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Identification of a new AT-rich-element binding factor PsATF1 and its combined effect with PsGBF on the activation of PsCHS1 promoter

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1. ABSTRACT

Chaltone synthase (CHS) is a key speed-limiting enzyme in the phenylpropanoid pathway which plays an important role in plant defense response against pathogens. In the *PsCHS1* promoter, there is an AT-rich element (ATRE) which is required for the maximal elicitor-mediated activation. However, the transcription activator of the ATRE and its regulation mechanism in pea keep unclear. In this paper, a new ATRE-binding factor was isolated from an elicitor-induced pea cDNA expression library and was designated as PsATF1. Electrophoretic mobility shift assay (EMSA) indicated the ATRE-specific binding activity of PsATF1. Beta-galactosidase assays in yeast cells suggested that PsATF1 possessed transcription-activating activity because PsATF1 activated the expression of the reporter gene even without the GAL4 activation domain (AD). The current study also examined the co-activation effects of PsATF1 with another transcription factor PsGBF on ATRE or *PsCHS1* promoter through a transient expression system. The present work reports that PsATF1 acts as a complete transcription activator and first indicates that there are combinated effects of PsATF1 with PsGBF on the activation of *PsCHS1* promoter. These results provide theoretical basis to the plant defense gene expression mechanism regulated by multiple activators.

2. INTRODUCTION

To resist pathogen's attack, plants exhibit many antifugal compounds such as phytoalexins (PA), lignin and phenolic compounds, which are produced by the phenylpropanoid pathway (1). Chalcone synthase (CHS) functions as a key rate-limiting enzyme for an early step in this pathway. In pea, CHS genes form a small multigene family with at least eight members which can be divided into two major groups, the elicitor-inducible (*PsCHS1, 2, 3, 4, 5 and 8*) and non-inducible group (*PsCHS6 and 7*), according to their elicitor-inducibility (2). The treatment of pea epicotyls with the elicitor of a pathogen *Mycosphaerella pinodes* induces the activation of the elicitor-inducible *CHS* genes (3), in which the kinetics is similar to that of samples treated with the reduced glutathione (GSH) (4). Therefore, GSH is considered as an exogenous elicitor. In the *PsCHS1* promoter, there are many elicitor-related *cis*-element such as Box I (-68 to -50), G-box (-86 to -81), Box II (-167 to -156), and AT-rich element (ATRE, -242 to -182) (5-8) (Figure 1A), which play important roles in the elicitor-induced activation of *PsCHS1*. Among them, ATRE is a 61 bp fragment containing two identical repeated sequences of TAAAATACT. It is reported the ATRE is required for the maximal elicitor-mediated activation (5), indicating the importance of the ATRE in plant defense response.

