Expression analysis of the polyphenol oxidase gene in response to signaling molecules, herbivory and wounding in antisense transgenic tobacco plants

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Abstract

Key message We provide evidence that the expression of the *PPO* gene was significantly reduced in response to wounding, MeJ and herbivory in transgenic tobacco under wound-inducible *OsRGLP2* promoter in an anti-sense orientation.

Abstract Polyphenol oxidase (PPO) genes play an important role in plant defense mechanisms against biotic and abiotic stresses. In the present study, a 655 bp core sequence of the potato *PPO* gene was placed under the control of wound-inducible *OsRGLP2* promoter in an anti-sense direction to evaluate its potential effects during biotic (*Trialeurodes vaporariorum*'s infestation) and various abiotic (wounding, MeJ, ABA) stresses. Transcriptional profiling of *PPO* gene by real-time PCR (qRT-PCR) in transgenic tobacco revealed a significant suppression (3.5-fold) of *PPO* in response to wounding than control plants after 24 h. In response to MeJ at different concentrations (100 μ M and 200 μ M), the *PPO* expression was greatly down-regulated by 4.7-fold after 6 h at 100 μ M MeJ, and a non-significant expression was observed with ABA treatment. Moreover, significant levels of *PPO* reduction (sixfolds) was found in whitefly feeding assay indicating that expression of potato *PPO* in an anti-sense orientation had down-regulated the *PPO* activity. This down-regulation of *PPO* by wounding, MeJ and whitefly infestation clearly links the specific expression of *PPO* in biotic and abiotic stresses. In the future, *PPO* gene suppression in transgenic plants using anti-sense potato *PPO* gene construct can be used to inhibit enzymatic browning in fruits and vegetables, e.g., potato.

Keywords Polyphenol oxidase · Wounding · Herbivory · Elicitors · Silencing

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Introduction

Plants are continuously exposed to various biotic and abiotic stresses. These stresses adversely affect plant growth and yield. Plants have evolved many different mechanisms to cope with stresses (Li and Steffens 2002). Among the biotic stresses, bacterial pathogens and insect pests are the most destructive for many crops such as tomato, sugar beet, legumes, cotton, maize, soybean, tobacco, pepper, alfalfa, potato, onion, sunflower, and citrus as well as many weeds (Bhonwong et al. 2009).

Polyphenol oxidases (PPO) are ubiquitous in plants and mainly related to defense (Constabel and Barbehenn 2008). They are present in all land plants except *Arabidopsis* (Tran et al. 2012). PPOs are plastid-localized nuclear-encoded enzymes that utilize molecular oxygen to oxidize phenols to quinones resulting in tissue browning, which is also observed during senescence, responses to pathogens and wounding (Mayer 2006). *PPO*-generated quinones alter

dietary proteins in plants making them non-nutritive (indigestible) for insects (Constabel and Ryan 1998). Moreover, the induced *PPO* activity in many plants was found useful to resist bacterial, fungal and insect pathogens (Campos et al. 2004). Transgenic approaches provide an opportunity to study the involvement of *PPO* in plant defense, as its role has been validated in many plants using *PPO* over-expression or down-regulation tools. Down-regulation of *StuPPO* in transgenic tomato increased the susceptibility of plants against insects, which gained weight by feeding and more leaf area consumption (Thipyapong et al. 2004a; Mahanil et al. 2008; Bhonwong et al. 2009). However, over-expression of hybrid poplar *PtdPPO* hindered forest tent caterpillar from gaining weight and consuming more leaf area than control and under-expressed hybrid poplar plants (Wang and Constabel 2004a).

PPO was also found to be induced by wounding, linking its involvement in plant defense mechanisms (Aziz et al. 2017). Plants synthesize many hormones as signaling molecules which trigger defense against various stresses. Plant defense against chewing insects is modulated by jasmonic acid (MeJ) and ethylene (Stotz et al. 2000). *PPO* induction in aspen and hybrid poplar by injuries and MeJ showed a signal-induced *PPO* defense in plants (Haruta et al. 2001; Wang and Constabel 2004b). Recently, strawberry *FaPPO* over-expression conferring fungal resistance was found to be induced by MeJ and other stresses (Jia et al. 2016). In view of the role of *PPO* in plant defense, a study was designed to test the effects of herbivory, mechanical wounding and hormone treatments (MeJ, ABA) on transgenic tobacco plants harboring an anti-sense *PPO* gene under the regulation of a wound-inducible promoter.

