TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2017) 41: 223-233

doi:10.3906/bot-1609-19

Characterization of rice polyphenol oxidase promoter in transgenic *Arabidopsis thaliana*

Wasim AKHTAR[1], Ejaz AZIZ[2], Hisashi KOIWA[3], Tariq MAHMOOD[2,*]
[1]Department of Biotechnology, University of Azad Jammu and Kashmir, Muzaffrabad, Pakistan
[2]Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[3]Department of Horticultural Sciences, Texas A&M University, College Station, TX, USA

Received: 20.09.2016 • **Accepted/Published Online:** 09.01.2017 • **Final Version:** 24.05.2017

Abstract: Polyphenol oxidase (PPO) enzymes are ubiquitous in plant kingdom and catalyze the oxidation of phenols to highly reactive quinones. The *PPO* gene plays an important role in plant defense mechanisms against biotic and abiotic stresses but the regulation of *PPO* promoter in stresses remains unclear. Here *Oryza sativa* (*OsPPO*) promoter was fused to firefly *Luciferase* (*LUC*) and β-glucuronidase (*GUS*) reporter genes separately to test the effects of wounding, abscisic acid (ABA), and methyl jasmonate (MeJ) applications on *PPO* promoter induction. To the best of our knowledge, this is the first report about rice PPO promoter induction in response to wounding and ABA characterized by RT-PCR. Transcriptional profiling of reporter genes by real-time (RT) PCR in transgenic *Arabidopsis* revealed ~2.5-fold induction under *PPO* promoter directed activity in response to ABA treatment. Moreover, a strong induction of 11-fold was observed in response to wounding in reporter gene transcripts under control of *OsPPO* promoter. Plant *cis*-acting regulatory DNA elements signal scanning of *OsPPO* promoter also showed homologies to ABA *cis*-regulatory element signaling complexes and wound responsive elements (W-boxes) residing within the 1020 bp promoter region. Wound and ABA inducibility of *OsPPO* promoter is a strong indicator of its role in the plant defense mechanism against abiotic and biotic stresses.

Key words: Characterization, wound induction, PPO, elicitors, transgenic *Arabidopsis*

1. Introduction

Plants act in response to pathogens attack through activation of many mechanisms, ultimately resulting in reduction of growth and control of pathogens. Polyphenol oxidases (PPOs) are copper metalloproteins that function in plant defense but also cause significant postharvest agricultural losses (Aniszewski et al., 2008). PPO enzymes are inert in thaylakoid and become active upon release from the thylakoid by disruption such as wounding, senescence, and attack by insect pests or pathogens. PPOs catalyze oxygen-dependent oxidation of phenols to quinones (brown phenomenon). These black and brown quinone adducts formed by PPO enzymatic activity are renowned and the reason for interest in the postharvest physiology of many vegetable and fruit crops (Mayer and Harel, 1979; Friedman, 1997). These quinones are highly reactive intermediates that undergo secondary reactions and ultimately bring about oxidative browning that accompanies plant senescence, wounding, and responses to pathogens (Thipyapong et al., 2004). Therefore, *PPO* gene activity ultimately enables quinones and reactive oxygen species to cope with stresses. Due to prominent wound and pathogen inducibilities, *PPO*s are significant in biological studies (Thipyapong et al., 1997).

PPO expression in transgenic plants provides a unique system to evaluate the involvement of *PPO* in plant disease resistance. The role of *PPO* in 'induced plant defense' has been validated in transgenic plants. *PPO* overexpressing lines showed increased resistance to bacterial pathogens (Li and Steffens, 2002; Richter et al., 2012). In some plant species *PPO* activity is strongly induced by insect attacks (Ruuhola et al., 2008). Using modified *PPO* expression in transgenic plants, a defensive role was established against insects (Barbehenn et al., 2007; Mahanil et al., 2008; Bhonwong et al., 2009) and pest infestations (Wang and Constabel, 2004; Richter et al., 2012; Chai et al., 2013). As anticipated, the downregulation or silencing of *PPO*s resulted in enhanced susceptibility to pathogens in transgenic tomato and potato lines (Thipyapong et al., 2004; Richter et al., 2012).

Ethylene, methyl jasmonic acid (MeJ), abscisic acid (ABA), and salicylic acid (SA) are universal signals that play a pivotal role in the growth and development of plants and defense to diseases and senescence. These signals interact in a mutually synergistic or in an antagonistic manner to overcome the attack of pathogen and herbivorous insects (Pieterse and Dicke, 2007; Spoel et al., 2007; Fan et al., 2009;

* Correspondence: tmahmood@qau.edu.pk

Pieterse et al., 2009). However, the promoters that regulate *PPO* gene expression in vegetative and reproductive development have not been studied so far. In the present study, *Oryza sativa* (*OsPPO*) promoter was cloned for its expression profiling in transgenic *Arabidopsis* in ABA, MeJ, and wounding stresses.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (Col. 0) plants were used for stable transformation. *Arabidopsis* seeds were grown on Murashige and Skoog (MS) media (Phyto Technology Laboratories) (Murashige and Skoog, 1962) and 10-day-old seedlings were shifted to soil pots. Plants were grown in growth room at 25 °C with 16 h light and 8 h dark cycles. Twenty-eight-day-old flowering plants were used for transformation.

2.2. Retrieval target gene promoter

Genomic DNA was isolated from *Oryza sativa* by cetyltrimethylammonium bromide (CTAB: Biochemica) method (Allen et al., 2006). *OsPPO* promoter ~1020 bp (Accession #JQ284399) was amplified by PCR with *Sma*I (NEB) restriction sites from rice genomic DNA using Phusion polymerase. The sequence of designed primer is given below:

Forward: GGCTGGTTCACTTGACAATTTCG
Reverse: GCACTGCGCTGTGAACTTGCA

PCR conditions used for amplification underwent pre-denaturation at 94 °C for 5 min following 35 cycles of denaturation at 94 °C for 30 s, annealing at gradient of 50 °C to 60 °C for 30 s, and extension at 72 °C for 40 s. The final extension was 72 °C for 3 min.

2.3. Vector construction

Amplified promoter was digested with *Sma*I (NEB) restriction enzyme and ligated into pEnOPTOEINTLUC gateway entry clone (EC) vector by T4DNA ligase (NEB). After electroporation into *E. coli*, strain DH10B, the correct ligated clones were confirmed by amplification of 1.3 Kb regions by colony PCR, restriction digestion by *Eco*RV (NEB), and sequencing (Figure 1A). This EC was combined with pMDC99 destination vector (Mann et al., 2012) through an LR reaction to make *OsPPOLUC* (*LUC*: *Luciferase*) expression vector.