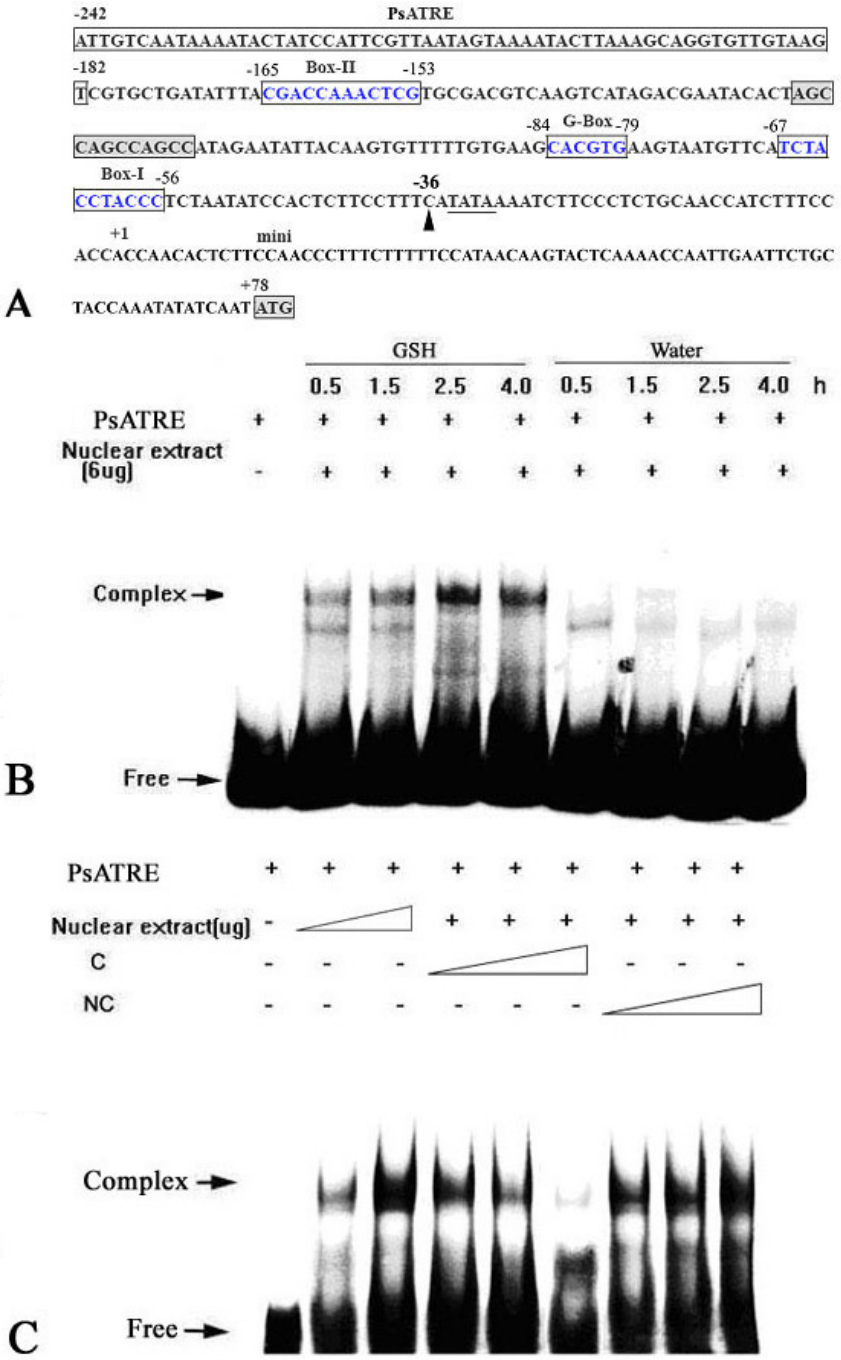

Figure 1. ATRE-binding activity of the pea nuclear proteins induced by GSH. (A). The location of the ATRE in the *PsCHS1* promoter. The ATRE element is an elicitor-induced 61bp sequence and locates in the site of -243~-182 in the *PsCHS1* promoter. (B). The DNA-protein complex was generated only when the G-box *cis*-element mixed with GSH-induced pea nuclear extracts. The pea epicotyls nuclear extracts treated by GSH or water at the indicated time points were mixed with ^{32}P-labeled ATRE sequence (PsATRE). Complex: the DNA-protein complexes; Free: free labeled probe. (C). The probe competitive and non-competitive experiments of ATRE. In the experiments, the competitive or non-competitive probes were unlabeled fragments of ATRE (C) or unlabeled fragments of pBS MCS (NC). Competitive probe competes with labeled ATRE to bind with the nuclear extracts while non-competitive probe does not. Triangles show the increasing amounts of proteins or DNA probes.

However, the transcription factors of the ATRE and complex regulation mechanisms of *PsCHS1* gene have not been elucidated.

In this study, an ATRE-binding factor PsATF1 is isolated through the yeast one-hybrid system. The ATRE-binding and transcription-activating activities of PsATF1 are proved. In addition, the present work characterizes the molecular interaction between the PsATF1 and ATRE or the full length PsCHS1 promoter *in vivo* and first reportes the combinated activation effect of PsATF1 and PsGBF on the *PsCHS1* promoter.

3. MATERIALS AND METHODS

3.1. Material

Pea seeds were planted in vermiculite and grown for one week at room temperature without light. Tobacco plants were grown in a growth chamber under a 16 h light/8 h dark regime at 25℃. PolyATtract mRNA Isolation Systems were purchased from Promega, Madison, USA. The BD Matchmaker™ Library Construction & Screening Kits and -His, -Leu, -Ura, and -His/-Leu/-Ura DO supplements were bought from Clontech, Palo Alto, USA. One-hybrid plasmids pHISi and pLacZi were kind gifts from Professor Fan Chen of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. *Escherichia coli* strain DH5α and expression plasmid pGADT7-Rec were kept in our laboratory.

3.2. Electrophoretic mobility shift assay (EMSA)

Pea epicotyls were cut and soaked in GSH (50 mM) for 0.5 h, 1 h, 1.5 h, or 2 h, and the epicotyls' nuclear extracts were prepared as described by Wang et al. (9). The gel mobility shift assay was performed with 5% native polyacrylamide gel electrophoresis (PAGE) in 1x TBE buffer at 80V for 1 h. Double-strand ATRE was formed as a probe by the annealing of the oligonucleotides PsATRE-S (5'-ATTGTCAATAAAATACTATCCATTCGTTAATAGTAAAATA-3') and PsATRE-A (5'-ACTTACAACACCTGCTTTAAGTATTTTACTATTAACGAAT-3') and labeled with ^{32}P. Unlabeled ATRE and unlabeled DNA fragments of pBluescriptSK(-) (pBS) multiple cloning sites (MCS) were used as DNA competitors or non-competitors, respectively. Before electrophoresis, the reaction mixture, containing 2 μl of 5x binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 50 mM Tris-HCl (pH 7.5)], 1 μl of poly(dI-dC) (1 μg/μl), 1 μl of probe DNA (additional 1μl competitor probe or non-competitor probe), and 5 μl of pea nuclear extracts (6μg) or yeast crude proteins, was incubated at 25℃ for 30 min. After electrophoresis, the gel was dried and autoradiographed.

