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RESEARCH ARTICLE

Whitefly and aphid inducible promoters of *Arabidopsis thaliana* L.

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Abstract. Lack of regulated expression and tissue specificity are the major drawbacks of plant and virus-derived constitutive promoters. A precise tissue or site-specific expression, facilitate regulated expression of proteins at the targeted time and site. Publically available microarray data on whitefly and aphid infested *Arabidopsis thaliana* L. was used to identify whitefly and aphid-inducible genes. The qRT-PCR further validated the inducible behaviour of these genes under artificial infestation. Promoter sequences of genes were retrieved from the Arabidopsis Information Resources database with their corresponding 5′UTR and cloned from the *A. thaliana* genome. Promoter reporter transcriptional fusions were developed with the beta-glucuronidase (GUS) *gusA* gene in a binary expression vector to validate the inducible behaviour of these promoters in eight independent transgenic *Nicotiana tabaccum* lines. Histochemical analysis of the reporter gene in T_2 transgenic tobacco lines confirmed promoter driven expression at the sites of aphid and whitefly infestation. The qRT-PCR and GUS expression analysis of transgenic lines revealed that abscisic acid largely influenced the expression of both aphid and whitefly inducible promoters. Further, whitefly-specific promoter respond to salicylic acid and jasmonic acid (JA), whereas aphid-specific promoters to JA and 1-aminocyclopropane carboxylic acid. The response of promoters to phytohormones correlated to the presence of corresponding conserved *cis*-regulatory elements.

Keywords. aphids; whitefly; inducible promoter; sap-sucking insects; biotic stress.

Introduction

Plants have evolved a wide range of adaptations in response to many biotic and abiotic stresses to improve their survival and reproduction. Among various biotic interactions, plant–pest interactions are the key factors in stabilizing both natural and man-managed ecosystem (Couldridge *et al.* 2007). More than one million phytophagus insects utilize aerial and underground plant parts as their food source, either by chewing or by sucking the plant's phloem sap. Phytophagus insects such as phloem sap-sucking aphids (*Myzus persicae*) (Bai *et al.* 2010) and

whiteflies (*Bemisia tabaci*) (Nikos *et al.* 2011) are the pests of many temperate and glasshouse grown crops. These insects not only assimilate the nutritious sap of phloem but also transmit viral diseases (Kempema *et al.* 2007) resulting in yield losses in almost all crops. To recover from these yield losses, genetically modified crops, by incorporating accurate tissue-specific expression system for the desired agronomic important gene(s), is the best alternate strategy (Potenza *et al.* 2004). Thus, the regulated transgene expression at the site and time of insect attack is required to minimize the potential adverse effects of transgene expression on nontargeted organisms and physiology of plants themselves.

The most commonly used promoter in developing transgenic plants is a virus-derived CaMV35S promoter, which is expressed constitutively in all the plant parts (Odell *et al.* 1985). Similarly, other virus-derived constitutive promot-

ers are also commonly used, such as Cassava vein mosaic virus (CsVMV) (Verdaguer *et al.* 1998), Australian banana streak virus (BSV) (Schenk *et al.* 2001), Mirabilis mosaic virus (MMV) (Dey and Maiti 1999) and Figwort mosaic virus (FMV) (Sanger *et al.* 1990; Maiti *et al.* 1997). Unnecessary, the overexpression of the transgene in all plant parts, all the time, may lead to unexpected consequences on plant growth and development. Sometimes, transgene silencing occurs for the foreign promoters result in the stable suppression of gene activity throughout the plant or affect the specificity of a promoter (Kloti *et al.* 2002). The silencing process was observed to be less prevalent when constitutive promoters from a plant were used (Potenza *et al.* 2004). Several groups have used different plant promoters for transgenes expression such as actin (Act2) of *A. thaliana* (An *et al.* 1996), rice *actin 1* (McElroy *et al.* 1991; Zhang *et al.* 1991), maize *ubiquitin 1* (pUbi) (Christensen *et al.* 1992), and *UbiU4* of *Nicotiana sylvestris* (Plesse *et al.* 2001). Similarly, maize *ubiquitin 1* and rice sucrose synthase (*RSs1*) gene promoter were used to express the snowdrop lectin *Galanthus nivalis* leaf agglutinin (GNA) against the brown plant hopper (*Nilaparvata lugens*) (Sudhakar *et al.* 1998).

Phloem specific expression of insecticidal protein would be the better strategy to control and enhance the expression of protein in the phloem sap for higher activity against sucking pests. Researchers already used phloem-specific promoters such as *RolC* (Chakraborti *et al.* 2009), maize sucrose synthase-1 promoter (Yang and Russell 1990); these promoters were also used to express *Alium sativum* leaf agglutinin (ASAL) against aphids (Saha *et al.* 2007). The promoters of pumpkin PP2 (Guo *et al.* 2004) and Commelina yellow mottle virus (Matsuda *et al.* 2002) were also reported to be phloem-specific promoters. These promoters are phloem specific, but their heterologous source and constitutive nature are still the concerns. Thus, the identification of sap-sucking insect-inducible plant promoters to target transgene expression specifically in phloem sap may ideally prove to be a powerful technique for sap-sucking insect resistant transgenic plant development. In the present study, we identified three promoters in *A. thaliana* and conceptually demonstrated their expression in transgenic plants after infestation with aphids and whitefly.

