nitrogen and samples were homogenized in 150-200µl of extraction buffer (50mM $Na_2H_2PO_4$ pH 7.0, 10mM EDTA, 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol and 0.1% Triton X). The protein concentration was measured by Pierce BCA protein quantification assay kit (Thermo Scientific). 10µg protein (sample volume 60 µl) of each sample was used for GUS assay. 90 µl of GUS reaction mixture (extraction buffer containing 2mM 4-MUG) was added to the samples and incubated at 37^0C in the dark for 30 minutes. The reaction was stopped by adding 190 µl of stop buffer (200 mM sodium carbonate pH 9.5) to 10 µl aliquot of sample reaction in 96- well plate. The GUS activity was determined with Varioskan Flash (Thermo Scientific) plate reader at 365 nm excitation wavelength and 445 nm emission wavelength with 12nm bandwidth.

Results

***In-silico* analysis**

The analysis revealed numerous *cis*-element and TFs binding motifs belonging to plants on both positive and negative strands, apart from the TATA and CAAT boxes, which are commonly present in the enhancer region of the promoter (table 1). The identified *cis*-elements had homology with several known hormone responsive elements, such as ABA response elements (P$ABRE), Brassinosteroid (BR) response element (P$BRRE), Jasmonate response element (P$JARE), (P$GCCF) GCC BOX family which bind ethylene responsive factors (ERFs), P$DOFF, a transcription factor of the DNA binding with one finger class, a family of transcription factors involved in many fundamental processes in higher plants, including responses to light and phytohormones as well as roles in seed maturation and germination through interaction with the pyrimidine box of the GA responsive complex (GARC). Abscisic acid (ABA), a phytohormone functions in many plant developmental processes, including bud dormancy and also in mediating adaptive plant responses to environmental stresses. Brassinosteroids (BR) are involved in plant development and physiology and are integrated into a complex signaling network intervening the levels of other phytohormones. Jasmonates are signaling molecules that orchestrate plant responses to Biotic and Abiotic stress. It also regulates wound response and pollen maturation in *Arabidopsis*. MYB factors in plants have been implicated in JA signaling pathways that bind to (T/C) AAC (T/G) G and G (G/T)T(A/T) G(G/T)T type of DNA sequences and are mostly documented in regulating the biosynthesis of primary and secondary metabolites though they are also known to play roles in abiotic and biotic stress.

DNA binding proteins ASYMMETRIC LEAVES1 (AS1) and AS2 target elements (P$ASRC), which acts as repressor complex were also found in the promoter regions. The AS1/AS2 complex is known to form a loop in the promoter of *KNOX* gene, and by recruiting histone regulator A (HIRA) by forming a complex which can repress the KNOX gene, which is required for the development of lateral organs. Several Circadian control elements (P$CCAF) were also found within the promoter indicating a circadian regulation of the gene. We also found *cis*-element with sequence homology to transcription repressor KANADI (P$KAN1), which is a key regulator of abaxial identity, leaf growth, and meristem formation in *Arabidopsis thaliana*. It also acts as a transcriptional repressor and among its targets are genes involved in auxin biosynthesis, auxin transport, and auxin response. Several Homeobox (P$AHBP) protein binding elements were also found in the promoter region. Some of these elements of interest were Homeobox-leucine zipper protein REVOLUTA (REV, IFL1 (P$REVOLUTA.01) and HD-ZIP class III protein which is known to act antagonistically to KANADI regulating auxin biosynthesis and auxin-related genes. Transcriptional repressor BELLRINGER, WUSCHEL-related homeobox 13 interacts with homeobox protein and acts as a repressor.

Multiple stress response elements were also identified in the promoter such as elements responsible for Dehydration response (P$DREB), heat (P$HEAT) and salt (P$SALT) response. The heat shock factor (HSF1) which is the central regulator of the heat stress response and is required for stimulating the transcription of the heat shock genes. Other identified *cis*-regulatory elements include the targets of NAC-domain containing transcription factors. Recent reports showed that GmNAC30 and GmNAC81 are involved in programmed cell death response by regulating the expression of VPE.