3.3. Yeast expression plasmids construction and one-hybrid screening

The double-stranded DNA fragment containing ATRE (ATREx1) or mutated ATRE (mATREx1) were constructed respectively by annealing the oligonucleotides PsATRE-S and PsATRE-A, or mPsATRE-S (5'-ATGGaagAgggggggCTATCCATTCGTTAATAggg-3') and mPsATRE-A (5'-AcgccCAACACCTGCTTTAAGccccccccTATTAACGAATGG-3') (the lowercase letters mean the mutant nucleotide residues). Then, they were inserted into pBS three times to form pBS-ATREx3 and the pBS-mATREx3. After that, ATREx3 or mATREx3 were cut from the above plasmids and inserted into yeast one-hybrid vectors pHISi and pLacZi to form the plasmid pHISi-ATREx3, pHISi-mATREx3, pLacZi-ATREx3 and pLacZi-mATREx3. Then, pLacZi-ATREx3 and pHISi-ATREx3 were sequentially transformed into yeast strain YM4271 to obtain YM4271 [pLacZi-Gx3][pHISi-Gx3] according to the Yeast Protocols Handbook of Clontech (Clontech, Palo Alto, USA.).

Pea epicotyls were cut and soaked in 50 mM GSH for 0.5 h, 1 h, 1.5 h, and 2 h, and then total RNA was extracted using the guanidinium isothiocyanate method (10). The mRNA was purified from the total RNA using PolyATtract mRNA Isolation Systems (Promega, Madison, USA). Then, cDNA library construction and the yeast one-hybrid screening were carried out according to the user manual of BD Matchmaker™ Library Construction & Screening Kits (Clontech, Palo Alto, USA.), in which the *Sma* I linearized pGADT7-Rec and the double-strand DNA (dscDNA) library were co-transformed into YM4271 [pLacZi-ATREx3][pHISi-ATREx3] and the transformants were spread on a SD/-His/-Leu/-Ura medium with 1.5 mM 3-AT.

For the transcription-activating assay of PsATF1, the GAL4 AD domain in pGADT7-Rec was deleted or replaced by *PsATF1* cDNA, thereby producing the yeast expression vector pGAL or pGAL-PsATF1. Subsequently, pGAL and pGAL-PsATF1 were transformed into YM4271 [LacZi-ATREx3] or YM4271 [LacZi-mATREx3] and the activation of the *lacZ* reporter were quantified by the beta-galactosidase assays according to Clontech's Yeast Protocols Handbook.

3.4. Construction of plant expression vectors and the transcription-activation assay in tobacco

The mini promoter (-44~+78) and full length promoter (-242~-+78) of *PsCHS1* were amplified respectively with the primers miniCHSp-S (5'-GGGCTTCCTTTCATATAAAATCTTCC-3') and miniCHSp-A (5'-TAGTGGATCCATATTGATATATTTGGTAGCAGAATTCA-3'), or Full-S (5'-AAGCAGGTGTTGTAAGTCG-3') and Full-A (5'-TAGTGGATCCATATTGATATATTTGGTAGC-3'). Then, the cDNA fragments replaced the 35S promoter in the plant expression vector pCAMBIA1301 and produced the reporter vectors pR-mini and pR-Full. To form the ATRE reporter plasmids, ATREx1 or ATREx3 were cut from pBS-ATREx1 or pBS-ATREx3 and inserted into the upstream of the mini promoter in pCAMBIA1301, which resulted in the reporter plasmids pR-ATREx1 or pR-ATREx3. For the construction of the effector plasmid pE-PsATF1 and pE-PsGBF, the full length cDNA of *PsATF1* and *PsGBF* were respectively inserted into the downstream part of the 35S promoter of pCAMBIA1301.

The reporter plasmids pR-n (pR-mini, pR-Full, pR-ATREx1, and pR-ATREx3) and the effector plasmid pE-PsATF1 or pE-GBF were transformed into tobacco leaves in the indicated combinations, and then the transient expression assays were carried out according to Yang et al. (11). Thirty-six hours after transformation, parts of the leaves were collected individually and applied in the fluorometric GUS assays as described by Jefferson et al (12). The difference in fluorescence intensity in the tobacco leaves was evaluated as 4-MU pmol/mg protein per minute by quantified scanning.

3.5. Northern blot hybridization

Different time points of the total RNAs from GSH- or water-treated pea epicotyl were isolated. Then, 10 μg of total RNA of each time was separated on a 1.2% denaturing agarose gel. The cDNA fragments of *PsATF1* or *PsCHS1* were used as probes, and Northern blot hybridizations were carried out according to the Bio-Rad Zeta-GT membrane manual (Bio-Rad, Hercules, Calif.).