Materials and methods

Selection of candidate genes and construct preparation

The *.cel* files of the GEO database (GSE6516 and GSE5525) were selected and analysed as explained in the result section. A number of genes were significantly affected by infestations of aphids and whitefly (figure 1 and table 1 (sheets 1–6) in electronic supplementary material at http://www.ias.ac.in/jgenet/). About 549 genes were

upregulated in whitefly infestation while 372 and 480 genes were upregulated at 48 and 72 h at postaphid infestation respectively (figure 1 in electronic supplementary material). About 599 genes were downregulated in whitefly infestation while 490 and 960 genes were downregulated at 48 and 72 h postaphid infestation respectively (figure 1 in electronic supplementary material). List of significantly affected genes after whitefly and aphid infestation at different time points is provided in table 1 in electronic supplementary material, includes genes upregulated after 21 days of whitefly infestation (sheet 1 in table 1 of electronic supplementary material), downregulated genes (sheet 2 in table 1 of electronic supplementary material), aphid 48 h infestation upregulated genes (sheet 3 in table 1 of electronic supplementary material), downregulated genes (sheet 4 in table 1 of electronic supplementary material), aphid 72 h infestation upregulated genes (sheet 5 in table 1 of electronic supplementary material), downregulated genes (sheet 6 in table 1 of electronic supplementary material) and commonly deferentially expressed gene between aphid and whitefly infestation (sheet 7 in table 1 of electronic supplementary material).

Three genes, namely W250 (AT1G19250, flavin-containing monooxygenase), A360 (AT3G48360, speckle-type POZ protein), and A080 (AT2G40080, early flowering 4), were selected (table 2 in electronic supplementary material), validated with quantitative reverse transcription polymerase chain reaction (qRT-PCR), and used for promoter cloning and transgenic plant development. The 1 kb promoter regions of the selected genes with their respective 5′UTRs were retrieved from the TAIR database (http://www.arabidopsis.org/). All three selected promoters were amplified by using *A. thaliana* genomic DNA as a template and AccuPrime Pfx DNA polymerase (cat. no. 12344-024, Invitrogen, Carlsbad, USA). Primer sequences used for the amplification of promoters and used elsewhere in this study are listed in table 3 of electronic supplementary material. PCR products containing *Sal*I and *Bam*HI restriction sites at their ends were first cloned into pSK$^+$ for sequencing. These promoters from pSK$^+$ clone were subcloned into a binary vector pBI 101 upstream to a GUS reporter gene (Clontech, http://www.clontech.com). Tobacco transformation was done as established by Horsch *et al.* (1985). Transgenic tobacco seeds (T_1) were collected and grown on kanamycin (300 mg/L) for positive plant selection. Seeds of the eight independent transgenic lines of T_2 generation were grown for a maximum of 8 weeks for further experiments.

Plant growth conditions and insect infestation

A. thaliana plants of a Col-0 background were grown on vermiculite and solarite TC mix (Keltech, Bengaluru, India) in a 10-inch plastic pot and kept at 4°C for 3 days. After 3 days, the sown seeds were transferred to a culture

room that was maintained at 16 h light / 8 h dark cycle at 22°C. Three to four week-old plants were selected for the experiments. Transgenic tobacco plants were grown in a greenhouse under standard field irrigation and photoperiod.

The culture of aphids (*M. persicae* and *M. nicotinae*) and whiteflies (*B. tabaci*) was maintained on potted *A. thaliana* and *N. tabaccum* plants in the insectry at $26 \pm 2°C$ and 70% relative humidity (Upadhyay *et al.* 2011). Newly emerged whiteflies and second instars aphid nymphs were used for the experiments.

Ten aphids (*M. persicae*) per plant were released on *A. thaliana*. After 2, 24, 48, 72 and 96 h of aphid infestation, aphids were removed with a fine brush and the leaves were used for RNA isolation.

For aphid (*M. nicotinae*) infestation treatment of the T_2 transgenic tobacco plant, leaf disks of a 10-mm diameter were cut, transferred to agar plate, and challenged with 15 aphids for 48 h in parallel to noninfested control leaf disks. After 48 h of aphid infestation on leaf disks, aphids were removed and leaves were used in GUS analysis (both fluorimetric and histochemical).

Fifteen whiteflies (*B. tabaci*) per plant were released on *A. thaliana*. After 7, 14 and 21 days of whitefly infestation, whiteflies were removed and RNA was isolated at each time point with control noninfested plants in biological triplicates. Time points of the highest induction of genes were identified in *A. thaliana* leaves, and they were selected for the infestation experiment in transgenic tobacco leaf.

For whitefly infestation, the leaf disk of the transgenic plant was challenged for 7 days with 10 to 15 whiteflies in parallel to the control (Upadhyay *et al.* 2011). All the experiments were performed in biological triplicate and experimental duplicate conditions.

Phytohormone treatment

Hundred milligrams of leaves from *A. thaliana* plants were cut and dipped in Hoagland media containing 1 mM salicylic acid (SA) (Onate-Sanchez and Singh 2002), 100 μM meJA (Onate-Sanchez and Singh 2002), 100 μM abscisic acid (ABA) (Zhang *et al.* 2008a), and 5 μM 1-aminocyclopropane carboxylic acid (ACC) (Staal *et al.* 2011) in parallel to the control Hoagland media. After 2, 6, 12, 18, 24 and 48 h of treatment, the RNA was isolated. Time points of the highest expression of selected genes in *A. thaliana* leaves were selected for the phytohormone treatment in transgenic tobacco leaf disks. All the experiments were performed in the biological triplicate condition.