Similarly *OsPPOGUS* (*GUS*: *β-glucuronidase*) expression vector was prepared by digesting the *Luciferase* gene from *Os*PPOLUC entry clone and amplified *GUS* gene (1.8 Kb) was ligated in place of the *Luciferase* gene to create the *Os*P*POGUS* entry clone. Again correct ligated clones were confirmed by colony PCR, *Eco*RV (NEB) digestion, and sequencing (Figure 1B). The *OsPPOGUS* entry clone was combined with pMDC99 destination vector through LR reaction and *OsPPOGUS* expression vector was designed. *OsPPOLUC* and *OsPPOGUS* expression vectors (Figure 2) were finally transformed into *Agrobacterium tumefaciens* GV1301 separately and both expression vectors were confirmed again by PCR and *Eco*RV (NEB) digestion while *OsPPOLUC* was confirmed by *Luciferase* expression as well.

2.4. Transformation

GV3101 strain harboring *OsPPOLUC* and *OsPPOGUS* vectors was grown separately in Luria Broth (LB) liquid media (Kanamycin 50 mg/L, Thermo Fisher Scientific). *A. tumefaciens* culture was harvested and re-suspended in transformation solution (5% Sucrose + 0.03% SilwetL-77). Floral buds of *Arabidopsis* were sprayed with *A. tumefaciens* strain GV 3101 harboring *OsPPOLUC* and *OsPPOGUS* transformation solutions separately.

2.5. Procurement of T1 and T2 generations

Mature seeds (T0) were harvested from plants and tested on hygromycin (40 mg/L, Thermo Fisher Scientific) selection. Hygromycin-resistant seedlings (T0) were collected from selection and transferred to hygromycin-free MS media and shifted to soil. T1 seeds were finally used to get T2 lines. For final stress treatment and real-time PCR analysis, hygromycin-resistant T2 lines (homozygous) were selected.

2.6. Wound induction

Ten-day-old T2 plants were subjected to mechanical wounding by forceps. Half of the leaves on each plant were injured while the other leaves remained unwounded. Wounded plants were kept on MS media for 12, 24, 36, and 48 h, respectively, along with controlled unwounded transgenic plants. After wounding the plants were used for *LUC*/*GUS* expression and total RNA isolation.

2.7. ABA and MeJ treatments

Solutions of ABA (Sigma-Aldrich) and MeJ (Sigma-Aldrich) were prepared from 1000-fold concentrated stock in combination with 0.01% SilvetL-77 (Merck) to facilitate infusion. Control solution (distilled water in combination with 0.01% SilvetL-77) was also used. Eleven transgenic T2 lines, almost 25 to 30 seeds per plate, were grown on MS media for 10 days. Then the plants were sprayed with 50 µM, 150 µM, 250 µM, 350 µM, and 450 µM ABA and MeJ solutions, respectively, along with control transgenic plants. Sprayed plants were kept for 24 h after spraying and then used for *LUC*/*GUS* expression and RNA isolation.

2.8. GUS and LUC assay

For *GUS* staining experiments 5 µL of 0.1 M X-Gluc solution was added to 1 mL of *GUS* buffer. Then treatments plants were immersed in *GUS* solution and incubated at 37 °C overnight. After that the plants were destained with ethanol to remove chlorophyll contents and relative *GUS* expression was checked for each stress treatment from intensity of *GUS* staining. For the *LUC* assay treated

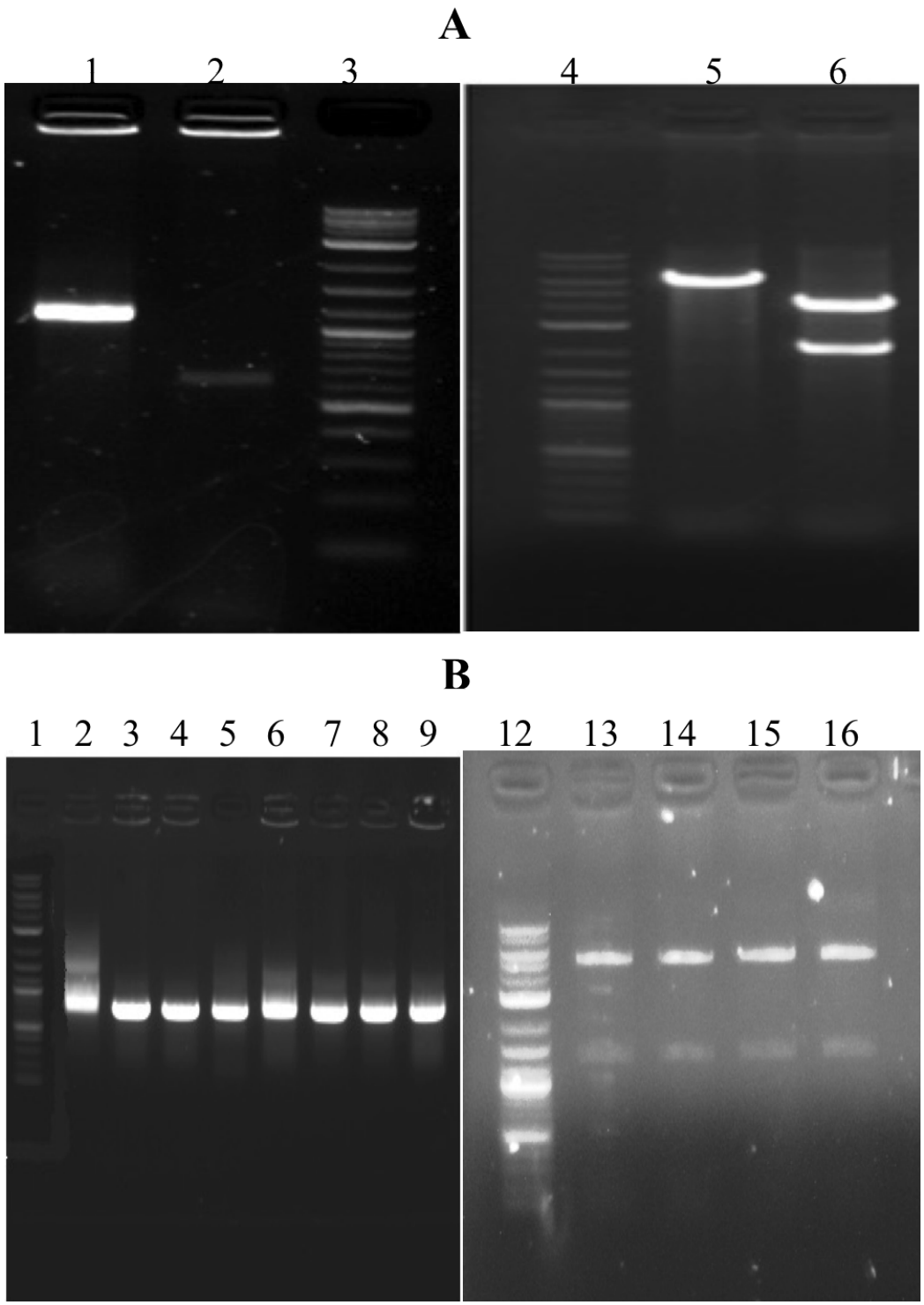


Figure 1. PCR confirmation of correct ligation of *OsPPO* in *OsPPOLUC* entry clone. (A): Amplification of *Os*PPO promoter in lane 1 by vector specific primers and PCR positive clones digested with *Eco*RV showed correct restriction pattern of right ligation in lane 6. Lanes 3 and 4: 2 log DNA ladder (NEB). (B): Confirmation of recombined *OsPPO*GUS entry clones by PCR in lanes 2–9 and confirmation of *OsPPO* ligation in *OsPPO*GUS entry clone by restriction digestion with *Eco*RV in lanes 13–16. Lanes 1 and 12: 2 log DNA ladder (NEB).