4. RESULTS

4.1. Sequence-specific DNA-protein interaction between GSH-induced nuclear extracts and the AT-rich *cis*-element of the *PsCHS1* promoter

To test whether ATRE is GSH-induction-related *cis*-element in *PsCHS1* promoter (see Figure 1A for the location of the ATRE in *PsCHS1* promoter), the interaction between the ATRE and GSH-induced pea nuclear extracts were analyzed through EMSA, in which the end-labeled ATRE fragment was incubated with GSH-induced pea nuclear extract and subjected to a nondenaturing PAGE. The major retarded band was observed only in GSH-induced proteins, in which the peak value occurred at 2.5 hour after GSH induction (Figure 1B). The amount of this complex was increased following the increasing amount of the nuclear extracts and it disappeared by competing with an excess of unlabeled ATRE (Figure 1C). This result indicates that there are one or more nuclear factors interacting specifically with the ATRE element and participating in the activation of the *PsCHS1* promoter.

4.2. Isolation and characterization of the *PsATF1* gene

To isolate the ATRE-binding factors from pea nuclear extracts, the yeast one-hybrid system was carried out. A GSH-induced pea cDNA library was screened and several positive clones were obtained from 8.0 x 10^6 colonies. Then, a new gene was isolated from a positive clone and was designated *PsATF1* (*Pisum sativum* AT-rich element binding Factor 1, GenBank accession: AY822466). According to the deduced amino acid sequences, PsATF1 belongs to bZIP-family factor because there is a bZIP domain (the basic and leucine zipper region) in position 168-232. Figure 2A shows the blast results of PsATF1 and some Arabidopsis bZIP proteins, indicating that the bZIP domain of the above proteins are highly conserved (Figure 2A). To further explore the relationships between these bZIP-type proteins, evolutionary distances among the protein sequences were calculated and a phylogenetic tree was constructed. As shown in Figure 2B, PsATF1 is most closely related to AtbZIP51 in group I and is more distantly related to AtbZIP60 in group X.

4.3. DNA-binding and transcription-activating activities of PsATF1

To characterize the DNA-binding activities of PsATF1, in vitro EMSA were carried out. Figure 3A shows the ATRE-binding activities of the total protein extracted from the yeast, which were transformed with pGADT7-Rec-PsATF1, and that the binding abilities were enhanced with the amount of the protein increasing The results reflect the specific ATRE-binding activity of PsATF1.

Furthermore, the transcription-activating activity of PsATF1 was analyzed through the X-gal assays in yeast cells. As shown in Figure 3B, not only PsATF1 with the GAL4 AD domain but also PsATF1 alone could activate the reporter genes in strain YM4271 [pLacZi-ATREx3]. However, no matter AD domain exists or not, fusion proteins of PsATF1 never activated the mutated ATRE (mATRE) (Figure 3B). These results suggest that PsATF1 possesses both ATRE-specific-binding and transcription-activating activities in yeast cells. Therefore, PsATF1 might be an integrated plant transcription factor.

4.4. Interactions between PsATF1 and ATRE or the full length promoter *in vivo*

To prove that PsATF1 acts as a trans-acting factor, *in vivo* transcription-activating activity of PsATF1 was analyzed in transiently transformed tobacco leaves through agro-infiltration. Figure 4A showed the diagrams of reporter and effector plasmids. Figure 4B presented that the GUS expression level downstream the single copy of the ATRE (ATRE x1) (sample 4) was activated by PsATF1 to 12-fold higher than that downstream of the *PsCHS1* minimal promoter (mini) (sample 2). Then, with the increase of the number of the *cis*-element (ATRE x3) (sample 6), the GUS expression level could also be improved to 26-fold that of mini. Furthermore, the interaction between PsATF1 and the full length *PsCHS1* promoter was also analyzed. As shown in Figure 4B, PsATF1 interacted with the full length *PsCHS1* promoter and activated the GUS expression level to 17-fold (sample 8) that of mini. The results suggest that PsATF1 recognizes the ATRE in *PsCHS1* promoter and activates the expression of the downstream reporter gene *in vivo*. PsGBF (*Pisum sativum* G-box-binding factor, GenBank accession: DQ399528), which we isolated using the G-box element as a bait, is a transcription factor of *PsCHS1* gene (data unpublished). Here, we also analyzed the co-activation effect of PsATF1 and PsGBF on the *PsCHS1* promoter. Figure 4B showed that PsATF1 alone activated the expression level of GUS gene to 17-fold (sample 8) that of the minin (sample 2) while PsGBF alone activated that to 18-fold (sample 9). However, when PsATF1 was co-transformed into tobacco with PsGBF, the GUS expression level was improved to 30-fold (sample 10) that of the mini; which was higher than that of PsATF1 alone (sample 8) or that of PsGBF alone (sample 9). The result reflects that there is co-activation effect of PsATF1 and PsGBF on *PsCHS1* transcriptional regulation when pea is induced by elicitor.

