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Cloning and functional analysis of the chitinase gene promoter in peanut

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ABSTRACT. Chitinase is an important pathogenesis-related protein in plants, and it can accumulate when induced by salicylic acid (SA) or other elicitors. Here, we found that chitinase mRNA levels were 4.5-times greater when peanut seedlings were sprayed with 1.5 mM SA, as compared to water. The upstream promoter sequence of the chitinase gene was cloned by TAIL-PCR and the potential cis-regulatory elements in this promoter were predicted by the cis-element databases PLACE and plantCARE. Elements in the promoter related to SA induction and disease resistance response included AS-1, GT1-motif, GRWAAW, TGTCA, W-box, and WB-box. The full-length promoter (P) and a series of 5'-deleted promoters (P_1-P_5) were cloned and then substituted for the 35S promoter of *pCAMBIA1301-xylA*, which carries the xylose isomerase gene as the selectable marker and GUS as the reporter gene. Six plant expression vectors (*pCAMBIA1301-xylA-P-pCAMBIA1301-xylA-*P_5) were obtained. The six expression vectors were then transferred into onion epidermal cells and peanut plants by *Agrobacterium*-mediated transformation. Both the full-length and deleted promoters resulted in GUS staining of the onion epidermis cells when

induced by SA. In onion epidermis cells, GUS enzyme activity was greater after SA induction. In transgenic peanut plants, GUS mRNA levels were greater after SA induction. Consideration of the cis-regulatory elements predicted by PLACE and plantCARE suggested that AS-1, GRWAAW, and W-box are positive regulatory elements in P_2 and P_3 and that GT1-motif and TGTCA are negative regulatory elements between P and P_2.

Key words: Peanut (*Arachis hypogaea* L.); Chitinase gene; SA induction; Inducible promoter; Deletion analysis

INTRODUCTION

Peanut plants are often attacked by pathogens that cause diseases such as mesh spot, leaf spot, black rot, rust, and bacterial wilt. These diseases reduce seed quality and yield; unfortunately, peanut germplasm resources with high resistance to disease are rare (Wang and Zhang, 2013). One solution to this lack of resistance in peanut germplasm is to transfer exogenous resistance genes into peanuts. Such transgenic peanut plants could defend themselves against pathogens through production of a variety of hydrolytic enzymes and pathogenesis-related proteins (PR proteins) (Vellicce et al., 2006; Jia et al., 2011). Chitinase is one of the proteins of interest in this context, as it is a PR protein that has a role in plant defense responses against fungal pathogens (Kellmann et al., 1996; Liu et al., 2007). Production of chitinase can be induced in plants by inoculation with pathogenic fungi and by exogenous application of salicylic acid (SA) (Kellmann et al., 1996; Qu et al., 2008).

The successful creation of transgenic plants with the required level and pattern of expression of the transferred exogenous gene is largely determined by the gene promoter sequences, which are important transcription regulatory elements. Constitutive expression promoters such as cauliflower mosaic virus 35S promoter (*CaMV35S*) and rice actin gene promoter (*Actin1*) have been widely used to drive the expression of transferred exogenous genes in all tissues and at all developmental stages of transgenic plants. The sustained expression of foreign genes, however, can suppress plant growth and development and can cause the accumulation of toxic substances in the plants (Moreno et al., 2005; Zhu et al., 2010). In contrast, an inducible promoter enables transferred exogenous genes to be expressed in specific tissues or at specific stages of development; thus, expression of the exogenous gene would be less likely to interfere with plant growth and development but would allow host cells to respond to specific environmental signals (Castresana et al., 1990; Chang et al., 2009). On this basis, an effective strategy for engineering disease resistance into plants is to identify and transfer appropriate inducible promoters into the plants and to confirm that the promoter supports the desired pattern of expression of the exogenous gene (Zuo and Chua, 2000).

Pathogen-induced promoters have been identified in the upstream regulatory sequences of some defense response genes. For example, the promoter for the beta-1,3-glucohexaose glycosidase gene in wild tobacco is induced by the bacterial pathogens *Pseudomonas syringae* and *Erwinia carotovora* (Castresana et al., 1990). In *Arabidopsis,* the promoter for the acid chitinase gene is induced by the fungal pathogen *Rhizoctonia solani*; the promoter is also induced by the fungal pathogen *Alternaria solani* in transgenic tomato (Samac and Shah, 1991). Similarly, the promoter for the osmotic regulation protein osmotin in potato can be induced by a chemical inducer and by the fungal pathogen *Phytophthora infestans* (Gong and Li, 2000; Pontier et al., 2001).