Table 1 : List of identified putative *cis* – elements in *AtyVPE* promoter analyzed MatInspector.

Matrix Family	Match Total	p-value	Detailed Family Information
O$PTBP	10	0.121141	Plant TATA binding protein factor
O$TF2D	1	0.038343	General transcription factor IID, GTF2D
P$ABRE	2	0.14338	ABA response elements
P$AHBP	18	0.016427	Arabidopsis homeobox protein
P$AHLF	4	0.146456	AT-hook containing transcription factors
P$ASRC	4	0.144673	AS1/AS2 repressor complex
P$BRRE	4	0.126237	Brassinosteroid (BR) response element
P$CAAT	3	0.148145	CCAAT binding factors
P$CARM	1	0.118453	CA-rich motif
P$CCAF	11	0.024666	Circadian control factors
P$CNAC	2	0.142989	Calcium regulated NAC-factors
P$DOFF	1	0.026454	DNA binding with one finger (DOF)
P$DPBF	2	0.039533	Dc3 promoter binding factors
P$DREB	2	0.113764	Dehydration responsive element binding factors
P$FLO2	2	0.145222	Floral homeotic protein APETALA 2
P$GARP	2	0.132208	Myb-related DNA binding proteins (Golden2, ARR, Psr)
P$GBOX	3	0.084108	Plant G-box/C-box bZIP proteins
P$GCCF	1	0.14809	GCC box family
P$GTBX	9	0.007873	GT-box elements
P$HEAT	5	0.099452	Heat shock factors
P$HMGF	3	0.12271	High mobility group factors
P$IBOX	4	0.091795	Plant I-Box sites
P$IDDF	1	0.140673	ID domain factors
P$JARE	2	0.145902	Jasmonate response element
P$KAN1	6	0.081	Transcription repressor KANADI
P$L1BX	6	0.049904	L1 box, motif for L1 layer-specific expression
P$LEGB	1	0.142166	Legumin Box family
P$LFYB	1	0.093105	LFY binding site
P$LICM	1	0.102623	Leaf and tiller angle increased controller (LIC) binding motif
P$LREM	5	0.125006	Light responsive element motif, not modulated by different light qualities
P$MADS	12	0.024666	MADS box proteins
P$MIIG	3	0.027342	MYB IIG-type binding sites
P$MSAE	1	0.145699	M-phase-specific activator elements
P$MYBL	5	0.002982	MYB-like proteins
P$MYBS	5	0.021954	MYB proteins with single DNA binding repeat
P$MYCL	1	0.079095	Myc-like basic helix-loop-helix binding factors
P$MYCS	1	0.035416	Cis-regulatory elements involved in the transcriptional activation of the arbuscular mycorrhizal (AM)-mediated inorganic phosphate transporter genes

Table 1 continued...

Table 1 continued...

P$NACF	4	0.023766	Plant specific NAC [NAM (no apical meristem), ATAF172, CUC2 (cup-shaped cotyledons 2)] transcription factors
P$NCS1	5	0.132488	Nodulin consensus sequence 1
P$NCS2	1	0.130922	Nodulin consensus sequence 2
P$NTMF	1	0.128634	NAC factors with transmembrane motif
P$PCDR	2	0.146484	Factors involved in programmed cell death response
P$PSPE	2	0.137071	Protein secretory pathway element
P$PSRE	2	0.146921	Pollen-specific regulatory elements
P$RAV5	1	0.060695	5'-part of bipartite RAV1 binding site
P$REMF	1	0.136885	B3 domain-containing REM family
P$ROOT	1	0.127449	Root hair-specific cis-elements in angiosperms
P$SALT	1	0.088219	Salt/drought responsive elements
P$SPF1	1	0.147806	Sweet potato DNA-binding factor with two WRKY-domains
P$STKM	3	0.131144	Storekeeper motif
P$SUCB	3	0.138624	Sucrose box
P$SWNS	3	0.146622	Secondary wall NACS
P$TCPF	1	0.128399	DNA-binding proteins with the plant specific TCP-domain
P$TOEF	1	0.132488	Target of early activation tagged factors
P$WBXF	2	0.119887	W Box family
P$WOXF	3	0.147404	WUS homeobox-containing protein family
P$WTBX	1	0.148074	WT-Box

Supplementary Table S1 : List of primers used for promoter cloning.