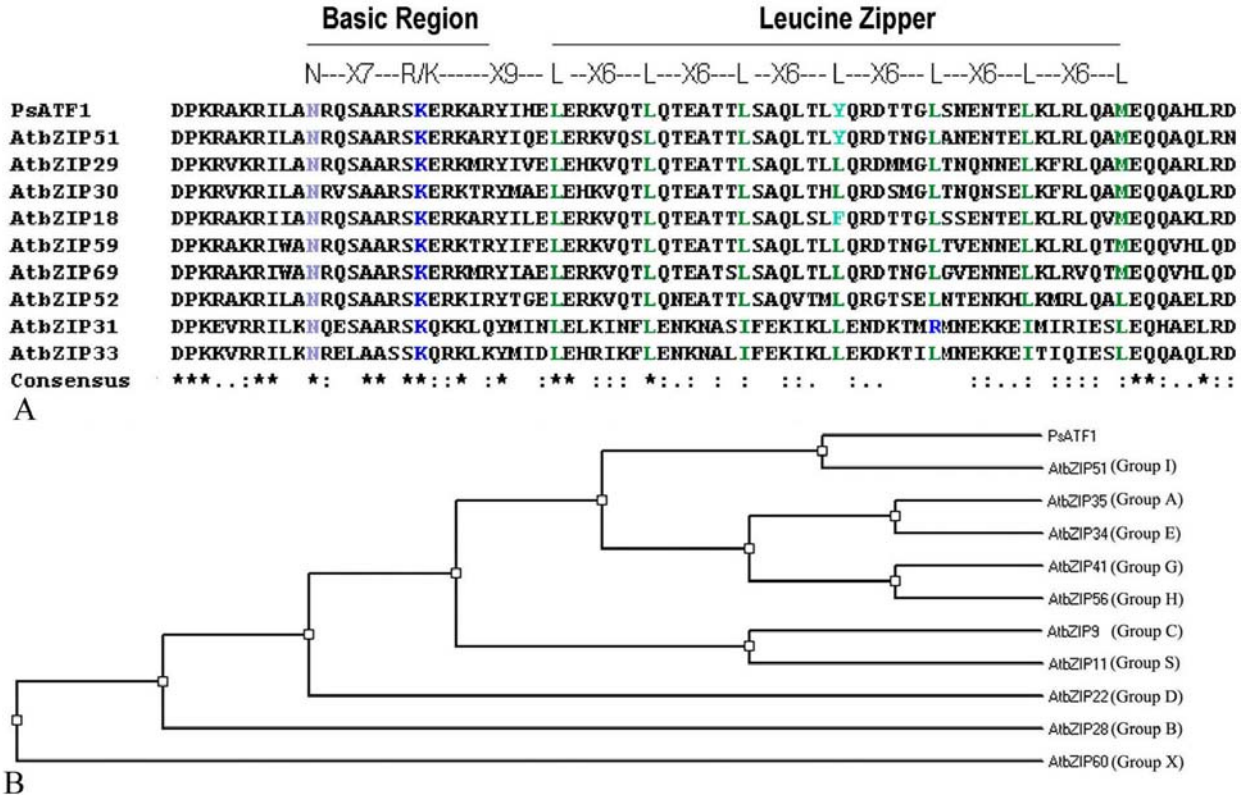

Figure 2. The comparison of the bZIP domain in PsATF1 with Arabidopsis bZIP proteins. (A). The comparison of conserved regions between PsATF1 and the other 9 Arabidopsis bZIP proteins. The conserved bZIP region has the common pattern of N-X7-R/K-X9-L-X6-L-X6-L-X6-L-X6-L-X6-L-X6-L-X6-L. Asterisks indicate positions which have single, fully conserved residues. (B). Phylogenetic tree of PsATF1 with other Arabidopsis bZIP proteins. There are 75 bZIP proteins in Arabidopsis, which are divided into 10 groups (Group A-I and Group S) by Jakoby, M. (20). Group X means the remaining several bZIP proteins not belonging to the above ten groups.

4.5. Transcription characteristics of *PsATF1* and *PsCHS1* induced by GSH

To determine whether PsATF1 is a regulator of *PsCHS1* in the phenylpropanoid pathway, the *PsATF1* and *PsCHS1* transcripts were characterized. Figure 5 shows that the maximum accumulation of *PsATF1* transcripts are observed 0.5 h after treatment with GSH, the peak emerging time is earlier than that of the *PsCHS1*, which is 4 h after being induced by GSH (Figure 5). The chronologicaol order of the two transcripts indicates that PsATF1 might be the regulator of PsCHS1.

5. DISCUSSION

The ATRE exists in many plant gene promoters and is bound by their binding proteins to regulate the expression of downstream genes. For example, soybean nodule nuclear factor NAT2 binds the ATRE in leghenmoglobin c3 and nodulin N23 gene (13, 14); French bean PNF-1 binds to the ATRE of several nodulin genes (15); and AT-1 of pea bind the ATRE in the promoters of light regulated genes RbcS or Cab (16). Among them, *CHS* and *PAL* (phenylalanine ammonia-lyase) in pea are the important genes related to fungus elicitor inductions. The ATRE in the two genes' promoters were detected to form complexes with elicitor-induced pea nuclear extracts, and was considered to be the elicitor responsive *cis*-elements (1, 5). However, the transcription factors of the ATRE in the two genes keep undiscovered.