Complementary DNA (cDNA) preparation and qRT-PCR

Ten μg RNA was used for DNaseI treatment (Ambion). DNaseI-treated RNA (2 μg) was used for the cDNA preparation by using SuperScript cDNA Synthesis Kit (Invitrogen). The qRT-PCR reaction was performed on the ABI 7500 Real-Time PCR Detection System (Applied Biosystems, Foster City, USA) by using SYBR Green PCR Master Mix (Applied Biosystems). Relative fold changes in expression were measured by using the $2^{-\Delta\Delta CT}$ method. The expression of *actin* gene (AT3G18780.2) was used as an internal control for data normalization of selected genes at all time points (Livak and Schmittgen 2001). The primer sequences used in this study are given in table 3 in electronic supplementary material.

Identification of cis-regulatory element

The promoter sequences were analysed by the PLACE analysis (PLACE, plant *cis*-acting regulatory DNA elements, http://www.dna.affrc.go.jp/PLACE/) for the theoretical identification of the *cis*-regulatory element-binding sites against the PLACE database (Higo *et al.* 1999).

Fluorimetric GUS assay and histochemical GUS analysis

Fluorimetric GUS assays were performed as described by Chaturvedi *et al.* (2006). For histochemical analysis, insect challenged and control leaf disks were coincubated at 37°C with 50 mM X-Gluc (5-bromo-4-chloro-3-indolylglucuronide) that was buffered overnight with 50 mM sodium phosphate buffer pH 7, 0.2% Triton X100, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide and 20% methanol.

Results

Identification of inducible genes and their validation with qRT-PCR

To identify aphid and whitefly-inducible genes, microarray expression profiles in the GEO database, namely GSE6516 for whitefly infestation of *A. thaliana* (Kempema *et al.* 2007) and GSE5525 for aphid infestation of *A. thaliana* (De Vos *et al.* 2005) were selected. The analysis was carried out with ArrayAssist software 5.2.2 (Stratagene, Santa Clara, USA) to select highly inducible genes in comparison to the control. We considered only those differentially expressed genes which showed fold change ≥ 2.0 and $P \leq 0.05$. Further, the results were also confirmed with MeV software (https://sourceforge.net/projects/mev-tm4/). Based on our analysis, one gene, namely flavin-containing monooxygenase (AT1G19250), coded as W250 from the whitefly dataset, and two genes, namely speckle-type POZ protein (AT3G48360), coded as A360 and early flowering 4 (AT2G40080), coded as A080 from the aphid-inducible dataset, were selected for further evaluation (table 2 in electronic supplementary material).
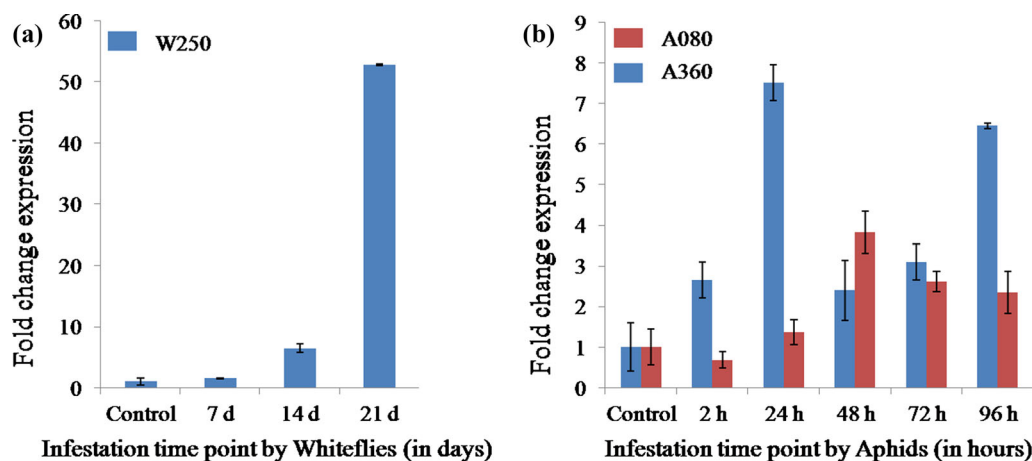
Figure 1. Expression patterns of selected genes established by qRT-PCR during whitefly and aphid infestation. Leaves of *A. thaliana* (Col-0) plants were challenged with whiteflies and aphids for 7, 14 and 21 d and 2, 24, 48, 72 and 96 h, respectively. Leaves of unchallenged plants were considered as controls. All the experiments were performed in biological and experimental triplicate condition.

To evaluate the aphid and whitefly-inducible behaviour of selected genes, RNA prepared from *A. thaliana*-infested plants were subjected to qRT-PCR analysis. More than a 50-fold induction of the W250 gene was observed in leaves of *A. thaliana* plants after 21 days of whitefly infestation (figure 1a). Thus, the qRT-PCR analysis validated the expression of the W250 gene, as determined earlier in the microarray result (table 2 in electronic supplementary material). In the case of aphid infestation, the expression of A360 was induced about eight-fold at 24 h whereas the expression of A080 was induced about four-fold at 48 h of infestation (figure 1b). The expression of A360 was also induced at a later time point of infestation, i.e. 96 h, which was more than six-fold compared with noninfested control plants. Thus, the expression of all the selected genes by qRT-PCR was found similar to that of microarray result (table 2 in electronic supplementary material).