In plants, defense responses can be induced by pathogen elicitors, i.e., recognition of the pathogen elicitor by the plant may cause the plant to produce proteins that resist infection. Elicitors are also experimentally useful because they can be used in place of pathogens to study promoter induction (Li et al., 2007). Promoters differ in many ways including regulatory element sequence, induction drive activity, and inductive response to the external environment. Therefore, increasing the expression of a defense-related gene depends on identifying and understanding the characteristics of its specific promoter.

The chitinase gene has been isolated and cloned from peanut (GenBank accession No. HQ439775). In this study, we sought to isolate the upstream promoter sequences of the chitinase gene, to predict the function of this promoter, and to confirm the key cis-regulatory elements. This information will provide the basis for induced high-level expression of the endogenous peanut chitinase gene by an elicitor.

MATERIAL AND METHODS

Plants and growing conditions

The peanut (*Arachis hypogaea* L.) variety *Xiyangsheng* and onion bulbs (*Allium cepa* L.) were used in this study. Peanut seeds were sown in autoclaved soil in 10 cm diameter clay pots (one plant per pot) and were kept in a greenhouse in an incubator at 25°C and 60% relative humidity, under 13 h light /11 h dark conditions. The same conditions were used for all experiments. Onions bulbs were obtained from the Plant Physiology Laboratory of Qingdao Agricultural University.

Treatment of peanut seedlings with SA to induce chitinase expression

Twenty-day-old peanut seedlings were sprayed with a solution of 1.5 mM SA and 0.1% Tween-20, pH 7.0, or with 0.1% Tween-20 alone (control). The SA or control solution was sprayed on both sides of each leaf until the entire leaf surface was wet. After 0, 12, 24, 36, 48, or 72 h, 3 - 4 leaves were collected, quickly frozen in liquid nitrogen, and stored at -80°C until required.

Extraction of total RNA and assessment of chitinase gene expression

Total RNA was isolated from the frozen peanut leaves and then reverse-transcribed into cDNA. Reverse transcription was performed using an ABI 7500 FAST Real-Time PCR System. The primers *Actin-F* and *Actin-R* were used to amplify the peanut reference gene *Ah-Actin*, and the primers *Ah-Chi-F* and *Ah-Chi-R* were used to amplify the peanut chitinase gene (*Ah-Chi*). The sequences of all primers used in this study are listed in Table 1. The amplification protocol was as follows: 95°C for 2 min; followed by 40 cycles of 95°C for 10 s and 60°C for 40 s; and then a slow increase to 95°C. A dissociation curve was then prepared. Each reaction was repeated. The relative level of expression of the target gene was calculated according to the $2^{-(\Delta\Delta Ct)}$ method of Livak and Schmittgen (2002).

Cloning the full-length promoter of the peanut chitinase gene

Three nested specific primers (*SP1*, *SP2*, and *SP3*) were designed using the 5'-end sequence of peanut chitinase cDNA (GenBank accession No. HQ439775). The upstream promoter

sequence, termed here *Ah-Chi-Pro*, was amplified by TAIL-PCR using *Xiyangsheng* genomic DNA as template. Two other primers, *Chi-F* and *Chi-R*, were designed using the sequence of the promoter *Ah-Chi-Pro*; their PCR amplification product is termed here *Ah-Chi-P*.

Table 1. Names and sequences of primers used in this study.

Name	Sequence(from 5' to 3')
Actin-F	GTGGCCGTACAACTGGTATYGT
Actin-R	ATGGATGGCTGGAAGAGAACT
Ah-Chi-F	GAGACAACAGGGAGGAACGA
Ah-Chi-R	ATCTGCCTTTATAGCTTGTCCA
SP1	ACCTGTTGTCTCGTGTGATGTTTGG
SP2	TGACGTCATCACCGGTTGTTCC
SP3	GCTTCAGGAATTCATTATAGAGAGGC
Chi-F	GGATCCAGCATTTCCCTACTTTACGTATTC
Chi-R	CCATGGCTGTTTTGAGTTTGGGATGTAC
Chi-P_1	GGATCCATGATAGTTCTGTCTTAG
Chi-P_2	GGATCCGAGTCACCATTTTGTTAGAACGCTT
Chi-P_3	GGATCCTGAGAGCAAAACTTATTCTTCTT
Chi-P_4	GGATCCGTAGACTCAGAAATTCGTTGGGGAT
Chi-P_5	GGATCCTGCTGACATAATAATAGACATTG

GGATCC is the *BamH*I restriction enzyme cutting site, and CCATGG is the *Nco*I restriction enzyme cutting site.