P	GTGTCTAGAACAAAACCCACTTGCTCTAATCAAAG
P1	GTGTCTAGAGGAATGGTGTGTGTCGTTG
P2	GTGAGATCTACCATCCATCTTAACTCTGATAAGCAATC
P3	GTGTCTAGACTCTGACTAAGAGAGTATATTCAG
P4	GTGTCTAGAAGTAGTGAATTTAGTCCGAAAAC
P5	GTGTCTAGAGACATGATCACAAGAAATAGTGC
P6	GTGTCTAGAATGGGATTTGTGAAACAAATGAAAG
P7	GTGTCTAGAGAAGGAAGCAACATCACGCAACTG
P6	GTGTCTAGAGTCTAAATGTGCAAATATATCC
R	GTGAGATCTACCATTAGCCGGCGAGTGATGATGA

Further cis-regulatory element (P$PCDR) involved in cell death response were also identified.

Functional characterization of *AtγVPE* promoter

To validate the function and expression pattern of *AtγVPE* gene promoter, we cloned approximately 1.2 kb upstream region from the start codon of *γVPE* gene sequence. We constructed eight deletion mutations from the 5' end of cloned in to pCAMBIA1304 upstream to mGFP:GUS reporter gene (fig. 1). The full-length *γVPE* promoter (1.2 kb) fused with mGFP:GUS reporter gene constructs, as well as all eight deletion sequences, drove GUS expression in examined tissues (fig. 2). The β-glucuronidase (GUS) activities revealed that all the promoter fragment of the *γVPE* gene were functional in tobacco (fig. 2), but the level of GUS activity observed under full-length, as well as deletion fragments of the *γVPE* promoter, was lower as compared to the CaMV35S promoter (positive control) (fig. 2). Further to understand the regulatory mechanism of *AtγVPE* gene, the GUS activity of full-length *γVPE* promoter was compared with the eight deletion mutants. The results indicated that all the seven deletion fragments (P1 to P7), except the smallest fragment (P8), showed significantly higher GUS activity in comparison to the full-length promoter (fig. 2). It was found that there was a gradual increase in GUS activity as we proceeded deletion from the 5' end and the fragments getting smaller up to -587bp (P4) promoter fragment (fig. 2). The -349bp promoter fragment (P6) showed highest GUS activity when compared to all the deletion fragments as well as full-length promoter. The P6 promoter fragments showed around three-fold increased GUS activity in comparison to the full-length promoter (P). It indicated that the *γVPE* promoter performed as an inducible promoter (fig. 2).