Materials and methods

Plant material

Tobacco seeds (*Nicotiana tabacum* cv. Xanthi) obtained from National Agriculture Research Centre (NARC), Pakistan, grown in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) were used in the present study. Tobacco plants were grown in a growth chamber under control conditions of 16:8 dark and light cycle at 27 °C.

Antisense polyphenol oxidase gene vector construction

PCR-amplified potato *PPO* copper-binding domain region (accession No. A27686.1) was directly ligated into the T/A cloning vector pTZ57R/T (Fermentas). The cloned *PPO* gene from T/A cloning system and the expression backbone vector p1391Z_*OsRGLP2* (Mahmood et al. 2013) were restricted with *EcoRI* and *AvrII* restriction enzymes. The recombinant plasmid (antisense *PPO* gene inserted in p1391Z_*OsRGLP2*) was prepared by ligating the digested and eluted *PPO* gene-specific insert downstream to wound-inducible *OsRGLP2* promoter (already available in p1391Z) in antisense orientation and was named RGLP2P-AsPPO. The RGLP2P-AsPPO expression cassette (Fig. 1a) was transformed into *E. coli* (DH5α) by electroporation and the isolated plasmid was confirmed by PCR and *EcoRI*/*AvrII* double digestion. Further, the verified RGLP2P-AsPPO plasmid was electroporated into the *Agrobacterium tumefaciens* strain EHA101. The plasmid of antibiotic (50 mg/L kanamycin)-resistant clones was again confirmed via PCR using gene-specific primers and restriction digestion.

Fig. 1 **a** A schematic representation of the RGLP2P-AsPPO construct. p1391Z_*OsRGLP2* vector with antisense-targeted region of potato *PPO* gene (under the control of *OsRGLP2* promoter) **b** qRT-PCR primers representation from endogenous tobacco *PPO* gene. *NtPPO1* qRT-PCR primers targeting the proximal coding region of

the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene

Tobacco transformation

Agrobacterium tumefaciens-mediated transformation methodology was applied to achieve genetic transformation (Horsch et al. 1989). The *A. tumefaciens* strain EHA101 harboring the RGLP2P-AsPPO expression cassette was used. The transformants that survived on selection medium (Hygromycin 50 mg/L) were cultured on simple 1/2 MS medium for root induction. Plant transformation was verified by PCR using *PPO* gene-specific primers and hygromycin primers.

Wound induction

To study the effect of wounding on the induction of *PPO*, the leaves of un-transformed and transgenic plants were mechanically injured (with the help of forceps) and placed for 12 and 24 h on solid MS medium. After wounding treatment, the wounded leaves were collected, frozen in liquid nitrogen and stored at -80°C for RNA isolation.

MeJ and ABA treatments

To examine the expression in response to hormone treatments, transgenic and non transgenic tobacco plants were treated with a solutions of ABA (Sigma-Aldrich) and MeJ (Sigma-Aldrich). Both ABA and MeJ were dissolved in ethanol (96%) and their final solution was prepared in distilled water. Transgenic and non-transgenic tobacco plants were sprayed with 100 μM or 200 μM of ABA and MeJ solutions, respectively, and harvested after 6 and 12 h. Control plants were also sprayed with Millipore water. The treated samples were stored at -80°C till further use.

Biotic stress

Expression of the *PPO* gene was studied in response to insect infestation using adult whiteflies (*Trialeurodes vaporariorum*) on transgenic plants. The whiteflies were collected by aspiration from whitefly populations maintained on tobacco plants growing in controlled conditions ($26 \pm 2^{\circ}\text{C}$ and relative humidity 60–80%). Transgenic as well as control (non-transgenic) tobacco plants growing in pots with up to seven fully expanded leaves stage were exposed to whiteflies. The attack of whiteflies on tobacco plants was by their choice. For RNA isolation, fully expanded leaves from the middle region of the tobacco plants were harvested after 2, 3 and 5 days of infestation and stored at -80°C .