Function prediction of the peanut chitinase gene promoter

The relevant cis-elements within *Ah-Chi-P* were predicted using the plant cis-acting regulatory element databases PLACE (http://www.dna.affrc.go.jp/PLACE/) (Higo et al. 1999) and plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2001). **(Links ok)**

Cloning a series of 5'-deleted sequences of the peanut chitinase gene promoter

According to the predicted cis-acting elements within *Ah-Chi-P*, five forward primers, *Chi-P_1* to *Chi-P_5*, and one reverse primer, *Chi-R*, were designed; each primer includes a *BamH*I restriction site (Table 1). The primers were used to amplify 5'-deleted sequences of *Ah-Chi-P*. The PCR amplification products were sequenced. The five promoters with 5'-deleted sequences were named P_1 to P_5.

Construction of plant expression vectors

35S promoter of *pCAMBIA1301-xylA* had been removed by digestion with both *BamH*I and *Nco*I, and further substituted by the full-length promoter (*Ah-Chi-P*) and the five deleted promoters (P_1 to P_5) respectively. The derived combination plasmids were named *pCAMBIA1301-xylA-Chi-P* to *pCAMBIA1301-xylA-Chi-P_5* and were transferred into *Agrobacterium* EHA105.

GUS transient expression in transformed onion epidermal cells

Fresh onion bulbs were cut into 0.25-cm^2 pieces and pre-cultured in MSB_5 medium in the dark for 24 h; the onion pieces were then immersed in a solution containing *Agrobacterium* EHA105 and a plasmid (*pCAMBIA1301-xylA-Chi-P* to *pCAMBIA1301-xylA-Chi-P_5*) for 15 min, then cultured in the dark for 3 days. Transformed onion epidermal cells and control cells were treated with 5.0 mM SA or distilled water for 84 h and then stained for GUS activity by incubating overnight

in X-gluc, 0.1 mM phosphate buffer, pH 7.0, 1 mM EDTA, 5 mM potassium ferrocyanide, Triton X-100, and N,N-dimethylformamide at 37°C, followed by decolorizing in 70% ethanol. GUS enzyme activity in transformed onion epidermal cells was detected as previously described (Ma et al. 2010).

Analysis of GUS expression in transgenic peanuts

Plasmids *pCAMBIA1301-xylA-Chi-P* to *pCAMBIA1301-xylA-Chi-P*$_5$, were separately transformed into cotyledon explants of the peanut variety Huayu-23. Transformed plants were selected on SEM culture medium containing 20 g/L xylose and 10 g/L sucrose as described previously (Ding et al., 2012).

PCR amplification was performed using the primers *GUS-F* and *GUS-R* and the genomic DNA of the transformed peanut plants as template. The amplification protocol was as follows: 94°C for 5 min; 32 cycles of 94°C for 50 s, 56°C for 50 s, and 72°C for 40 s; and a final extension at 72°C for 10 min. The amplification products were separated by 1% agarose gel electrophoresis.

Transformed plants and control plants were treated with 5.0 mM SA for 72 h. The primers *Ah-Actin-F* and *Ah-Actin-R* were used to amplify the *Actin* reference gene, and the primers *GUS-F* and *GUS-R* were used to amplify the GUS gene. Real-time fluorescent quantitative PCR was performed as described above.

RESULTS

Expression level of peanut chitinase gene after SA induction

After treatment with SA, the level of *Ah-Chi* mRNA in peanut leaves increased and, at 72 h after treatment, was more than 4 times greater than in water treated controls, getting a very significant difference ($P < 0.01$) (Figure 1). Thus, the promoter of the peanut chitinase gene is SA-inducible.

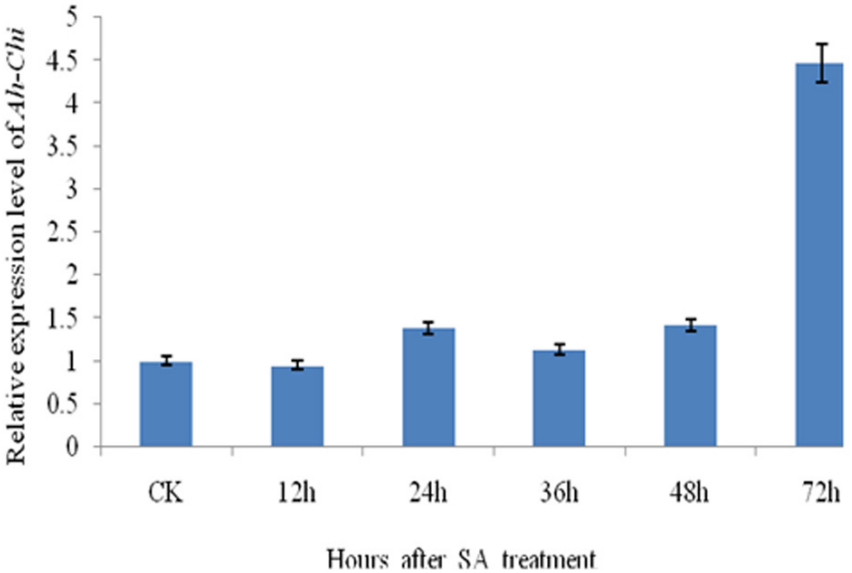

Figure 1. mRNA expression level of Ah-Chi in peanut after treatment with 1.5 mM SA. Data are reported as means + SEM.