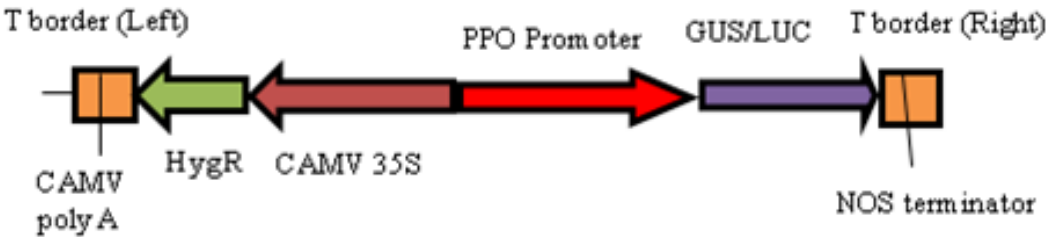


Figure 2. A schematic representation of *OsPPOLUC*/*OsPPOGUS* construct. The *OsPPO* promoter was cloned in gateway entry clone and finally combined in pMDC99 backbone destination vector.

plants were sprayed with 2 mM Luciferine in petri plates and kept for 2–3 min. Then the plates were placed in a charged coupled device camera (CCD) for checking *LUC* expression.

2.9. RNA isolation and cDNA synthesis

The ABA, MeJ, and wound stress treated plants along with the control were subjected to total RNA isolation as described by Oñate-Sánchez and Vicente-Carbajosa (2008). Ground plant tissue was treated with cell lysis solution, homogenized quickly by vortexing, and left at room temperature for 5 min. Then after addition of protein DNA precipitation solution, cell lysate was mixed up gently, incubated at 4 °C for 10 min, and spun down. Isopropanol was added to supernatant, mixed by inverting, and centrifuged for 5 min at 4 °C. By carefully removing supernatant, the pellet was washed with 70% ethanol, dried, and resuspended in autoclaved distilled water. DNase (Promega: M6101) was added and incubated at 37 °C for 30 min. Ammonium acetate (7.5 M) was added along with ethanol, mixed well, and spun down for 20 min at 4 °C. The pellet was washed with 70% ethanol, dried, and finally resuspended. Total RNA quality was checked on 1.5% agarose gel. Total RNA was quantified by NanoDrop (ND 1000). Using 1 µg of total RNA a reverse transcriptase reaction was carried out to synthesize cDNA using Goscript RT enzyme (Promega: A5000).

2.10. RT-PCR

For RT-PCR (Roche Light Cycler-480) operation the EvaGreen protocol was followed using EvaGreen qPCR master mix, template DNA, primers, and RNase-free water. Five times diluted complimentary DNA (cDNA) was used for real-time PCR screening. All primers were started with denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s in standard protocol duration, 3 s in fast protocol duration, and annealing at 60 °C for 60 s in standard protocol duration and 30 s in fast protocol duration. Melting curve analysis was carried out according to the instrument's guidelines. *LUC* and *GUS* genes were used as target and the 18S gene was used as internal control for wounding, MeJ, and ABA stresses. Amplification of the target and internal control genes was confirmed on 2% gel (Supplementary Figure). The fold change values were calculated by threshold crossing point (CP) values for stress-treated samples along with controls (Supplementary Tables 1–4).

2.11. Statistical analysis

All treatments' wounding, ABA, and MeJ applications were performed with three replicates. One way ANOVA ($P < 0.05$) was applied for statistical analysis of the data. The relative expression level was calculated according to the formula $2^{-\Delta\Delta Ct}$.

3. Results and discussion

3.1. Induction of OsPPO promoter by wounding

To analyze *OsPPO* promoter induction in response to wounding, reporter genes' transcript levels were quantified after 12, 24, 36, and 48 h. Transcript level revealed that wounding induced *OsPPO* promoter activity, which ultimately directed the reporter genes mRNA levels (up to 3–11-fold) as compared to the control (unwounded). Among the different time interval treatments, 36 h resulted in maximum transcript levels of *LUC* reporter gene (Figure 3A) and *LUC* expression (Figure 3B), which started declining after 48 h. These results were also supported by higher mRNA levels of the *GUS* reporter gene (Figure 4A) and *GUS* expression assay (Figure 4B) under *OsPPO* promoter activity after 36 h of wounding. Wounding was induced on half of the leaves of a plant and unwounded leaves of the same plant also showed induction of *OsPPOGUS* and *OsPPOLUC* construct activities (Figures 3B and 4B), indicating a possible role of *PPO* in insect resistance (Haruta et al., 2001). *OsPPO* promoter is not an early responsive promoter as it induced after 36 h, which is very close to aspen (*Poplus tremuloide*) *PPO* gene wound response (Haruta et al., 2001). Relative expression of pineapple (*Ananas comsus*) *PPO* gene promoter (Zhou et al., 2003) after 48 h is also a quite late wound response. Similarly, hybrid poplar (*Poplus trichocarpa* × *Poplus deltoides*) *PPO* gene (Constabel et al., 2000) and artichoke (*Cynara cardunculus*) *PPO* gene were also induced by wounding (Quarta et al., 2013).

Wound induction of *OsPPO* promoter suggests a possible role of the *OsPPO* gene in plant defense. This notion was also supported by experimental data in transgenic tomato overexpressing *PPO* gene, which increased resistance to