Here we obtained a new ATRE-binding protein named as PsATF1. Based on amino acid comparisons, PsATF1 appears to belong to the bZIP-type transcription factor family. Plant bZIP proteins preferentially bind to DNA sequences with an ACGT core such as A-box (TACGTA), C-box (GACGTC) and G-box (CACGTG) (17), in which the DNA-protein binding specificity is regulated by flanking nucleotides. But there are other examples of nonpalindromic binding sites (18, 19). In Arabidopsis genome, there are 75 bZIP-type transcription factors, which were divided into 10 groups due to the basic region or other conserved motifs (20). Because members of a given group share a similar DNA-binding basic region, most of them probably recognize similar *cis*-element (20). According to the amino acid comparisons, PsATF1 is most similar with AtbZIP51, which is one member of group I.

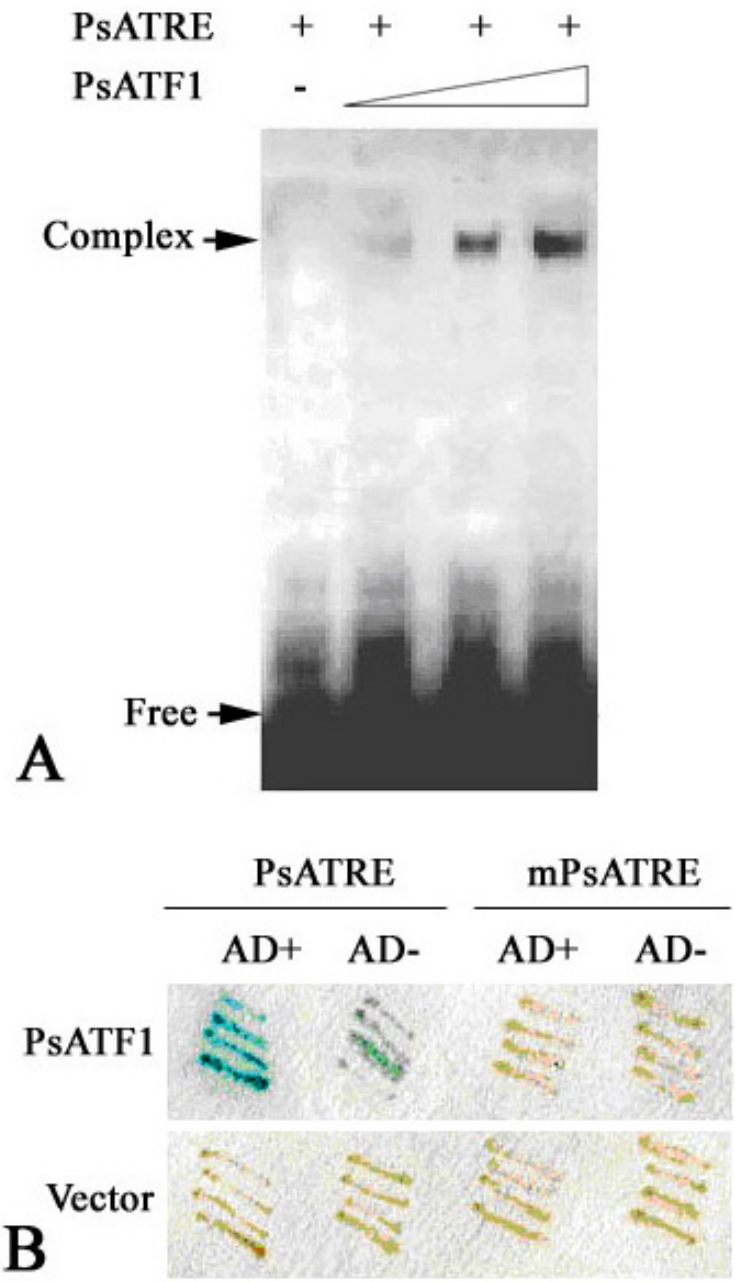

Figure 3. ATRE-specific binding and transcription-activating activities of PsATF1. (A). In EMSA, the expressed PsATF1 in yeast cells showed specific binding activity with ATRE *in vitro*. PsATF1: total proteins from yeast cells with pGADT7-Rec-PsATF1; Triangles show the increasing amounts of the proteins. (B). PsATF1 showed ATRE-specific binding and transcription-activating activities in yeast cells. The beta-galactosidase assay indicates that PsATF1 in yeast activates the reporter gene with or without the yeast GAL4 AD domain, but no matter AD exists or not, PsATF1 could not activate the reporter gene downstream of mATRE. ATRE and mATRE: yeast cells with ATREx3 or mATREx3; AD+ and AD-: yeast cells containing the expression vector with or without GAL4 AD domain; PsATF1: pGADT7-Rec-PsATF1; Vector: pGADT7-Rec.