Cloning of the promoter of the peanut chitinase gene

TAIL-PCR amplification was conducted using a random combination of the three nested specific primers, *SP1* to *SP3*, with one of the four random primers, *AP1* to *AP4*. A single 1710 bp specific band was obtained when *SP1* to *SP3* were paired with *AP3* in the third round of amplification; no specific bands were obtained if *SP1* to *SP3* were paired with *AP1*, *AP2*, or *AP4* (Figure 2a). As the sequence of the 1710 bp band matched that of the 5'-end of the peanut chitinase gene, the amplification product was considered to be the upstream promoter sequence for the peanut chitinase gene and was named *Ah-Chi-Pro*. A 1650 bp band was generated using primers *Chi-F* and *Chi-R*, and named *Ah-Chi-P*. This amplification product was inserted into the *pMD18-T* vector to obtain the recombinant plasmid *pMD18-T-Ah-Chi-P*; construction of the plasmid was verified by PCR and double-digestion with *BamH*I and *Nco*I, which produced the 1650 bp construct (Figure 2b,c).

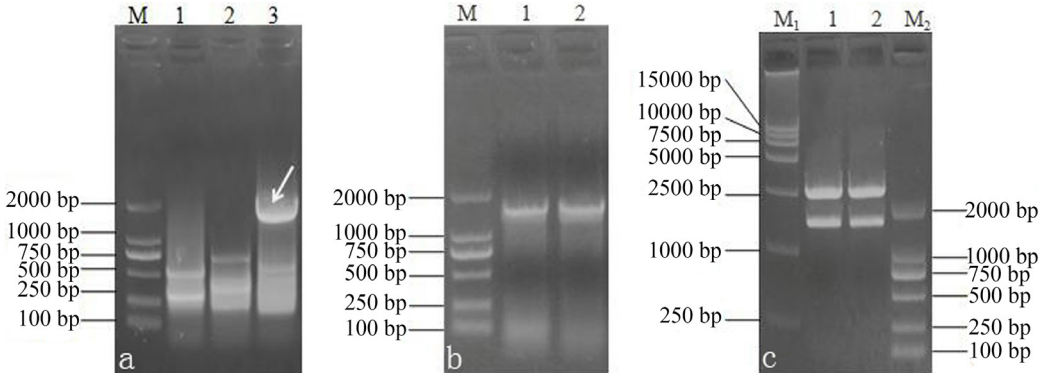

Figure 2. TAIL-PCR amplification of Ah-Chi-P and confirmation of recombinant plasmids pMD18-T-Ah-Chi-P. **a.** TAIL-PCR amplification by primer-pair SP1/AP3, SP2/AP3, and SP3/AP3 in three steps. M: DL2000; 1: product amplified by SP1 and AP3 in first step; 2: product amplified by SP2 and AP3 in second step; 3: product amplified by SP3 and AP3 in third step. **b.** Confirmation of recombinant plasmids by PCR. M: DL2000; 1, 2: product amplified by primers Chi-F and Chi-R. **c.** Confirmation of recombinant plasmids by BamHI and NcoI digestion. M1: DL15000; 1, 2: Products digested by BamHI and NcoI; M2: DL2000.

Analysis of the predicted function of the peanut chitinase gene promoter

Online prediction and analysis using PLACE and PlantCARE databases showed that the *Ah-Chi-P* promoter region contained approximately 90 regulatory elements distributed across 401 sites. These elements included typical regulatory elements such as a CAAT-box and a TATA-box. Also predicted were regulatory elements related to pathogen-induced reactions: the GT1-motif (GAAAAA), TGTCA, TTGCA, and TGAC, which play an important role in pathogen-induced gene expression (Park et al., 2004); AS-1 (TGACG), which is mainly involved in SA-induced gene expression (Redman et al., 2002); and W-box, WB-box (TTTGACY), and EIRE (TTCGACC), which respond to elicitors (Laloi et al., 2004). Other cis-acting elements found in *Ah-Chi-P* included WAACCA and YAACKG, which are related to drought-resistance, and TACGTGTC, ACGTGKC, and TACGTGTC, which are related to ABA-induced reactions (Lu et al., 2009; Graeber et al., 2010). The major elements responsible for pathogen and SA responsiveness were present in different sites of the promoter (Table 2 and Figure 3).