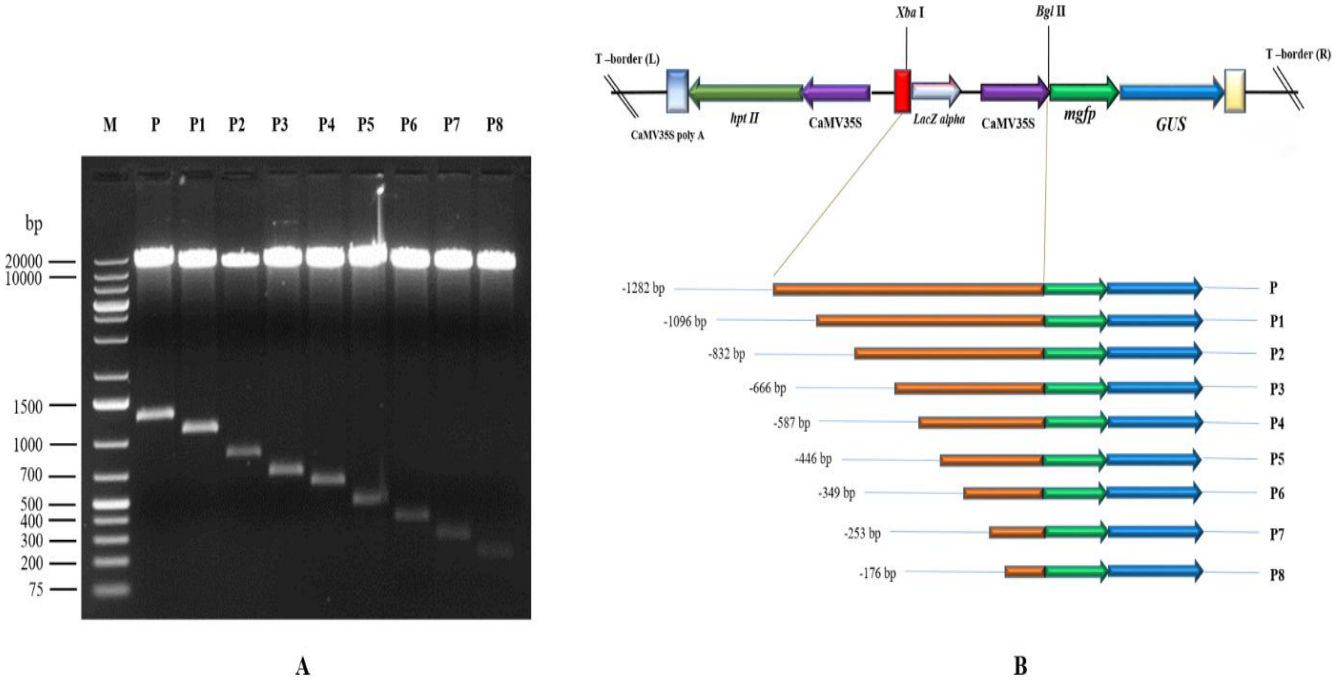

Fig. 1 : Cloning of *AtγVPE* promoter and its deletion fragments in binary vector pCAMBIA1304. (**A**) restriction digestion of *AtγVPE* full-length promoter and deletion fragments clones by *Xba* I and *Bgl* II. Lane M, 1kb marker; Lanes P to P8, chimeric plasmids (**B**) schematic representation T-DNA region of binary vector with full length and deletion promoter fragments. The name of chimeric plasmid (right) and size of fragments (left) indicated relative to the start codon of *AtγVPE* gene.