The lysine residue in PsATF1 basic region replaces the highly conserved arginine (N-x_7-R to N-x_7-K, where x represents an amino acid residue), which is also the main character of Group I proteins distinguished from other group proteins (20). This amino acid might determine the specific binding site of bZIP proteins because this type of basic region presents a higher affinity to non-palindromic binding sites. For example, tobacco RSG binds TCCAGCTTGA or TCCAACTTGGA (19) and tobacco VSF-1 binds to GCTCCGTTG (21). Therefore, we considered PsATF1 might bind non-palindromic element ATRE in pea.

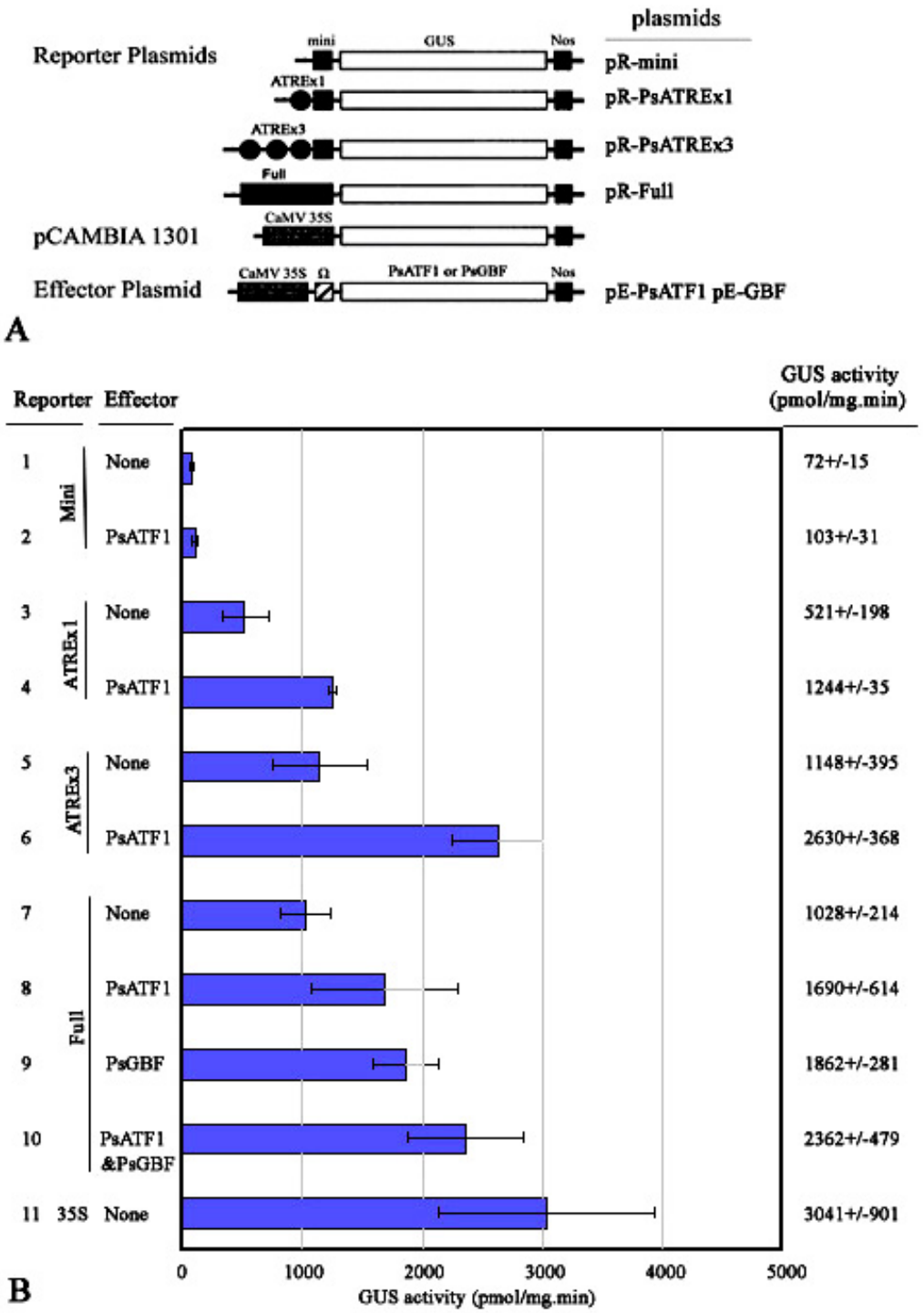

Figure 4. PsATF1/PsGBF interacting with the ATRE or the full length *PsCHS1* promoter in tobacco. (A). Construction of reporter and effector plasmids for transient trans-activation assays in tobacco leaves. In the trans-activation system, reporter and effector plasmids was designed as described in the Materials and Methods section. (B). The transcription regulation of PsATF1 and/or PsGBF to the ATREx1, ATREx3 or the full length PsCHS1 promoter (Full) were detected by the GUS expression assay in transient-transformation tobacco leaves. The data represent mean values of 4 independent transfections. Transfection without the effecter plasmid (none) or with pCambia1301 were used as controls. Mini: pR-mini; ATREx1: pR-ATREx1; ATREx3: pR-ATREx3; Full: pR-Full, None: expression plasmid without any factor gene; PsATF1: pE-PsATF1; PsGBF: pE-PsGBF; 1301: pCambia1301.