Table 2. Primary cis-acting elements which might be relevant to disease resistance and SA induction.

sequence range concerned elements	P-P1 1641-1513	P1-P2 1512-1384	P2-P3 1383-995	P3-P4 994-618	P4-P5 617-236	P5 235-31
TGACG (AS-1)	0	0	1	0	0	0
TGTCA	1	2	0	1	1	1
GRWAAW	2	2	3	6	2	4
GAAAAA	0	0	1	2	1	2
TTTGACY (WB BOX)	0	0	1	0	0	0
TTGAC (W BOX)	0	1	1	1	2	1
TGACT (W BOX)	0	0	1	0	2	1
TGACY (W BOX)	0	0	1	0	2	2
TGAC (WRKY710S)	1	2	2	1	3	3
Total	4	7	11	11	13	4

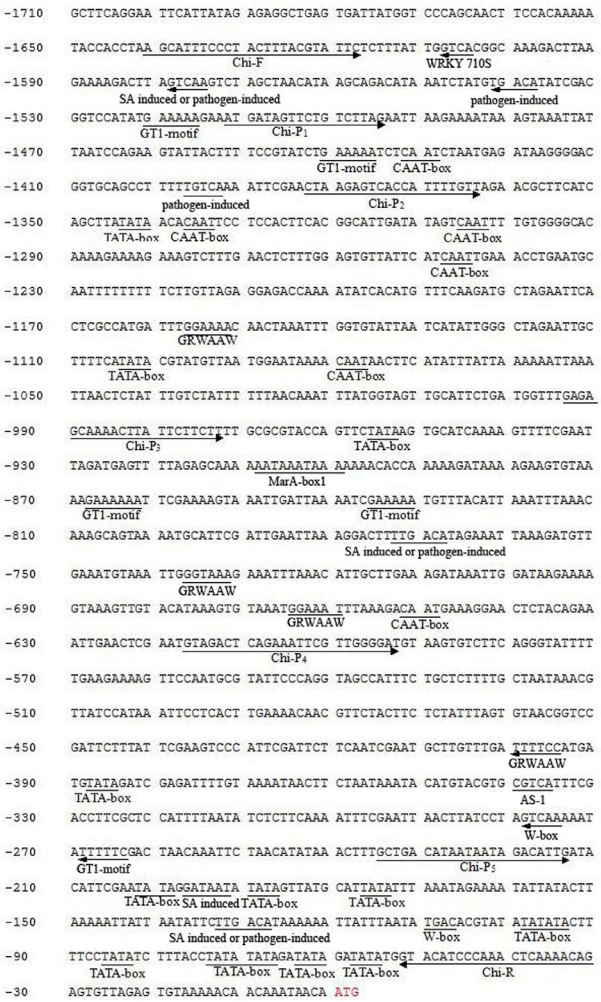

Figure 3. Function prediction for Ah-Chi-Pro sequences by databases PLACE and plantCARE. Long arrows indicate the orientation of the primer sequences. Short left-pointing arrows show putative cis-acting elements in the complementary chain. Underlined sequences indicate cis-elements in the positive chain.

Cloning of a series of 5'-deleted promoter sequences and expression vector construction

Plasmids *pCAMBIA1301-xylA-Chi-P* to *pCAMBIA1301-xylA-Chi-P*$_5$ were confirmed by PCR amplification and double-digestion with *BamH*I and *Nco*I, and corresponding target bands were obtained (Figure 4a, b).