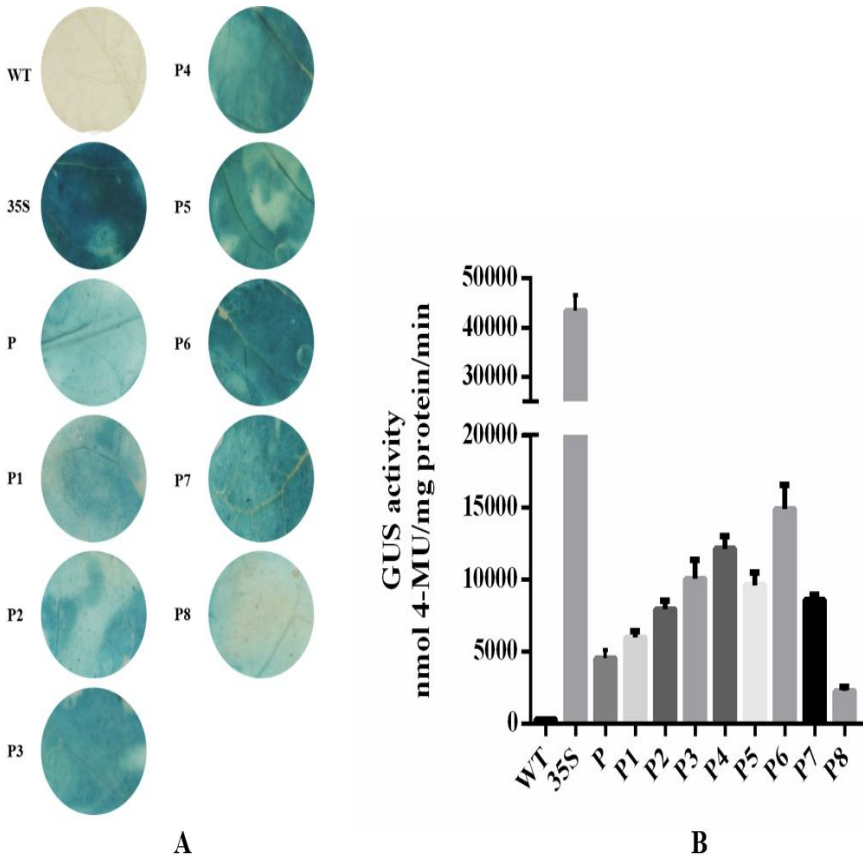

Fig. 2 : Transient GUS assay in *N. benthamiana* leaves. (**A)** GUS histochemical assay of *Agrobacterium* infiltrated leaves with promoter::*GUS* chimeric plasmids. (**B**) GUS activity measured in *Agrobacterium* infiltrated leaves. WT, mock; 35S, pCAMBIA 1304 vector; P to P8; *AtγVPE* promoter::*GUS* chimeric plasmids.