Validation of selected promoters in transgenic tobacco plants

Promoters of all the three selected genes were cloned upstream to the *gusA* gene in the pBI101 vector, and 12–14 independent transgenic tobacco lines were generated by using agrobacterium-mediated transformation. Tobacco was selected as a model plant system for studying the promoters isolated from *A. thaliana*. Evaluation of the inducible behaviour of promoters in a heterologous plant system will validate the instant feasibility of its use of the expression of insect-resistant proteins in crop plants. The expression pattern of selected promoters in tobacco will also reflect their utility in the crop improvement programme. Eight-week-old transgenic tobacco plants from eight independent transgenic (T_2) lines were selected for the fluorimetric experiments. All the assays were performed in biological triplicate and experimental duplicate

in parallel to the control. Transgenic plants expressing the GUS protein driven by P_{W250} showed average GUS activity 91 pmol/mg/min protein after seven days of whitefly infestation (figure 2a), which was significantly higher than the noninfested control. The histochemical staining of whitefly-infested transgenic leaves showed that the GUS expression of promoter P_{W250} was specifically at the site of whitefly infestation (figure 2d) in agreement with the quantitative data. Whereas in P_{A360}, the average GUS activity of 13 pmol/mg/min protein was observed after 48 h of aphid infestation (figure 2b), which was significantly higher compared with the noninfested control. Also, in the case of P_{A360}, histochemical staining for GUS activity was restricted on the site of aphid infestation (figure 2 in electronic supplementary material). However, transgenic lines of P_{A080} showed a high background level GUS activity, which was induced nonsignificantly as much as 417 pmol/mg/min protein after 48 h of aphid infestation (figure 2c). Further, significant GUS staining was observed in both aphid-infested and noninfested areas in the stained leaves of transgenic lines of the promoter P_{A080} (figure 2, e&f). Thus, these results validated the inducible expression of selected promoters of the site of aphid and whitefly infested transgenic leaves.

Expression profile of selected genes in response to SA, jasmonic acid (JA), ACC and ABA treatment

The expression of insect-inducible genes is regulated by phytohormones such as SA, JA, ABA and ethylene. Thus, phytohormones SA, JA, ABA and ACC (a nonvolatile precursor of ethylene) were selected to understand the regulatory nature of the selected promoters. The influence of these hormones on the expression of selected genes (W250, A360 and A080) was assessed by qRT-PCR after treatment with phytohormones in *A. thaliana*. The expression
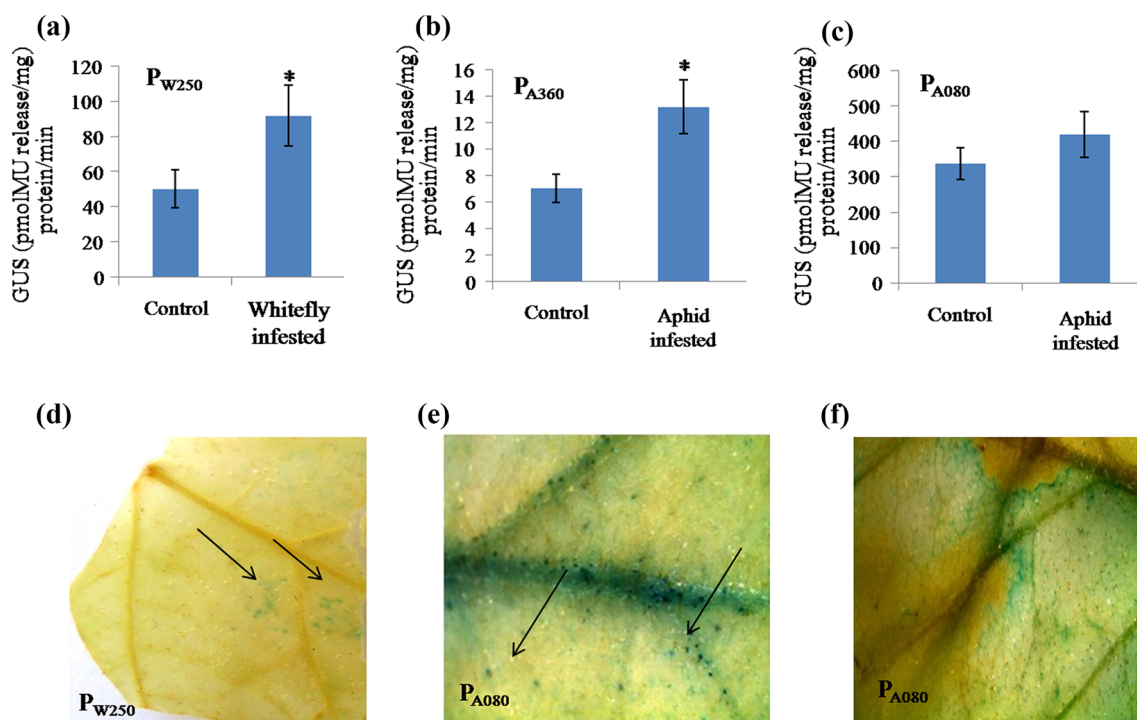
Figure 2. GUS expression pattern of selected promoters P_{W250}, P_{A360} and P_{A080} (fluorimetric: a, b, c; histochemical: d (P_{W250}), e, f (P_{A080}) in T_2 transgenic tobacco plants after 7 days of whitefly (P_{W250}) and 48 h of aphid (P_{A360} and P_{A080}) infestation). Mean $\pm$ SE were obtained from eight independent transgenic lines with biological triplicate and experimental duplicate. Bars labelled with stars indicate the significant differences as determined by *t*-test analysis ($P \leq 0.05$).

of selected genes was assessed at 2, 6, 12, 18, 24 and 48 h after phytohormone treatment.