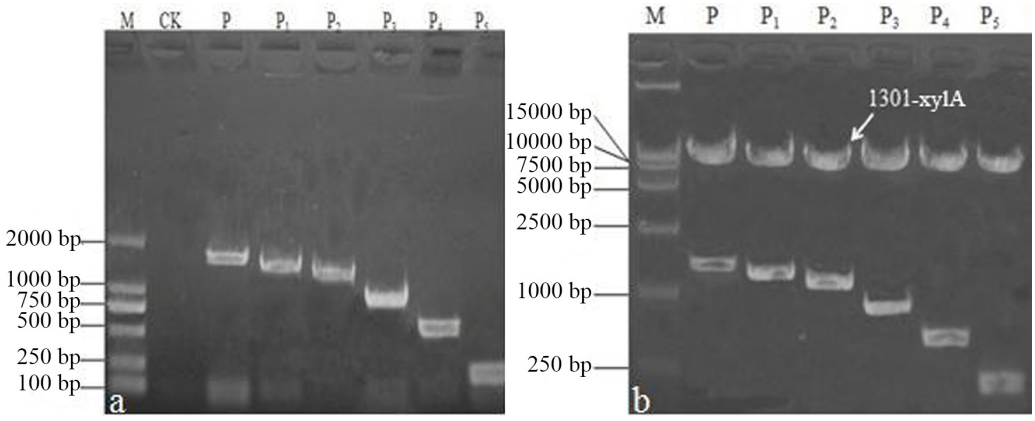

Figure 4. Confirmation of the recombinant plasmid *pCAMBIA1301-Ah-Chi-P* by PCR (a) and *BamH*I and *Nco*I digestion (b). **a.** M: DL2000; CK indicates the control that lacks a DNA template; P (full-length promoter), P1,P2, P3, P4,and P5 (serially deleted primers) are the PCR products amplified from *pCAMBIA1301-xylA-Chi-P*, *P1*, *P2*, *P3*, *P4*, and *P5*. **b.** M: DL15000; *P*, *P1*,*P2*, *P3*, *P4*, and *P5* are *BamH*I and *Nco*I double-digestion products of *pCAMBIA1301-xylA-Chi-P*, *P1*, *P2*, *P3*, *P4*, and *P5*.

GUS staining of transformed onion epidermal cells

In the absence of SA induction, non-transformed onion epidermal cells did not show GUS staining (Figure 5a) whereas onion epidermal cells transformed with the *CaMV35S* promoter (positive control) did show staining (Figure 5b). Onion epidermal cells transformed by *pCAMBIA1301-Ah-Chi-P* showed light staining without SA induction (Fig. 5c), but displayed deeper staining after SA induction (Fig. 5d). Thus, *Ah-Chi-P* is a promoter that can be induced by SA. Onion epidermal cells transformed with the 5'-deleted promoters P_1 to P_5 were also stained after SA induction (Figure 5e-i). The intensity of staining varied depending on the sequences deleted, indicating differences in the levels of activity driven by promoters P_1 to P_5.

Quantitative analysis of GUS activity in onion epidermal cells

GUS activity in onion epidermal cells transformed with the *CaMV35S* promoter was similar with or without SA treatment (Figure 6), indicating that the 35S promoter cannot be induced by exogenous SA. In contrast, GUS activity in cells transformed with P to P_5 promoters was higher after SA treatment than in its absence, indicating that the P to P_5 promoters could be induced by exogenous SA. GUS activity after SA induction was especially high in cells transformed with P_2 and P_3. The results suggested that there might be positive regulatory elements within P_2 and P_3, and also that there might be negative regulatory elements between the P and P_2 sequences.

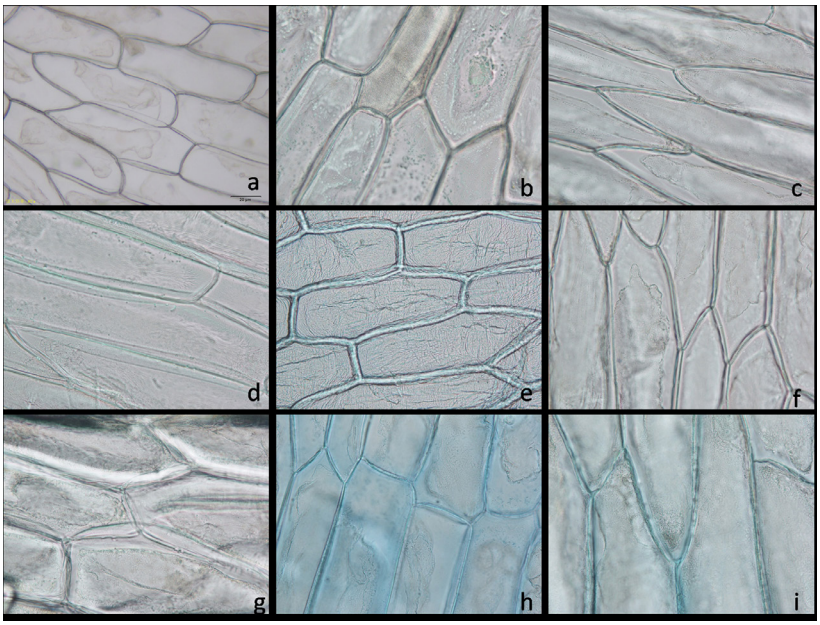

Figure 5. GUS staining of transformed onion epidermal cells. **a.-c.** Cells not induced by SA. **d.-i.** Cells induced by 5.0 mM SA for 84 h. **a.** Negative control: non-transformed cells. **b.-i.** Transformed cells driven by a positive control: 35S (**b**), *Ah-Chi-P* (**c-d**), *Ah-Chi-P*$_1$ (**e**), *Ah-Chi-P*$_2$ (**f**), *Ah-Chi-P*$_3$ (**g**), *Ah-Chi-P*$_4$ (**h**), and *Ah-Chi-P*$_5$ (**i**).