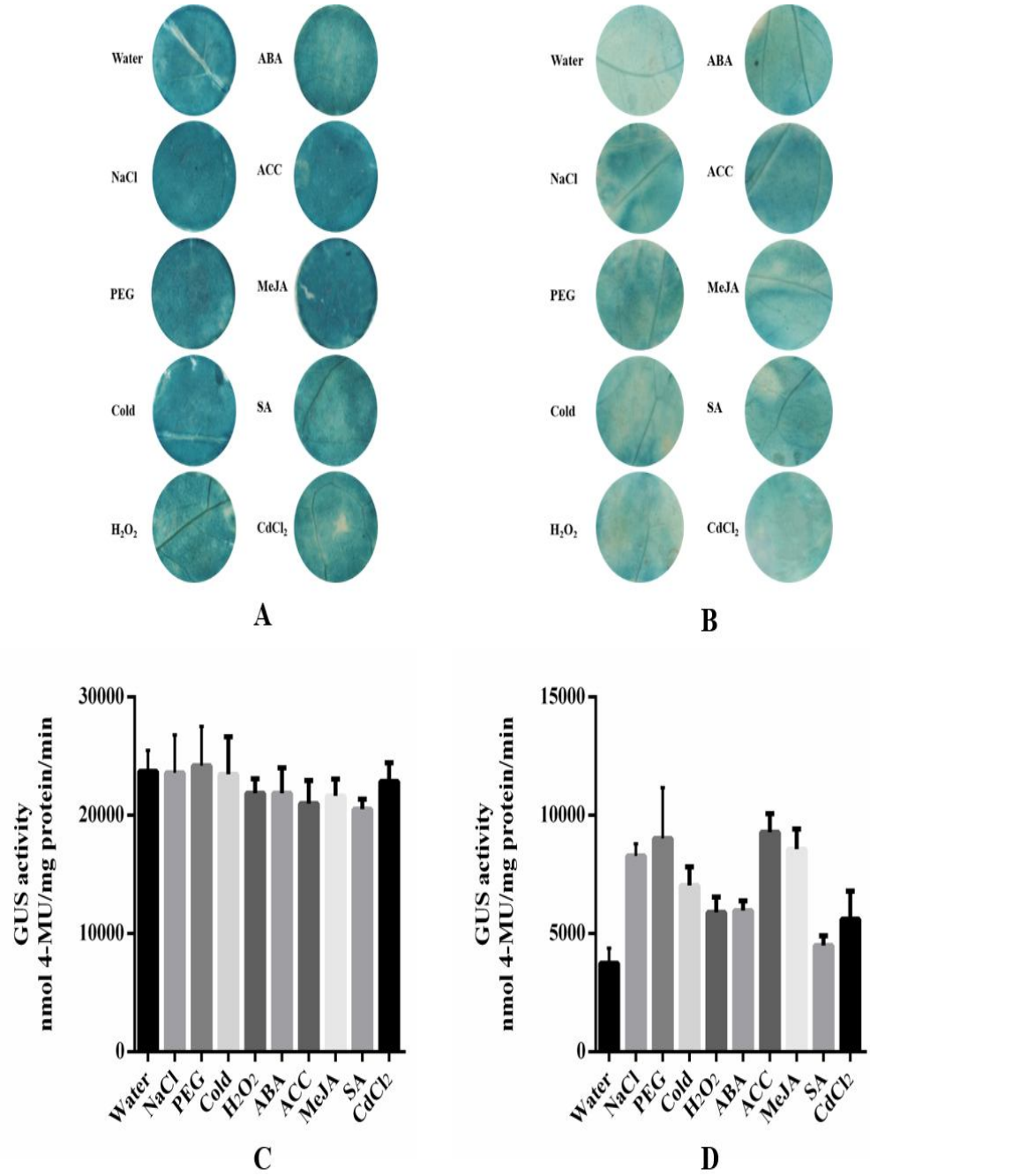

Fig. 3 : Transient GUS assay under stress condition. **(A)** GUS histochemical assay of *Agrobacterium* infiltrated leaves, with pCAMBIA 1304 vector plasmid, **(B)** with full *AtγVPE* promoter::*GUS* chimeric plasmid. **(C)** GUS activity measured in *Agrobacterium* infiltrated leaves, with pCAMBIA 1304 vector plasmid and **(D)** with full *AtγVPE* promoter::*GUS* chimeric plasmid.

Induced GUS activity of *γVPE* promoter under phytohormones

The *γVPE* promoter responded differently to the phytohormones treatment. The measurement of GUS activity was performed after 24 hours of ABA, ACC, MeJA and SA treatment. All the phytohormone treated samples showed significantly increased GUS activity in comparison to the water treated control sample. The ACC and MeJA treated sample showed approximately two-fold increased GUS activity, on the other hand, SA and ABA-treated sample showed a relatively low increase in GUS activity in comparison to control (fig. 3). However, the 35S promoter comparatively showed almost similar GUS activity in water and phytohormones treatment (fig. 3).

Abiotic stresses and heavy metal induces the *γVPE* promoter in tobacco leaves

The expression pattern of the *γVPE* promoter was assayed under different abiotic stresses such as salinity, drought, cold and heavy metal as well as under oxidative stress. Results revealed that the GUS activity of *γVPE* promoter was upregulated under these stress conditions (fig. 3). The increased GUS activity was higher in case of salinity, drought and cold in comparison to $CdCl_2$ and H_2O_2 treated sample. However, 35S promoter GUS activity was similar under stress conditions (fig. 3). These results indicated that the *γVPE* promoter is a stress-inducible promoter.