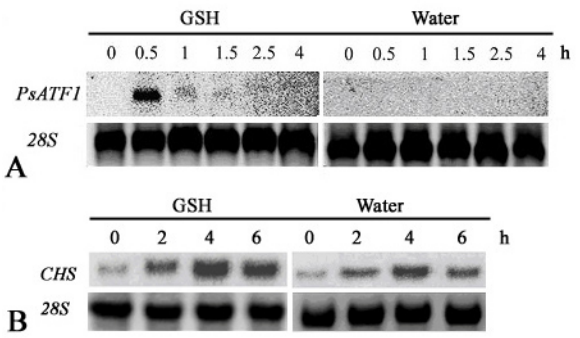

Figure 5. Northern blot hybridization of *PsATF1* and *PsCHS1*. Characterization of *PsATF1* (A) and *PsCHS1* (B) expression induced by GSH in pea epicotyls. Pea epicotyls were treated with 50 mM GSH or water at the times points given in hours (h). Abbreviations: PsCHS1: *Pisum sativum* Chalcone synthase 1; PAL: phenylalanine ammonia-lyase; EMSA: Electrophoretic mobility shift assay; ATRE: AT-rich element; bZIP: Basic/Leucine zipper; PsATF1: *Pisum sativum* AT-rich element binding Factor 1; PsGBF: *Pisum sativum* G-box binding factor; AD: activation domain; MCS: multiple cloning sites; pBS: pBluescriptSK(-) plasmid.

A complete transcription factor can bind and activate the specific *cis*-element. To test whether the PsATF1 functions as a transcription factor of ATRE, EMSA and β-galactosidase assays in yeast were carried out. Our results suggest that PsATF1 has both DNA-binding activity and transcription-activating activity. To analyze the transcriptional regulation mechanism of PsATF1 *in vivo*, the transient expression assays were performed in tobacco leaves, which indicate that PsATF1 activates the GUS gene downstream of ATRE *in vivo* and that the activation level is enhanced with the amounts of ATRE increasing. The results reflect that PsATF1 activates *GUS* expression through binding with the ATRE.

In plant, many bZIP proteins are linked to plant defense responses, such as the typical class of TGA/OBF family, which bind to as-1 *cis*-element and regulate the expression of some defense-responsive genes such as the PR-1 (22). However, although some bZIP transcription factors bind to the specific cis-elements *in vitro*, they do not regulate the downstream gene expression *in vivo* (23). To determine whether PsATF1 regulates the expression of *PsCHS1 in vivo*, we performed co-transformation of PsATF1 and *PsCHS1* full-length promoter in tobacco leaves. GUS gene expression levels indicated that PsATF1 interacted with the full length promoter *in vivo* and improved the expression level of downstream reporter gene. Moreover, the accumulation time of the *PsATF1* transcript preceded that of *PsCHS1*, which suggests that PsATF1 mediates GSH-induced activation of the *PsCHS1* gene. These results indicate that the bZIP protein PsATF1 is the transcription factor of *PsCHS1* gene and functions as an activator in the elicitor-induced defense responses.

In the control of gene expression, the *basal transcription factors*, which position RNA polymerase at the start of the protein-coding region of a gene, are essential for transcription but can not increase transcription rate by themselves. The task of increasing transcription rate falls to the *activators*, which determine some gene will be switched on and speed the transcription rate. *Activators* communicate with the *basal transcription factors* through *coactivators* (24). In recent years, more and more *activators* and *coactivators* were obtained to speed genes' transcription rate (25, 26). Here we analysis the combination effects of two *activators* PsATF1 and PsGBF on the activation of *PsCHS1* promoter. The results show that when the two *activators* co-act with the promoter, the expression level of the reporter gene is higher than that activated by the single factor, indicating that PsATF1 and PsGBF interact with the *basal transcription factors* through different *coactivators* and co-activated the expression of *PsCHS1* gene after elicitor induction. It also demonstrates that when plant is infected by fungus, kinds of activators gather around the *PsCHS1* promoter and co-activate the gene expression. As a result, *PsCHS1* transcripts accumulate to a much higher level in a short time.

In conclusion, the present works isolate a new ATRE-binding factor PsATF1 which belongs to the bZIP transcription factors family and is induced by GSH. PsATF1 possesses DNA specific-binding and transcription-activating characteristics when acting with the ATRE in the *PsCHS1* promoter. Furthermore, our works first indicate that PsATF1 is an activator of the *PsCHS1* gene and might co-activates the gene expression with PsGBF through different coactivators. Based on these findings, PsATF1 regulates the phenylpropanoid biosynthesis pathway and plays an important role in multiple regulations of plant defense genes' expression.

6. ACKNOWLEDGMENTS

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