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen samples, harvested from mechanical wounding, whitefly infestation assay,

ABA and MeJ treatments along with controls using Spectrum™ Plant Total RNA isolation kit (Sigma, USA). The first-strand cDNA was obtained using 3 μg of RNA with nonamer and random oligonucleotide primers using the SuperScript II reverse transcriptase (Invitrogen).

qRT-PCR analysis

Real-time PCR (qRT-PCR) was performed in 384-well plates using the ViiATM 7 Real-Time PCR System (Applied Biosystems, USA). The PCR reaction was carried out using Power SYBR® Green PCR master mix (6 μL), 1.2 μL of each forward and reverse primer (1.5 μM), 0.5–1.0 μL of cDNA and 2.6–3.1 μL of nuclease-free water. Three different sets of primers (Fig. 1b) were designed from endogenous tobacco *PPO* cDNA sequences for qRT-PCR analysis and 18S rRNA and actin were selected as reference genes (Table 1). qRT-PCR for all primers was started with denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. Melting curve analysis consisting of 95°C for 15 s, 55°C for 1 min and 95°C for 15 s. After successful amplification of targets, the cycle threshold (C_t) values were exported from the ViiATM 7 software and used as raw data for the analysis of qRT-PCR data. The R software and the HTqPCR (Dvinge and Bertone 2009) and Limma (Ritchie et al. 2015) add-on packages were used for the manipulation and analysis of the C_t values. The fold changes are shown on the log 2 scale.

Table 1 List of primers used in qRT-PCR for expression analysis of endogenous *NtPPO* gene in transgenic tobacco plants harboring anti-sense *PPO*

Primers	Primer sequence
18S	5'-GGTGGAGCGATTGTCTGGT-3' 5'-CAGGCTGAGGTCTCGTTCGT-3'
Actin	5'-CCTGAGGTCTTTTCCAACCA-3' 5'-GGATTCCGGCAGCTTCCATT-3'
<i>NtPPO1</i>	5'-ATGGACAGCGTTCCTATTACA-3' 5'-GGAGTACATTGCTAAATACCAGTTAGC-3'
<i>NtPPO2</i>	5'-AACCCGTTCCGTGTGAAAGTCC-3' 5'-CTTCGATTACGCACCGATGCCA-3'
<i>NtPPO3</i>	5'-TCTCAAAGCTGGACAGAGCC-3' 5'-CCATCTTCGTCAAGGACCCA-3'

18S 18S rRNA, *NtPPO* *Nicotiana tabacum* polyphenol oxidase, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene

Statistical analysis

The data are the mean of three independent replicates. The statistical differences were compared with a *t* test at a significance level of $P < 0.05$ (*) and $P < 0.01$ (**).

Results and discussion

Generation of transgenic plants

Transgenic plants were developed through *Agrobacterium*-mediated transformation method. After co-cultivation, small bud-like calli appeared on cut edges which increased in mass up to 14 days (Fig. 2). Small shootlets started to develop after 28 days, which were cut individually from callus and shifted to simple MS media in jar for rooting. Mature plants were developed in about 45 days, shifted to the soil and maintained in greenhouse under control conditions of 16:8 dark and light cycle at 27 °C.

Confirmation of transgenic plants

To confirm the insertion of transgene in transgenic plants, PCR was carried out with *PPO* gene-specific primers and hygromycin gene-resistant primers. High-quality PCR products of approximately 700 bp and 655 bp were obtained with Hyg and *PPO* primer sets, respectively (Supplementary Figs. 1 and 2).

Expression analysis in wounding

Plants being sessile continuously face environmental stresses including biotic and abiotic stresses. In abiotic stresses, wounding is common by attack of insect herbivory, wind hail or rain (Wang et al. 2014). Different defense-related genes are activated and up-regulated by wounding, pathogen infestation or predator attacks causing conspicuous changes in transcript accumulation and protein synthesis of those defense genes (Gulbitti-Onarici et al. 2009). Among these, *PPO* is considered important in plants defense mechanisms, as it is strongly induced by wounding, insect attacks and

Fig. 2 Regeneration of transgenic tobacco plants from leaf discs growing on selection media. **a** Calli emerging from cut edges of leaf discs. **b** 14 days old callus. **c** Regeneration of shoots from calli. **d** 28

days old shoot growing on selection media. **e** Shootlets growing on simple MS media for rooting. **f** Three-month-old transgenic plants at flowering stage growing in a greenhouse

Fig. 3 Relative expression analysis of *NtPPO* gene activity induced by wounding shown on the log2 scale. **a** After 12 h treatment. **b** After 24 h treatment. WT wild type, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments and statistically significant difference is indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

Table 2 Difference in the expression level of the *PPO* gene in response to wounding stress in transgenic plants and WT