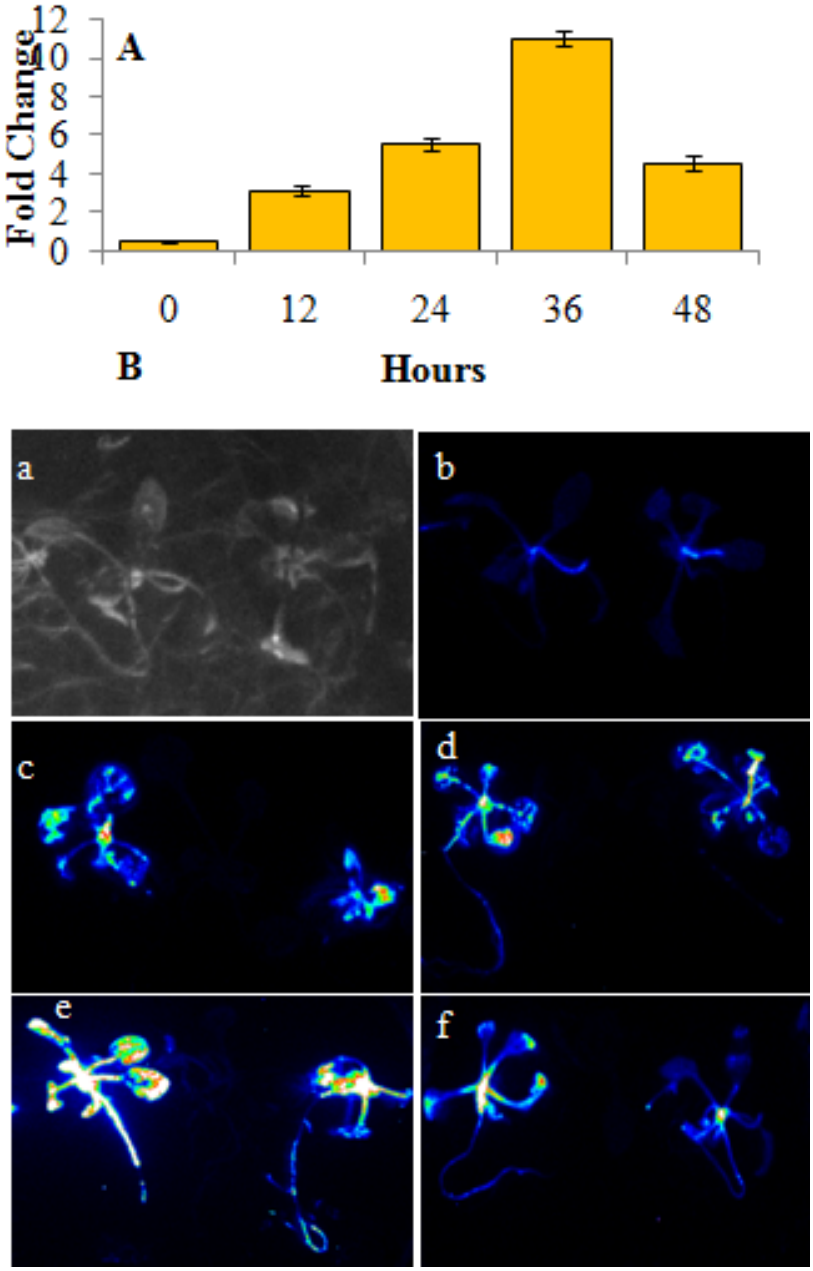


Figure 3. (A) Quantification of *OsPPOLUC* mRNA level induced by wounding. Ten-day-old T2 transgenic *Arabidopsis* lines were subjected to mechanical injury growing on MS media and RT-PCR was performed after intervals of 12 h to detect wound induced response of *OsPPO* promoter. (B) Wound induced *LUC* expression of *OsPPO* carried out on 10-day-old T2 transgenic *Arabidopsis* lines. Control plants (transgenic unwounded) (b). Mechanically injured plants were incubated on MS media and tested after 12 h (c), 24 h (d), 36 h (e), and 48 h (f) intervals. Wounds were produced on half the leaves on each plant (Key. a: bright field, b: control). The data shown are mean ± SE of three independent experiments ($P < 0.05$, $n = 3$).

Pseudomonas syringae (Li and Steffens, 2002). Likewise, *PPO* in dandelion was found to be strongly induced by *Botrytis cinerea* as well as in transgenic *Arabidopsis* with *PPO* overexpression also showing antibacterial activity against *P. syringae* (Richter et al., 2012). Overexpression of the *PPO* gene in transgenic Populus enhanced resistance

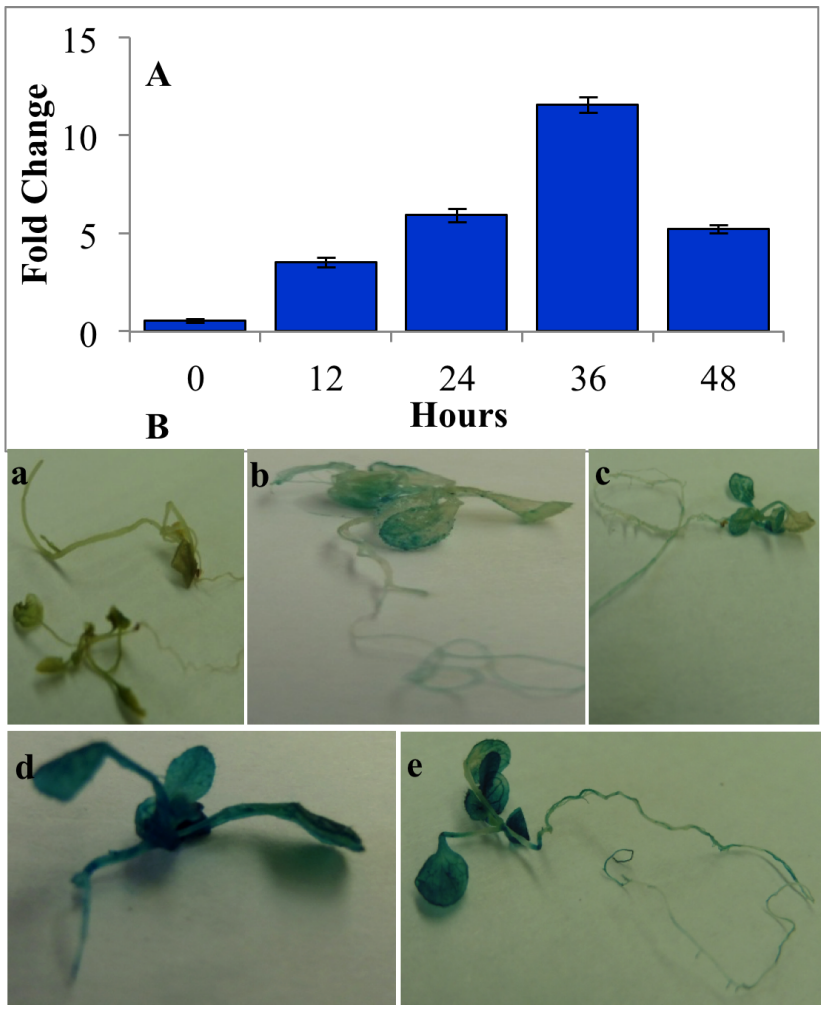


Figure 4. (A) Quantification of *OsPPOGUS* mRNA level induced by wounding. Ten-day-old T2 transgenic *Arabidopsis* lines were subjected to mechanical injury growing on MS media and RT-PCR was performed after intervals of 12 h to detect wound induced response of *OsPPO* promoter. (B) Wound induced *GUS* expression of *OsPPO* carried out 10-day-old T2 transgenic *Arabidopsis* lines. Control plants (transgenic unwounded) (a). Mechanically injured plants were incubated on MS media and tested after 12 h (b), 24 h (c), 36 h (d), and 48 h (e) intervals. Wounds were produced on half the leaves on each plant (Key. a: control). The data shown are Ωean ± SE of three independent experiments ($P < 0.05$, $n = 3$).

against the forest tent caterpillar (Wang and Constabel, 2004).