Discussion

***AtγVPE* promotor is an inducible promoter**

Expression of the gene in different tissues and organs is regulated during various growth and developmental stage, as a consequence of external stimuli at the level of transcription, post-transcription and post-translation. The activation and suppression of a gene are controlled by the interaction of transcriptional factors with the gene promoters and their contributing *cis*-acting elements. All these factors may either bind directly to the promoter sequence or in association with other proteins. The behavior of promoter to different stimuli is also affected by the copy number, inter-motif distance, their position and mutations in the core sequences of the *cis*-regulatory elements (Zou *et al.*, 2011). The full-length putative promoter (P), as well as the deletions (P1 to P8), fused with GUS, were able to drive the GUS expression though the level of expression varied in full length and various deletions (fig. 2). Earlier, Nishimura *et al*. (1999) have functionally validated the expression of an approximately 2 kb upstream region of the *γVPE* gene from *Arabidopsis thaliana*, cloned from -2036bp to +11 bp, have shown the gene to express spatially and temporally in vegetative organs with high expression in senescent tissues (Kinoshita *et al*., 1999). In the present study, the expression observed in the full-length promoter (1282 bp) was lesser than all the subsequent deletions from 5' end from P1 to P7, and then there was a drastic reduction in the expression on further deletion (P8). P6 was showing the maximum GUS expression (fig. 2). The reduced activity in the full-length promoter could be attributed to the presen ce of various repressor *cis*-elements including, AS1/AS2 repressor complex present in the promoter, upstream of P6. Guo *et al*. (2008) have shown that recruitment of the AS1 complex to a promoter is mediated by binding of AS1-AS2 to the c-Myb–related sequence CTGTT (Motif 1) and TCg/tTTGAAT (Motif 2) *cis*-elements and forming a loop in the promoter (34). It was indicated that the motif 2 stabilizes AS1 complex binding to the promoters. In the *AtγVPE* promoter, Motif 1 is present 82 bp away from start site. This motif 1 can interact with any of the four AS1/AS2 repressor elements in the promoter. However, according to the GUS analysis results, it is likely that the motif 1 does not interact with motif 2 that lies close to it. Thus, removal of the repressors elements presents before P5 construct restores the promoter activity. The reduced activity of P7 can be attributed to the presence of another repressor downstream of P6. It is, however, likely that P7 may contain the minimal core promoter region as further deletion showed little or negligible promoter activity. The lower expression of the full-length promoter can also be linked to the presence of KANADI box upstream of P4. It has been reported that the Myb-like domain in KAN1 binds the 6-bp motif GNATA (A/T) and that this motif alone is sufficient to repress the transcription of a linked reporter gene in in-vitro experiments (Huang *et al*., 2014). BELLRINGER and WUSCHEL too act as a repressor and down-regulates the expression of pectin methylesterase (PME5) and cell cycle genes respectively playing roles in phyllotaxis and lateral root formation (Peaucelle *et al*., 2011; Kong *et al*., 2016).

***AtVPE* upregulates in response to phytohormones, oxidative, heavy metal, and abiotic stress**

AtγVPE expression is known to upregulate in response to phytohormones, oxidative stress, heavy metals and abiotic stresses. The upregulation of vegetative *VPE* in response to phytohormones has already been studied in Arabidopsis (Kinoshita *et al*., 1999). Our promotor:GUS activity results also corroborate with the upregulated *γVPE* transcripts in response to phytohormones (fig. 3). The VPE homologs in *Oryza sativa* (*OsVPE*) are reported to upregulate under salinity and oxidative stress (Deng *et al*., 2011; Kim *et al*., 2014; Kadono *et al*., 2010). Here, we report that in addition to salinity and oxidative stresses, the VPE promoter is also induced by cold and drought stress as indicated by increased GUS activity under cold and drought stresses (fig. 3). Thus in this report, we infer that *γVPE* may be associated with cold-induced programmed cell death in plants as the cold stress-induced chromatin fragmentation and cell death has reported in tobacco BY-2 cell lines (Koukalova *et al*., 1997). Additionally, the GUS activity also increased under PEG-treatment indicating the possible new role of *γVPE* in drought stress. Aluminum toxicity is known to induce cell death in plants as well as human and often shows the typical apoptotic features (Hatsugai *et al*., 2015; Kochian, 1995). It has also been