Stress treatment	Target genes used	Plants tested	Expression level (mean ± SE) (12 h)	Expression level (mean ± SE) (24 h)
Wounding	<i>NtPPO1</i>	WT negative	0.00 ± 0.00	0.00 ± 0.00
		WT wounded	1.41 ± 0.04	3.52 ± 0.04
		Transgenic wounded	-0.90 ± 0.02*	-2.81 ± 0.02*
	<i>NtPPO2</i>	WT negative	0.00 ± 0.00	0.00 ± 0.00
		WT wounded	1.19 ± 0.05	3.52 ± 0.05
		Transgenic wounded	-1.25 ± 0.01*	-3.54 ± 0.02**
	<i>NtPPO3</i>	WT negative	0.00 ± 0.00	0.00 ± 0.00
		WT wounded	1.20 ± 0.02	2.91 ± 0.02
		Transgenic wounded	-1.37 ± 0.02**	-2.06 ± 0.01*

WT wild type, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene

Statistically significant difference is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$)

defense-related signals such as ABA, JA, MeJ and ethylene (Mayer 2006; Constabel and Barbehenn 2008). The role of *PPO* has been validated in transgenic plants revealing that *PPO* conferred resistance against bacterial, fungal and chewing insect herbivores (Thipyapong et al. 2007; Bhonwong et al. 2009; Jia et al. 2016).

In the present study, transgenic plants (*PPO* down-regulated) were mechanically wounded by forceps. Expression analysis of anti-sense *PPO* gene was carried out after 12 and 24 h intervals targeting proximal coding, core and terminal coding regions of the *NtPPO* gene (primers *NtPPO1*, *NtPPO2* and *NtPPO3*) for wild and transgenic plants (Fig. 1b; Table 1). RT-PCR analysis showed a significant reduction ($P < 0.05$; $P < 0.01$) in the expression of endogenous tobacco *PPO* gene compared with control tobacco plants after 12 h (Fig. 3a; Table 2). The proximal coding region of *PPO* showed less down-regulation than core and terminal coding regions, while control plants showed an up-regulation of the endogenous *PPO* gene upon mechanical injury. However, after 24 h *NtPPO2* showed greater down-regulation (3.5-fold) than *NtPPO1* and *NtPPO3*. Overall, wounding depicted higher down-regulation of endogenous *PPO* gene over time (Fig. 3b; Table 2).

Induced *PPO* expression is well studied in many environmental abiotic and biotic factors or stresses (Quarta et al. 2013). Other defense-related genes such as peroxidases and systemins also induced *PPO* up-regulation upon wounding indicating involvement of *PPO* in defense (Constabel et al. 1995). *PPO* induction and regulation in hybrid poplar, pineapple and rubber tree upon wounding were also reported earlier (Li et al. 2014), linking *NtPPO* down-regulation in transgenic tobacco in the present study. *PPO* down-regulation can weaken plant defense resulting in acquired leaf lesion necrosis accomplished by *PPO*-mediated cell death in walnut, leading to increased vulnerability against bacterial and insect attacks in tomato (Thipyapong et al. 2004a;

Bhonwong et al. 2009; Araji et al. 2014). *NtPPO* down-regulation reached up to twofold after 24 h, showing a slightly delayed response to injuries as also reported after 36 h in *Populus trichocarpa* (Haruta et al. 2001) and even after 2 days in pineapple (Zhou et al. 2003) and artichoke (Quarta et al. 2013).

Wound-induced *NtPPO* expression points *PPO* role in plant defense and can also be attributed to insect pest resistance due to its induction upon wounding and real herbivory (Haruta et al. 2001). Wound-induced *NtPPO* expression can be correlated to *PPO* over-expression which enhanced disease tolerance against *Pseudomonas syringae* in transgenic tomato (Li and Stiffen 2002) and transgenic *Arabidopsis* (Richter et al. 2012), in populus against insects (Wang and Constabel 2004a; Thipyapong et al. 2007; Bhonwong et al. 2009) and in transgenic strawberry with delayed fungal attack during fruit development (Jia et al. 2016). Soybean *Gm PPO* promoter exhibited an early response to *Phytophthora sojae* (Chai et al. 2013). Tran et al. (2012) reported that *PPO* is a part of defense in plants based on wound and pathogen-induced activities. The wound-induced down-regulation of *NtPPO* in our experiments can be attributed to a possible role in plant defense mechanism.