On the other hand, plants with *PPO* downregulation showed more susceptibility to pathogens (Richter et al., 2012; Thipyapong et al., 2004). Similarly altered *PPO* gene expression in transgenic plants showed anti-herbivory action against various insects such as tree feeding caterpillars (Constabel et al., 2000; Wang and Constabel, 2004; Barbehenn et al., 2007), lepidopteron insects (Thipyapong et al., 2004), cut worms (Mahanil et al., 2008), and bollworms and armyworms (Bhonwong et al., 2009). Plant *PPO* is considered a part of the defense mechanism due to its induction in wound and pathogen attacks (Tran et al., 2012).

3.2. Induction of OsPPO transcript in response to ABA and MeJ treatment

To validate the role of *OsPPO* promoter in response to ABA and MeJ, accumulation of *OsPPOGUS* and *OsPPOLUC* transcript levels was analyzed. RT-PCR showed that reporter gene mRNA upregulated ~2.5-fold induction under *OsPPO* promoter regulation in response to ABA application in both combinations with *LUC* (Figure 5A)

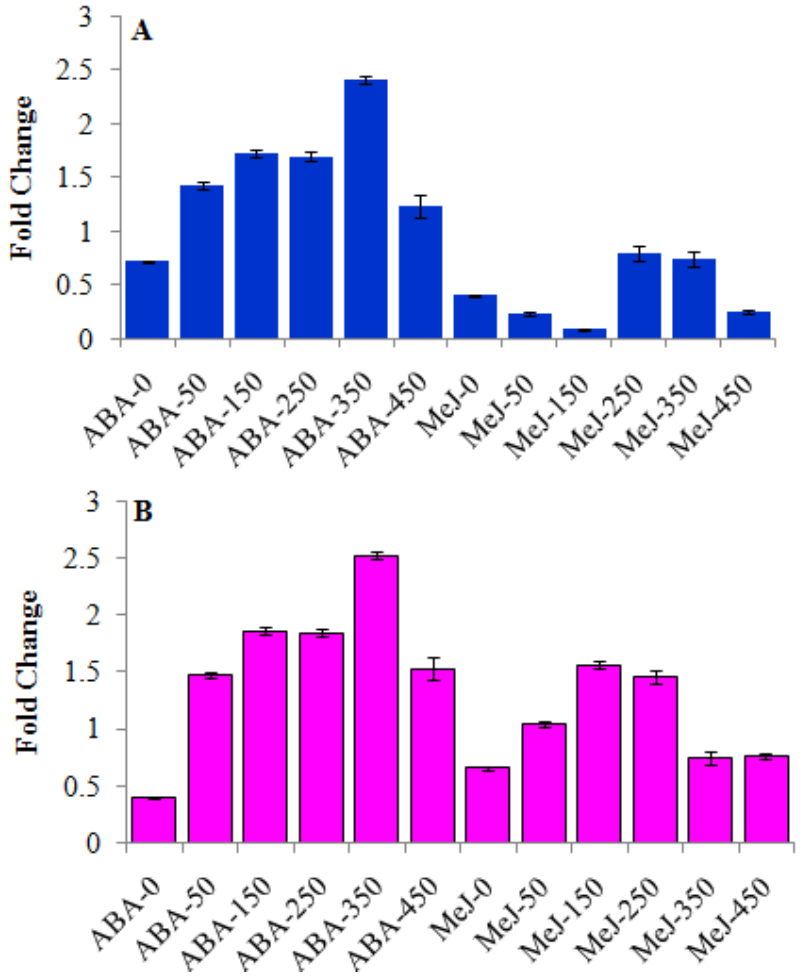


Figure 5. (A) RT-PCR analysis of *OsPPOLUC* activity by ABA and MeJ applications. Quantitative RT- PCR was carried out to detect *OsPPOLUC* transcripts level in 10-day-old T2 transgenic *Arabidopsis* lines by sprays of ABA and MEJ solutions (50 µM, 150 µM, 250 µM, 350 µM, and 450 µM) growing on MS media. (B) RT-PCR analysis of *OsPPOGUS* activity by ABA and MeJ applications. Quantitative real-time PCR was carried out to detect *OsPPOGUS* transcript level in 10-day-old T2 transgenic *Arabidopsis* lines by spray of ABA and MeJ solutions (50 µM, 150 µM, 250 µM, 350 µM, and 450 µM) growing on MS media. The data shown are mean ± SE of three independent experiments ($P < 0.05$, $n = 3$).

and *GUS* (Figure 5B) but not by MeJ. *LUC* (Figure 6A) and *GUS* (Figure 6B) activities also support this finding of *OsPPO* promoter induction with higher activity by ABA at 350 µM, while *LUC* (Figure 7A) and *GUS* (Figure 7B) activities remained unresponsive to MeJ treatments. Similar results were found in *Glycine max PPO* gene promoter, which showed 2.5-fold upregulation by ABA but not by MeJ (Chai et al., 2013). Moreover, banana *PPO* gene also remained unresponsive to MeJ (Gooding et al., 2001). Recently, ABA signal transduction was found to be involved in regulation of stomatal opening and closure to control pathogen and drought stress (Lim et al., 2015), which may indicate a possible involvement of *OsPPO* in plant defense through ABA signaling. Strawberry (*Fragaria* × *ananassa*) *FaPPO* was found to be regulated by ABA as well as fungal pathogen (Jia et al., 2015).

3.3. Signal scan (PLACE) shows cis-regulatory elements for ABA and wounding

The PLACE signal scan revealed the presence of sequences homologous to stress signaling or ABA responsive elements and wound responsive *cis*-elements (W-boxes) related wound induction (Figure 8). Such stress signals reside within *OsPPO* promoter including DPBF (2), MYB (2), MYC (4), and WRKY (8) homologous to stress