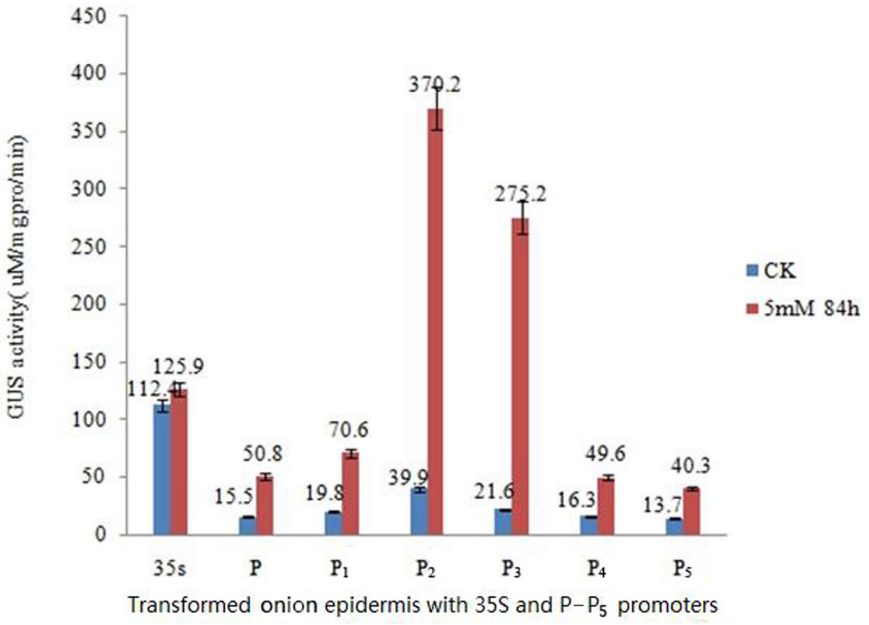

Figure 6. GUS enzyme activity in transformed onion epidermal cells after treatment for 84 h with water (blue bars) or 5 mM SA (red bars). Data are reported are means + SEM.

GUS expression in transgenic peanuts carrying the P to P_5 promoters

The generation of the T_0 transgenic peanuts was confirmed by PCR using the primers *GUS-F* and *GUS-R*, getting a 410 bp target fragment. In regenerated plants screened with xylose, 81% were confirmed to be transgenic. GUS mRNA levels in transgenic peanuts transformed with the *CaMV35S* promoter were similar with or without SA treatment (Figure 7). GUS mRNA levels in transgenic peanuts transformed with promoter P to P_5 were greater after SA treatment than in its absence. As was the case for GUS activity in transgenic onion epidermal cells, GUS mRNA levels increased sharply after SA treatment of peanuts transformed with the P_2 and P_3 promoters but increased much less in peanuts transformed with the P and P_1 promoters. Again the evidence indicates the presence of positive regulatory elements in the P_2 and P_3 promoters that are responsive to SA induction, and also the presence of negative regulatory elements in P and P_1 promoters.

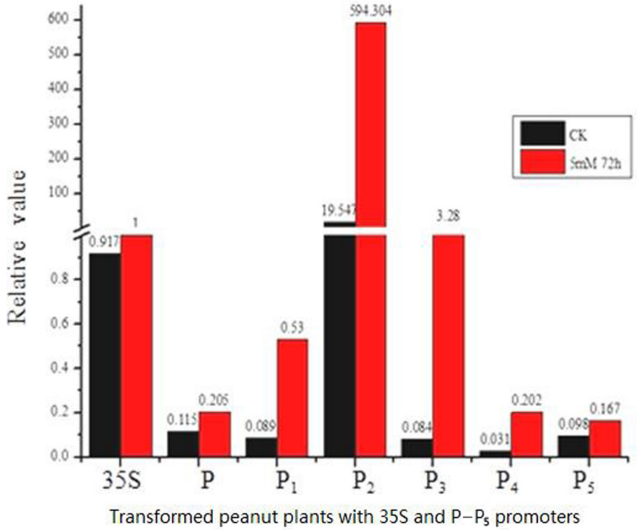

Figure 7. GUS mRNA levels in transformed peanut plants after treatment for 84 h with water (black bars) or 5 mM SA (red bars).

DISCUSSION

Promoters, which are located upstream of structural genes and have a binding site for RNA polymerase, regulate gene transcription by recognizing and binding to transcription factors. The processes involved in regulating eukaryotic gene expression are complex, and promoter-mediated control at the transcription level is an important aspect of the overall mechanism. Elucidating how promoters regulate transcription requires information from many sources, for example, cloning, confirmation of promoter function, and analysis of the interactions between promoter and transcription factor (Mu et al. 2013).