The expression of W250 was significantly induced after 48 h of SA treatment (figure 3a). In the case of JA treatment, the expression of W250 was found to be substantially higher after 12 h of treatment (figure 3b). The response of W250 toward ABA was gradual, and it attains maximum expression after 48 h of treatment (figure 3c). The treatment of ACC did not show any significant induction at any time points when we examined (figure 3d). Thus, we selected 48 h for SA, 12 h for JA, and 48 h for ABA as optimal time points for phytohormonal treatment of transgenic lines expressing P_{W250}. We observed significant induction in the GUS activity in transgenic lines treated with SA and JA (figure 3e). However, in the case of ABA treatment, although transgenic lines showed an elevated level of expression, it was not statistically significant. The expression of A360 was also examined post-treatment of SA, JA, ABA and ACC at different time points. The expression of A360 was found to be the highest at 24 h in all the treatments, including JA (figure 4a), ACC (figure 4b), and ABA (figure 4c). However, in the case of SA treatment, we did not observe any significant induction at any time points analysed (figure 4d). Thus, the 24-h time point was selected to evaluate the expression of P_{A360} in transgenic tobacco lines expressing GUS post-treatment of JA, ACC and ABA. The expression of P_{A360} was elevated in SA and JA treatments, but it was statistically significant only in

ABA treatment (figure 4e). The expression of A080 was also evaluated after treatment of SA, JA, ABA and ACC at different time points. The expression of A080 was not significantly affected by treatment of SA and JA; however, A080 does respond to ACC and ABA (figure 5, a–d). The expression of A080 was found highest after 6 h of ACC treatment and 48 h of ABA treatment; thus, these hormones and time points were further selected to evaluate transgenic lines expressing GUS under the control of P_{A080}. Transgenic lines showed elevated expression in response to 6 h of ACC treatment; however, this induction was not statistically significant considering the large variation in independent transgenic lines in response to ACC (figure 5e). However, ABA treatment for 48 h resulted in induced expression of GUS, which was statistically significant compared with the untreated control (figure 5e).

Cis-regulatory motif analysis of cloned promoter

All the cloned promoter sequences were subjected to the *cis*-regulatory motif analysis by using the PLACE (http://www.dna.affrc.go.jp/PLACE/) database (figure 6). The maximum numbers of conserved *cis* motifs identified were of MYCCONSENSUSAT (found in dehydration responsive genes) origin in all three cloned promoters (figure 6). All the cloned promoters also showed a higher number of ABA-responsive elements such as ABRELATERD1, ACGTATERD1, ABRERATCAL,
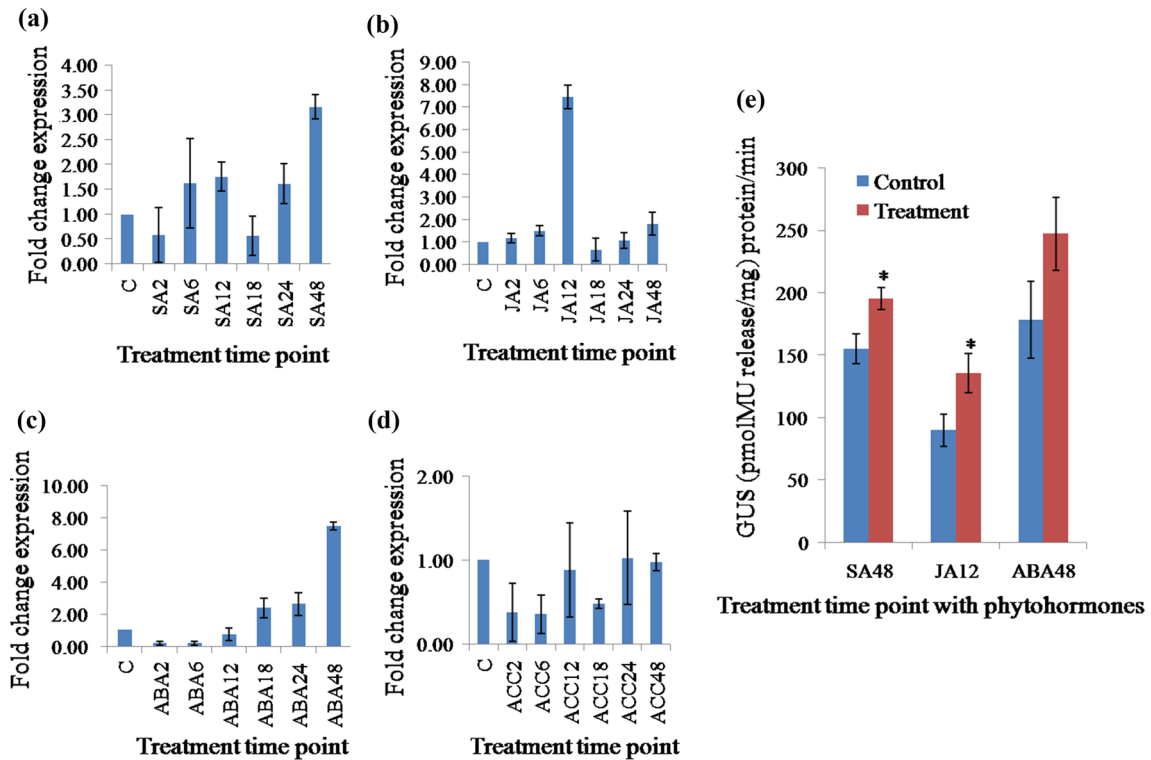
Figure 3. Expression pattern of the W250 gene in response to SA, JA, ABA and ACC treatment in *A. thaliana* (Col-0) leaves at different time points (a–d). The time point of maximum induction was selected for further hormone treatment to transgenic tobacco leaves. GUS activity of P_{W250} after SA (48 h), JA (12 h), and ABA (48 h) treatment (e). Bars labelled with * indicate the significant differences as determined by *t*-test analysis ($P \leq 0.05$).
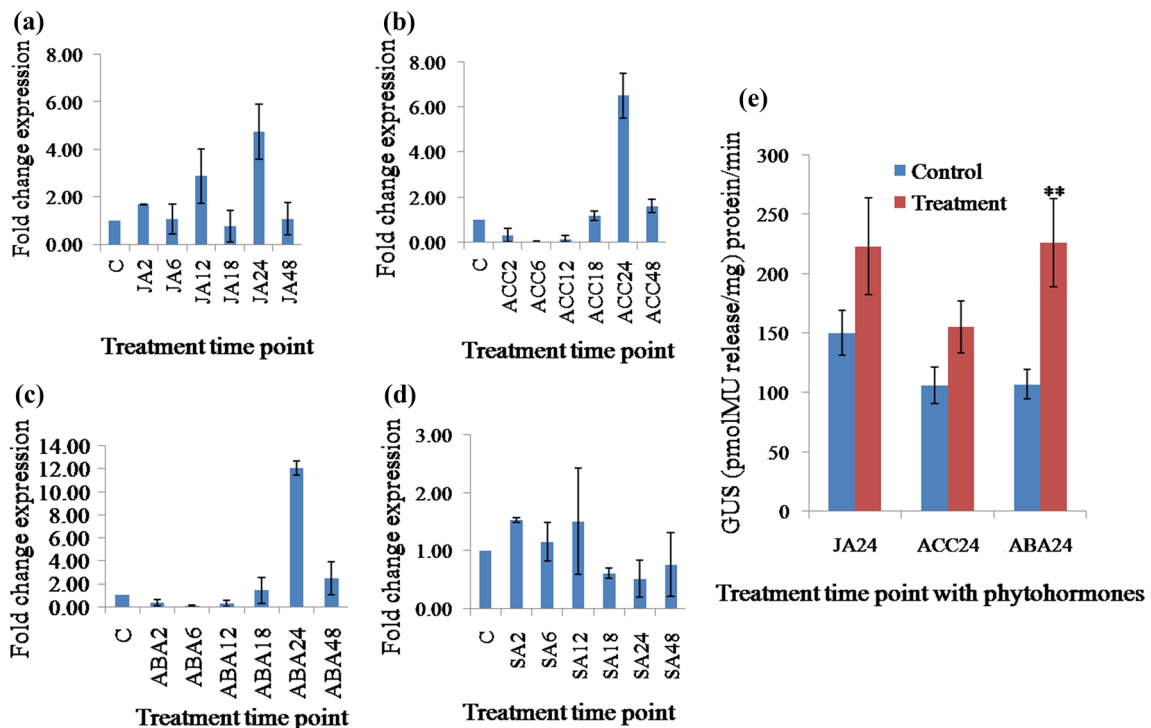