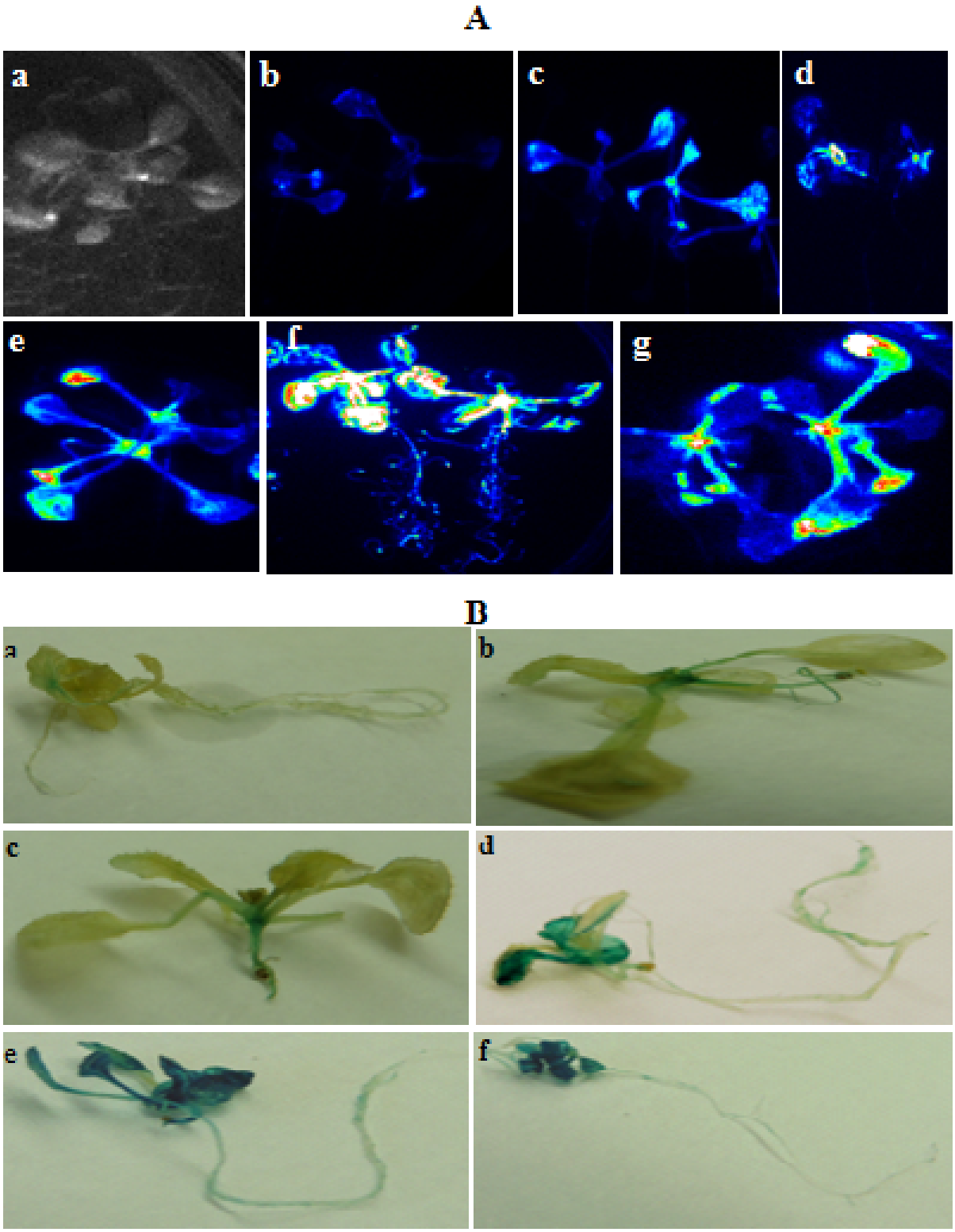


Figure 6. (A) *Luciferase* expression by *OsPPO* promoter fused with *LUC* reporter gene by ABA application. Transgenic *Arabidopsis* T2 lines were sprayed with different concentrations 50 µM (c), 150 µM (d), 250 µM (e), 350 µM (f), and 450 µM (g) of ABA on MS media for 24 h (a: Bright field, b: control, ABA: Abscisic acid). (B) *GUS* expression by *Os*PPO promoter fused with *GUS* reporter gene by ABA application. Transgenic *Arabidopsis* T2 lines were sprayed with different concentrations 50 µM (b), 150 µM (c), 250 µM (d), 350 µM (e) and 450 µM (f) of ABA on MS for 24 h. Plants were immersed in *GUS* staining solution overnight (Key. a: control plants, ABA: abscisic acid).

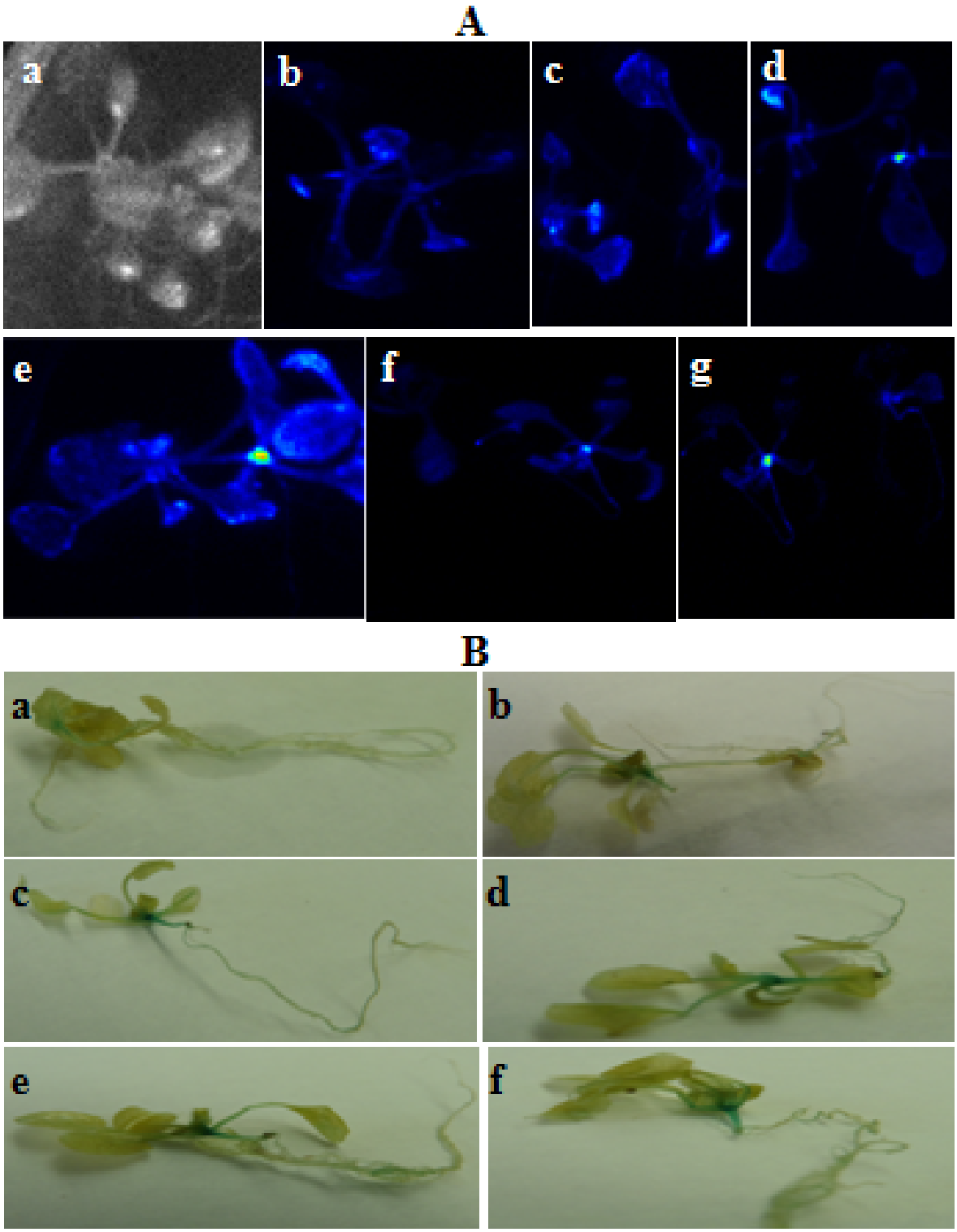


Figure 7. (A) *Luciferase* expression by *OsPPO* promoter fused with *LUC* reporter gene in MeJ application. Transgenic *Arabidopsis* T2 lines were sprayed with different concentrations of 50 µM (c), 150 µM (d), 250 µM (e), 350 µM (f), and 450 µM (g) of MJA and kept on MS media for 24 h (a: bright field, b: control, MeJ: methyl jasmonate). (B) *GUS* expression by *OsPPO* promoter fused with *GUS* reporter gene by MeJ application. Transgenic *Arabidopsis* T2 lines were sprayed with different concentrations 50 µM (b), 150 µM (c), 250 µM (d), 350 µM (e), and 450 µM (f) of MJA on MS for 24 h. Plants were immersed in *GUS* staining solution overnight (Key. a: control, MeJ: methyl jasmonate).