In this study, we cloned the upstream promoter sequence of the peanut chitinase gene using TAIL-PCR, and a series of 5'-deletion promoter sequences were obtained by PCR amplification. Transcription initiation by the full-length promoter or these 5'-deletion promoter sequences was studied using GUS enzyme activity in transformed onion epidermal cells and GUS mRNA levels

in transformed peanut plants. Out results indicated that the promoter of the peanut chitinase gene responded to treatment with SA and that GUS enzyme activity and GUS mRNA levels after SA induction differed depending on the promoter sequence used in the assay.

Overall, our results suggested that the promoter of the peanut chitinase gene contains both positive and negative regulatory mechanisms. A similar conclusion was made with respect to the soybean GmDREB3 promoter and the endosperm-specific ALP type-B gene promoter (Sun et al. 2008). Regarding regulation of expression of the peanut chitinase gene, the negative regulatory elements in the promoter possibly interfere with interactions between transcription factors and positive regulatory elements; this antagonism might affect expression of downstream genes. It is also possible that the positive and negative regulatory elements jointly affect the formation of a transcription initiation complex, thus affecting expression of downstream genes.

In transgenic peanuts with different lengths of promoter sequence, SA treatment increased GUS expression to a variable extent depending on the promoter present. The same effect was found for GUS enzyme activity in transgenic onion epidermal cells. The P_2 and P_3 promoters showed the strongest responses to SA, suggesting that they might contain key cis-elements responsive to SA induction. Predictive analysis of promoter structure using PLACE and PLANTcare databases indicated that the full-length promoter P contains 90 types of regulatory elements at 401 different sites: 32 regulatory elements were located between P and P_1, 35 between P_1 and P_2, 92 between P_2 and P_3, 98 between P_3 and P_4, 84 between P_4 and P_5, and 60 within P_5. These include typical regulatory elements such as CAAT and TATA boxes, a TATTCT box that is responsive to light, a tissue-specific expression element TGHAAARK, an ACGTG responsive to dehydration and dark, a TACGTGTC responsive to ABA, a TGACG responsive to jasmonic acid, SAR-binding domains MarAbox1 (AATAAAYAAA), MarTbox (TTWTWTTWTT), and 5'-UTR (Py-rich) (Lu et al. 2009; Graeber et al. 2010). Also detected were elements involved in disease resistance and SA induction: the disease-resistance elements TGTCA, GAAAAA (GT1-motif), and TGAC; the SA-induction elements TGACG (AS-1 element) and GRWAAW; W-Box elements (TTGAC, TGACT, TGACY) responsive to both pathogen and SA; and a TTTGACY element (WB-box) responsive to an elicitor (Somssich, 1994; Zhen et al., 2000; Kundu et al., 2011; Maita et al., 2015). The various GUS mRNA levels generated by the different promoters (Figures 6 and 7) lead us to speculate that the high level of expression associated with P_2 might be related to its AS-1 element. GUS mRNA level was low with P_3, P_4 and P_5, which lacks the AS-1 element. This indicates that the AS-1 element plays a key role in the response of the chitinase promoter to SA. A GRWAAW element was detected within all of the P_1 to P_5 promoters and might be another element responsive to SA. P_3 contained 12 GRWAAW elements, more than in the P_4 and P_5 promoters. It is possible that the higher level of GUS expression driven by P_3 compared to P_4 and P_5 promoters is also associated with its high number of GRWAAW elements. The WB-Box and W-Box are considered AS-1-like elements, as they contain a TGAC sequence as their core motif. Six WB-Box and W-Box elements were present within P_2-P_3, and they might contribute to the increased transcriptional activity driven by P_2. The W-Box can specifically bind with transcription factors of the WRKY family, which regulate the expression of PR-proteins after induction by exogenous SA (Yu et al. 2001). P and P_1 showed a much weaker response to SA induction than P_2, indicating that P and P_1 might contain negative regulatory elements. In addition to the positive GRWAAW and W-Box elements, P and P_1 also contain a GT1-motif and TGTCA elements. The GT-1 light-responsive element acts as a negative regulatory element within the GalUR promoter in strawberry (Agius et al., 2005). Possibly, it also acts within P- $P_1$and P_1- P_2 as a negative regulatory element for chitinase expression in peanut. TGTCA may be another negative regulatory element. Thus, in addition to the presence of positive

elements, the high promoter activity of P_2 might be related to the absence of a TGTCA element.

In summary, the promoter region of the peanut chitinase gene contains a series of important regulatory elements. CAAT and TATA are indispensable for gene transcription and play a key role in transcription initiation. The AS-1, W-box, and GRWAAW elements might have a positive regulatory effect and be involved in the response to SA. The GT1-motif and TGTCA might be negative regulatory elements. However, as there are so many regulatory elements within each of the promoters, it is difficult to identify unambiguously the exact function of each element. Clarifying these functions will require additional investigations using single point mutations and yeast hybrid technology.

Conflicts of interest

The authors declare no conflict of interest.

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