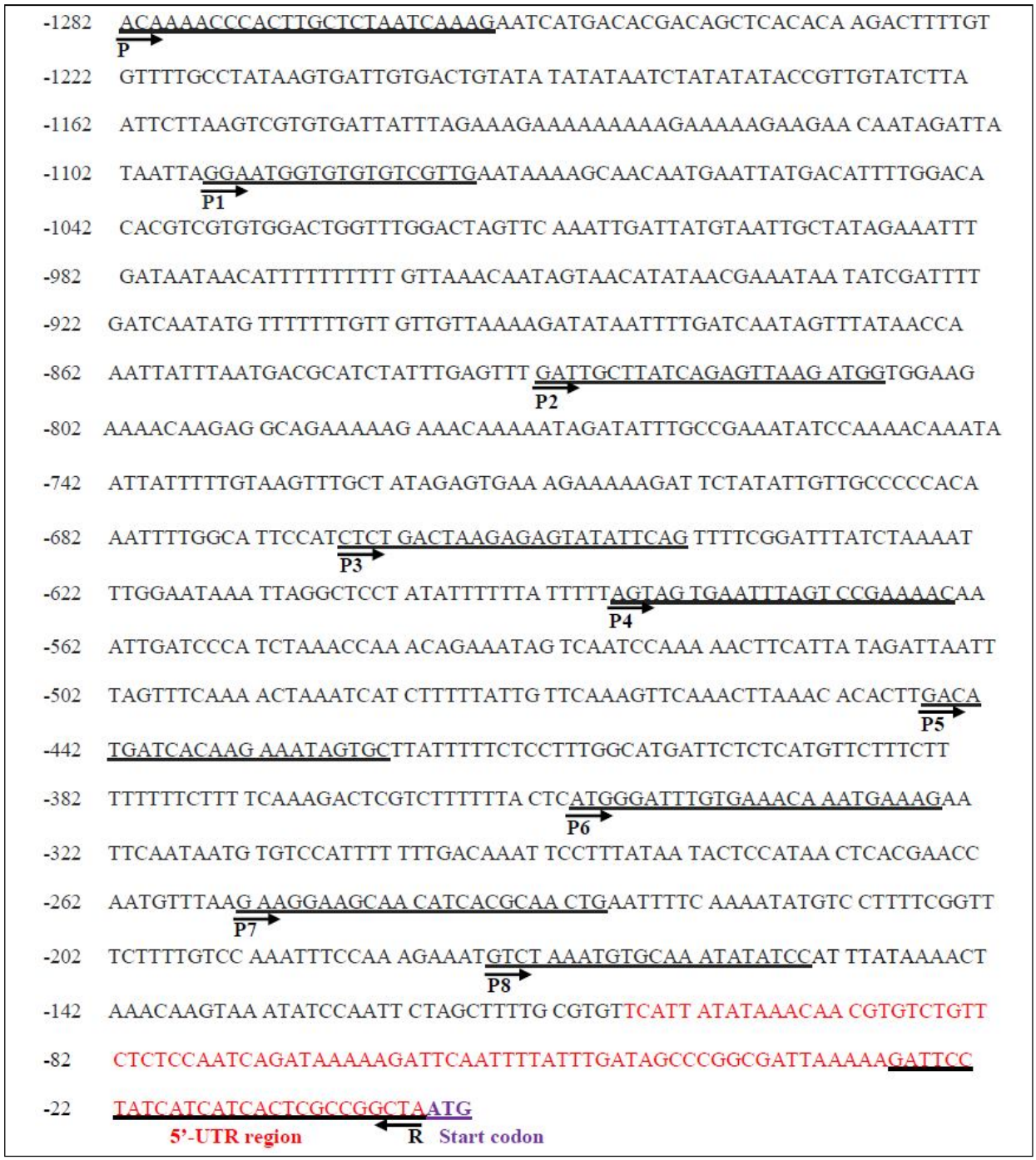

Fig. S1 : Promoter sequence of *AtγVPE*. Sequence showing 1282 upstream region of *AtγVPE* transcription start codon as obtained from TAIR database. Underlined sequences indicated with chimeric plasmid name from P-P8 used as forward primer sequences. R denoted as reverse primer sequence.

shown that in tomato suspension cultured cells Cadmium induce cell death and enhanced caspase-1-like activity (Sanitadi and Gabbrielli, 1999). The increased GUS activity in response to $CdCl_2$ may link VPE to the heavy metal-induced PCD in plants.

Conclusion

The vacuoles mediated cell death has been studied elaborately in the past two decades, and VPEs are known to be actively participating in various types of external stimuli induced cell death in plants. However, there is vague information about the regulatory mechanism of VPEs at transcriptome level. Several studies have confirmed the localization of VPE in the vacuole, our findings, the upregulation of *AtγVPE* promoter in response phytohormones and stress may give the basic idea of

transcriptional regulation of VPE. The numerous identified putative transcription factors binding sites, especially repressor elements from *in-silico* result further gives insight into the spatial and temporal expression of VPEs.

Conflict of interest : Authors declared that they have no conflict of interest.

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