Figure 8. 1020 bp region of *OsPPO* promoter. MYC (CANNTG), WRKY (TGAC), DPBF (ACACNNG), and MYB (YAACKG) elements are capitalized while W-boxes (TGACY) are italicized and elements residing on-strand are underlined.

inducible or ABA responsive elements. Occurrence of ABA and W-boxes in *OsPPO* promoter can mediate such induction activities (Kim et al., 1997; Abe et al., 2003; Xie et al., 2005).

These ABA and wound responsive motifs further affirm *OsPPO* induction by ABA and wounding (Oh et al., 2005; Chai et al., 2013). Presence of W-boxes responsible for wound induced expression noted in the 1020 bp region of *OsPPO* promoter is consistent with ERF3 gene rapid activation by wounding stress (Nishiuchi et al., 2004). *OsPPO* promoter sequence data are also comparable with wound responsive elements as in artichoke *PPO* promoter (Quarta et al., 2013).

ABA or stress responsive regulatory sites such as DPBF, MYB, MYC, and WRKY suggest *OsPPO* promoter can regulate such induction activities (Kim et al., 1997; Abe et al., 2003; Xie et al., 2005). Chai et al. (2013) reported the presence of MYB and MYC *cis*-regulatory elements in *Glycin max PPO* promoter, which was found to be ABA and *Phytophtora* inducible. Structural analysis of *OsPPO* promoter confirms the key motifs (DPB, MYB, MYC, WRKY, and W-boxes) for ABA and wound signaling.

In conclusion, during the expression profiling of reporter genes derived by *OsPPO* promoter, it was observed that this promoter performs a wide range of functions in response to different hormonal applications or stresses. *OsPPO* induction by wounding and ABA indirectly indicates its role in environmental biotic and abiotic stresses such as insect, pathogen, and drought resistance. The *OsPPO* promoter derived expression of *LUC* and *GUS* genes in wounding is consistent with PPO inductions by insect and herbivores. These results are helpful in potential understanding of plant responses to such stressors. The presence of stress responsive *cis*-regulatory elements residing within the 1020 bp region of *OsPPO* promoter also reveals its potential in different environmental stresses. These findings may also help in the study of regulatory motifs responsible for *OsPPO* gene expression and its role in the plant defense mechanism against insects or pathogens.

Acknowledgment
We acknowledge the financial support to Higher Education Commission, Pakistan, for funding International Research Initiative Program to conduct research work.

References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003). Arabidopsis *AtMYC*2 (*bHLH*) and *AtMYB*2 (MYB) function as transcriptional activators in abscisic acid signaling. The Plant Cell 15: 63-78.

Allen G, Flores-Vergara M, Krasynanski S, Kumar S, Thompson W (2006). A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. Nat Protoc 1: 2320-2325.

Aniszewski T, Lieberei R, Gulewicz K (2008). Research on catecholases, laccases and cresolases in plants. Recent progress and future needs. Acta Biol Cracov Bot 50: 7-18.

Barbehenn RV, Jones CP, Yip L, Tran L, Constabel CP (2007). Limited impact of elevated levels of polyphenol oxidase on tree-feeding caterpillars: assessing individual plant defenses with transgenic poplar. Oecologia 154: 129-140.

Bhonwong A, Stout MJ, Attajarusit J, Tantasawat P (2009). Defensive role of tomato polyphenol oxidases against cotton bollworm (*Helicoverpa armigera*) and beet armyworm (*Spodoptera exigua*). J Chem Ecol 35: 28-38.

Chai C, Lin Y, Shen D, Wu Y, Li H, Dou D (2013). Identification and functional characterization of the Soybean *GmaPPO12* promoter conferring *Phytophthora sojae* induced expression. PloS one 8: e67670.

Constabel CP, Yip L, Patton JJ, Christopher ME (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiol 124: 285-296.

Fan J, Hill L, Crooks C, Doerner P, Lamb C (2009). Abscisic acid has a key role in modulating diverse plant-pathogen interactions. Plant Physiol 150: 1750-1761.

Friedman M (1997). Chemistry, biochemistry, and dietary role of potato polyphenols. A review. J Agr Food Chem 45: 1523-1540.

Gooding PS, Bird C, Robinson SP (2001). Molecular cloning and characterisation of banana fruit polyphenol oxidase. Planta 213: 748-757.

Haruta M, Pedersen JA, Constabel CP (2001). Polyphenol oxidase and herbivore defense in trembling aspen *(Populus tremuloides*): cDNA cloning, expression, and potential substrates. Physiol Plantarum 112: 552-558.

Jia H, Zhao P, Wang B, Tariq P, Zhao F, Zhao M, Wang Q, Yang T, Fang J (2015). Overexpression of polyphenol oxidase gene in strawberry fruit delays the fungus infection process. Plant Mol Biol Rep 1-15.

Kim SY, Chung HJ, Thomas TL (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the *Dc3* promoter using a modified yeast one-hybrid system. The Plant Journal 11: 1237-1251.

Li L, Steffens JC (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta 215: 239-247.

Lim CW, Baek W, Jung J, Kim JH, Lee SC (2015). Function of ABA in stomatal defense against biotic and drought stresses. Int J Mol Sci 16: 15251-15270.

Mahanil S, Attajarusit J, Stout MJ, Thipyapong P (2008). Overexpression of tomato polyphenol oxidase increases resistance to common cutworm. Plant Sci 174: 456-466.

Mann DG, LaFayette PR, Abercrombie LL, King ZR, Mazarei M, Halter MC, Poovaiah CR, Baxter H, Shen H, Dixon RA (2012). Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. Plant Biotechnol J 10: 226-236.

Mayer AM, Harel E (1979). Polyphenol oxidases in plants. Phytochemistry 18: 193-215.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15: 473-497.

Nishiuchi T, Shinshi H, Suzuki K (2004). Rapid and transient activation of transcription of the *ERF*3 gene by wounding in tobacco leaves: possible involvement of NtWRKYs and autorepression. J Biol Chem 279: 55355-55361.