Figure 4. Expression pattern of the A360 gene in response to JA, ACC, ABA and SA treatment in *A. thaliana* (Col-0) leaves at different time points (a–d). The time point of maximum induction was selected for further hormone treatment to transgenic tobacco leaves. GUS expression of P_{A360} after JA (24 h), ACC (24 h), and ABA (24 h) treatment (e). Bars labelled with stars indicate the significant differences as determined by *t*-test analysis ($P \leq 0.05$).
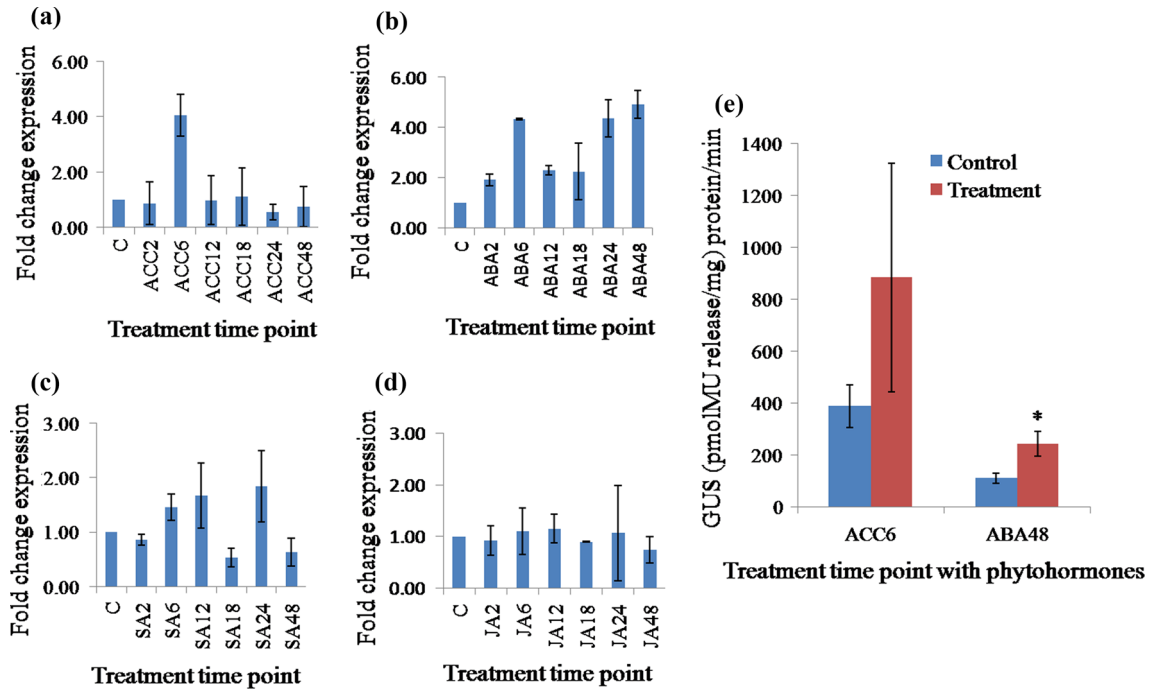
Figure 5. Expression pattern of A080 gene in response to ACC, ABA, SA and JA treatment in *A. thaliana* (Col-0) leaves (a–d). The time point of maximum induction was selected for further hormone treatment to transgenic tobacco leaves. GUS activity of P_{A080} after ACC (6 h) and ABA (48 h) treatment (e). Bars labelled with stars indicate the significant differences as determined by *t*-test analysis ($P \leq 0.05$).
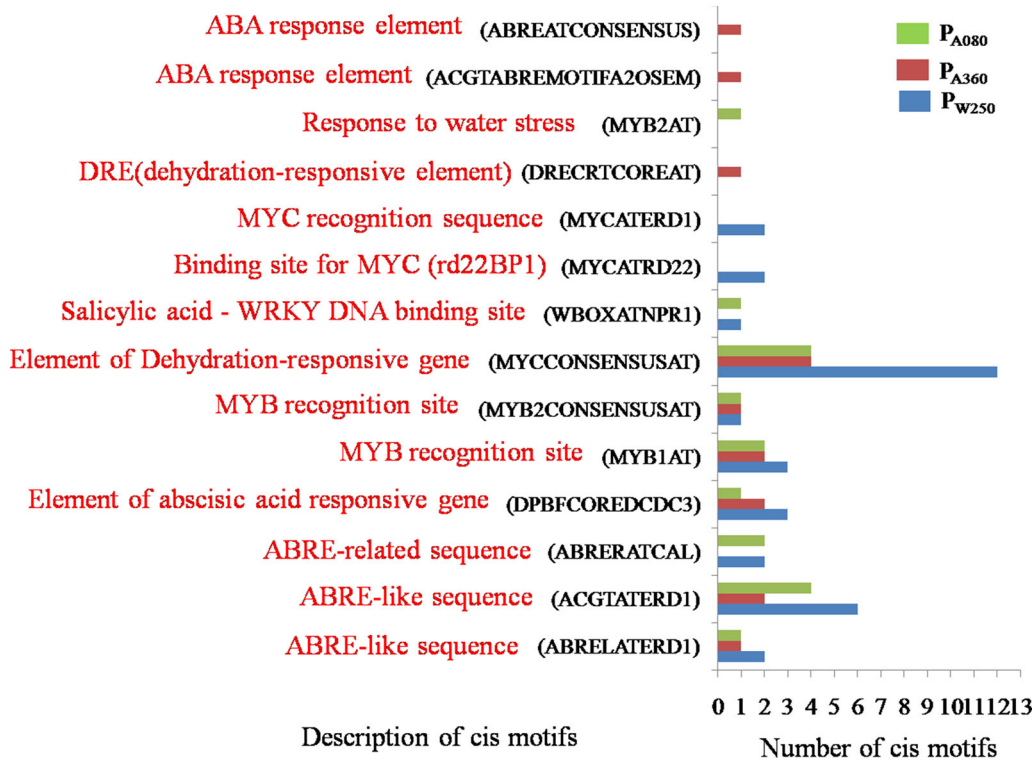

Figure 6. List and number of phytohormones and stress-responsive *cis* elements in cloned promoter.

DPBFCOREDCDC3, MYB1AT, MYB2CONSENSU-SAT and MYCCONSENSUSAT, and probably responsible for ABA-induced expression either in *A. thaliana* (figures 3c, 4c and 5b) or in transgenic tobacco lines (figures 3e, 4e and 5e). P_{W250} also showed conservation of SA-responsive *cis*-regulatory element WBOXATNPR1

(figure 6) that agrees with its SA-induced expression (figure 3, a&c), thus corroborating our results. We did not find any JA-responsive *cis*-regulatory element in the cloned promoter fragment of P_{W250} but the induction of the native gene was observed in JA at 12 h in *A. thaliana* (figure 3b) and transgenic tobacco lines (figure 3e). Other than the ABREs mentioned earlier, three more motifs, ABREATCONSENSUS, ACGTABREMOTIF2OSEM and DRECRTCOREAT, were found to present exclusively in P_{A360}. In accordance, P_{A360} showed relatively early ABA induction in native *A. thaliana* (figure 4c) and transgenic tobacco lines (figure 4e). Along with several ABREs found in the two promoters mentioned earlier, an additional MYB recognition sequence MYB 2AT was found to be present in P_{A080}. The abundance of ABREs in P_{A080} was also mirrored in its ABA-inducible expression in the case of both *A. thaliana* (figure 5b) and transgenic tobacco lines (figure 5e).

Discussion

In this study, we cloned and characterized one whitefly and two aphid-inducible promoters from *A. thaliana*. The whitefly and aphid inducible genes were identified by analysing publically available datasets (GSE6516 and GSE5525). All the three selected genes were well validated for their expression by qRT-PCR in *A. thaliana* (figure 1), indicating the selection of genes by microarray profiles represents their true expression patterns. The selected gene W250 (AT1G19250) encodes FMO, which is reported to be involved in the plant–pathogen interaction (Mishina and Zeier 2006). The gene A360 (AT3G48360), known as *BT2*, a protein with BTB, TAZ, and calmodulin-binding domains, is an essential component of the TAC1-mediated telomerase activation pathway (Ren *et al.* 2007) and it is localized in the nucleus and cytosol (Robert *et al.* 2009). The gene A080 (AT2G40080) encoded a small protein known as ELF4 and is essential for circadian clock function, seedling de-etiolation, photoperiod perception, and flowering (Khanna *et al.* 2003; Doyle *et al.* 2005; Kim *et al.* 2013). The expression of A360 and A080, in response to aphid infestation, is interesting and suggests crosstalk of hormonal pathways that are responsible for telomerase activation and flowering with that of plant insect interactions.