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK (2005). Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol 138: 341-351.

Oñate-Sánchez L, Vicente-Carbajosa J (2008). DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. BMC Research Notes 1: 1.

Pieterse CM, Dicke M (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci 12: 564-569.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009). Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5: 308-316.

Quarta A, Mita G, Durante M, Arlorio M, De Paolis A (2013). Isolation of a polyphenol oxidase (PPO) cDNA from artichoke and expression analysis in wounded artichoke heads. Plant Physiol Bioch 68: 52-60.

Richter C, Dirks ME, Gronover CS, Prüfer D, Moerschbacher BM (2012). Silencing and heterologous expression of *ppo*-2 indicate a specific function of a single polyphenol oxidase isoform in resistance of dandelion (*Taraxacum officinale*) against *Pseudomonas syringae* pv. *tomato*. Mol Plant Microbe In 25: 200-210.

Ruuhola T, Yang S, Ossipov V, Haukioja E (2008). Foliar oxidases as mediators of the rapidly induced resistance of mountain birch against *Epirrita autumnata*. Oecologia 154: 725-730.

Spoel SH, Johnson JS, Dong X (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. PNAS 104: 18842-18847.

Thipyapong P, Hunt MD, Steffens JC (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. Planta 220: 105-117.

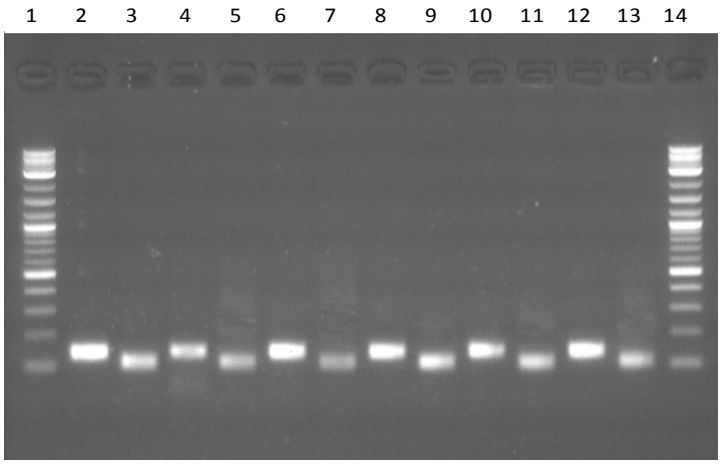
Thipyapong P, Joel DM, Steffens JC (1997). Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development. Plant Physiol 113: 707-718.

Tran LT, Taylor JS, Constabel CP (2012). The polyphenol oxidase gene family in land plants: Lineage-specific duplication and expansion. BMC Genomics 13: 395.

Wang J, Constabel CP (2004). Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). Planta 220: 87-96.

Xie Z, Zhang ZL, Zou X, Huang J, Ruas P, Thompson D, Shen QJ (2005). Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol 137: 176-189.

Zhou Y, O'Hare TJ, Jobin-Decor M, Underhill SJ, Wills RB, Graham MW (2003). Transcriptional regulation of a pineapple polyphenol oxidase gene and its relationship to blackheart. Plant Biotechnol J 1: 463-478.



Supplementary Figure. RT-PCR amplification of *GUS* and 18 S internal control gene. Lanes 1 and 14: 2 log DNA ladder (NEB). All even number wells are indicative of *GUS* gene and all odd number amplicons showing 18 S internal control genes.

Supplementary Table 1. Threshold crossing point (CP) values of *LUC* target gene fused to *OsPPO* promoter in T2 transgenic *Arabidopsis* lines after wounding.

No.	Wounding	CP values of target gene	CP values of internal control gene
1 2	Control 0 h	26.93, 27.08 27.95, 27.85	10.12, 10.30 10.17, 10.02
3	12 h	26.15, 26.50	10.97, 11.38
4	24 h	26.40, 26.15	11.98, 11.91
5	36 h	25.11, 25.01	11.67, 11.78
6	48 h	26.41, 26.10	11.88, 11.42

Supplementary Table 2. Threshold crossing point (CP) values of *GUS* target gene fused to *OsPPO* promoter in T2 transgenic *Arabidopsis* lines after wounding.

No.	Wounding	CP values of target gene	CP values of internal control gene
1 2	Control 0 h	21.29, 21.34 21.69, 21.22	9.84, 9.72 8.91, 8.21
3	12 h	19.92, 19.93	10.11. 10.01
4	24 h	19.18, 19.15	10.11, 10.02
5	36 h	18.22, 18.09	10.07, 10.02
6	48 h	19.37, 19.49	10.18, 10.12

Supplementary Table 3. Threshold crossing point (CP) values of *LUC* target (reporter) gene fused to *OsPPO* promoter in T2 transgenic *Arabidopsis* lines after application of different concentrations of ABA and MeJ.

No.	Hormone	CP values of target gene	CP values of internal control gene
1	Control	27.23, 27.11	9.88, 9.56
2	ABA 0 µM	27.76, 27.50	10.81, 10.58
3	ABA 50 µM	27.92, 27.89	10.01, 9.94
4	ABA 150 µM	27.05, 27.15	10.48, 10.39
5	ABA 250 µM	27.09, 27.11	10.55, 10.28
6	ABA 350 µM	27.13, 27.10	10.77, 11.10
7	ABA 450 µM	28.13, 28.03	10.89, 10.97
8	MeJ 0 µM	28.92, 2848	10.14, 10.09
9	MeJ 50 µM	28.22, 28.10	10.64, 10.79
10	MeJ 150 µM	26.84, 26.71	9.99, 9.82
11	MeJ 250 µM	27.49, 27.63	10.59, 10.72
12	MeJ 350 µM	28.22, 28.58	9.45, 9.82
13	MeJ 450 µM	28.13, 28.47	10.16, 10.50

Supplementary Table 4. Threshold crossing point (CP) values of *GUS* target gene fused to *OsPPO* promoter in T2 transgenic *Arabidopsis* lines after application of different concentrations of ABA and MeJ.

No.	Hormone	CP values of target gene	CP values of internal control gene
1 2	Control ABA 0 µM	20.69, 20.82 21.75, 21.88	11.50, 11.78 11.30, 11.44
3	ABA 50 µM	20.60, 20.82	12.28, 12.19
4	ABA 150 µM	20.77, 20.91	12.74, 12.81
5	ABA 250 µM	21.10, 21.15	12.89, 13.01
6	ABA 350 µM	20.92, 20.89	13.28, 12.92
7 8	ABA 450 µM MeJ 0 µM	20.50, 20.81 21.87, 21.98	12.25, 12.30 12.13, 12.26
9	MeJ 50 µM	21.10, 21.07	12.20, 12.16
10	MeJ 150 µM	20.93, 20.81	12.62, 12.50
11	MeJ 250 µM	20.86, 20.65	12.32, 12.40
12	MeJ 350 µM	20.91, 21.01	11.61, 11.17
13	MeJ 450 µM	20.94, 20.99	11.12, 11.18