The GUS estimation showed that P_{A080} is the strongest among all the selected promoters, as GUS activity driven by this promoter reaches a maximum of 400 pmol/mg/min protein after aphid infestation (figure 2c). The highest expression of P_{A080} might also be due to its higher level of background expression. Since histochemical analysis of aphid-infested leaves of transgenic lines indicated that the expression of P_{A080} was not strictly restricted on the site of infestation (figure 2, e&f), which was in sharp contrast to that observed in the case of P_{W250} and P_{A360} (figure 2d;

figure 2 in electronic supplementary material). We identified that fold induction in all three selected genes in response to whitefly or aphid infestation was significantly higher (figure 1a) in *A. thaliana* as compared with their respective promoters in transgenic tobacco lines (figure 2, a, b&c). This higher gene expression may be due to better sensitivity and linearity of qRT-PCR than the enzymatic GUS activity. It is also noteworthy that for each promoter, several transgenic lines are evaluated; thus, the expression of promoters is strongly influenced by their position of integration in the genome (Horner *et al.* 1995; Kiran *et al.* 2006; Chaturvedi *et al.* 2007; Lodhi *et al.* 2008; Tiwari *et al.* 2008; Srivastava *et al.* 2014).

Insect attacks lead to the modulation of plants phytohormonal pathways to cope with attacking insects (Kempema *et al.* 2007). SA-related and JA-related pathways play an important role during the infestation of whitefly and aphid, respectively (Kempema *et al.* 2007). Our previous result showed that the aphids and whiteflies modulated the expression of phytohormonal pathway related genes in cotton plants (Dubey *et al.* 2013). The expression of W250 (FMO1) responds to 12 h of JA treatment; whereas its expression increased in the later stage of SA and ABA treatments (figure 3). An enhancement in the expression of W250 in response to SA treatment complements the earlier published report, where the authors reported it as an SA marker gene for cell death, involvement in lesion and SAR development (Pieterse *et al.* 1996; Brodersen *et al.* 2005; Graaff *et al.* 2006; Mishina and Zeier 2006; Zhang *et al.* 2008b). The expression of P_{A360} (BT2) was reported to be downregulated after infection with the *Plasmodiophora brassicae* in *A. thaliana* (Siemens *et al.* 2006) and induced by other stresses such as drought, high salt and cold (Fujita *et al.* 2007). Exogenous sugars decreased A360 expression, whereas exogenous nitrogen increased its expression (Mandadi *et al.* 2009). Sap-sucking insects, including aphids, are secondary sinks of sugar in plants and deprivation of sugar through aphids may be the reason of induction of A360 genes in aphid-infested plants (figure 1b). Induction of A360 by both ACC and ABA at the same time point, i.e. 24 h (figure 4, b&c), and induction of A080 by both ACC and ABA at 6 h (figure 5, a&b) indicate the involvement of ABA and ACC cross-talk in the regulation of A360 and A080 genes. The expression profile of selected genes in response to SA, JA, ACC and ABA indicates that both whitefly and aphid can induce selected genes via different phytohormone pathways.

In our study, we identified potential *cis*-regulatory elements conserved in the cloned promoters (figure 3 in electronic supplementary material). These *cis*-acting elements were earlier reported to play their respective role in regulating the expression of those genes. One of the important *cis*-regulatory motifs identified in all the cloned promoters was 'MYCCONSENSUSAT'; this particular motif was previously reported to regulate ABA-induced expression in the *rd22* gene in *A. thaliana* (Abe *et al.* 2003).

We also identified additional ABA-responsive elements such as ABREATCONSENSUS and ACGTABREMO-TIFA2OSE in P_{A360}; both these motifs are a part of ABA and drought response (Choi *et al.* 1999; Huang *et al.* 2008). Drought-induced upregulation of ABA signalling decreases SA-dependent response but increases the JA-dependent response in wild-type *Medicago truncatula* after the infestation of the pea aphid (Guo *et al.* 2016). Other than ABREs, 'MYCATERD1' and 'MYCATRD22' are the two MYC recognition sequences exclusively present in the P_{W250} that are also known to have a distinct role in ABA and drought inducible expression (Abe *et al.* 1997; Simpson *et al.* 2003). In the case of P_{A080}, an extra ABA-responsive motif MYB2AT was identified that was reported for the regulation of ABA response in the *Atmyb2* gene in *A. thaliana* (Urao *et al.* 1993). Besides, we also identified conserved WBOXATNPR1 in the P_{W250} promoter (figure 6), which explains its SA-induced expression (figure 3, c&e); this particular motif was previously reported for SA-induced expression of the *NPR1* gene in *A. thaliana* (Yu *et al.* 2001). However, further detailed investigations involving site-directed mutagenesis of these elements and expression of the mutant version of these promoters in plants will be needed to substantiate the role of these *cis*-regulatory motifs in the regulation.

In conclusion, well characterized inducible promoter identified in this study can be used to regulate the expression of the insecticidal protein in fine-tune manner. These promoters are strictly expressed at the site of whitefly and aphid infestation, making them suitable for biotechnological interventions. Both the whitefly and aphid inducible promoters are regulated by ABA, whereas whitefly inducible promoters specifically induced by SA and aphid induced promoters are regulated by JA.

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