Expression analysis in response to ABA

To elucidate the activity of the suppressed *PPO* gene in response to exogenous ABA application, transgenic tobacco (anti-sense potato *PPO* gene) along with wild-type plants was treated with 100 or 200 μM of ABA for 6 and 12 h. The qRT-PCR analysis was carried out for wild-type and transgenic tobacco plants. Transcripts accumulation showed a significant down-regulation ($P < 0.05$; $P < 0.01$) of *PPO* gene in transgenic plants treated with different concentrations of ABA at different time intervals compared to WT plants. In the presence of 100 μM ABA, there was a slight up-regulation of 0.5-fold in WT plants, while a slight down-regulation of 0.3-fold was observed in transgenic plants after 6 h (Fig. 4a; Table 3). Twelve hours after using similar ABA concentration, wild-type treated plants showed an accumulation of *PPO* mRNA up to 0.6-fold and reduced expression of *PPO* gene in transgenic treated plants up to 0.5-fold (Fig. 4b; Table 3).

However, at 200 μM ABA after 6 h, the *NtPPO* gene showed transcript elevation level up to 0.8-fold in wild-type and down-regulation of 0.9-fold in transgenic plants (Fig. 5a). Similarly, after 12 h at 200 μM , *PPO* gene regulation remained unchanged after 6 h (Fig. 5b). Overall, all *PPO* gene regions showed similar transcript accumulation in response to ABA. Previously, Song et al. (2011) treated tomato plants with exogenous ABA and observed non-significant *PPO* activity between wild-type and transgenic tomato which supports our results. The antagonistic

Fig. 4 Relative expression analysis of *NtPPO* gene activity in response to 100 μM ABA treatment shown on the log2 scale. **a** After 6 h treatment. **b** After 12 h treatment. WT wild type, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments, and statistically significant difference is indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

interaction between ABA and JA or salicylic acid (SA) showed resistance in *Arabidopsis* against *F. oxysporum*, in agreement with our study (Anderson et al. 2004). Likewise, elevated levels of ABA in tomato plants were negatively correlated with SA-dependent defense pathway (Audenaert et al. 2002).

Furthermore, lack of significant *PPO* induction by exogenous ABA application in wild-type plant also supports the fact that ABA restrained the expression of several defensive genes such as *PPO* (Mohr and Cahill 2007). However, exogenous application of ABA resulted in induction of other defense-related genes such as *POD*, phenylalanine ammonia-lyase (*PAL*) and *PPO* that improved tomato's resistance against *A. solani* (Song et al. 2011), whereas elevated level of ABA in *Arabidopsis* was linked with positive as well as negative role in disease resistance

Table 3 Difference in the expression level of the *PPO* gene under different concentrations of ABA and MeJ in transgenic plants and wild type (WT)

Pytohormones	Target genes used	Plants tested	Expression level at 100 μ M concentration (mean $\pm$ SE) (6 h)	Expression level at 100 μ M concentration (mean $\pm$ SE) (12 h)	Expression level at 200 μ M concentration (mean $\pm$ SE) (6 h)	Expression level at 200 μ M concentration (mean $\pm$ SE) (12 h)
ABA	<i>NtPPO1</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.33 $\pm$ 0.02	0.38 $\pm$ 0.04	0.48 $\pm$ 0.02	0.39 $\pm$ 0.05
		WT treated	0.43 $\pm$ 0.01	0.51 $\pm$ 0.01	0.72 $\pm$ 0.01	0.83 $\pm$ 0.04
		Transgenic treated	− 0.22 $\pm$ 0.01*	− 0.43 $\pm$ 0.01*	− 8.4 $\pm$ 0.03*	− 0.54 $\pm$ 0.03*
	<i>NtPPO2</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.38 $\pm$ 0.01	0.48 $\pm$ 0.02	− 0.41 $\pm$ 0.04	− 0.43 $\pm$ 0.03
		WT treated	0.44 $\pm$ 0.02	0.49 $\pm$ 0.01	0.79 $\pm$ 0.02	0.78 $\pm$ 0.05
		Transgenic treated	− 0.29 $\pm$ 0.01**	− 0.45 $\pm$ 0.02*	− 0.89 $\pm$ 0.01*	− 0.93 $\pm$ 0.05**
	<i>NtPPO3</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.31 $\pm$ 0.02	0.45 $\pm$ 0.04	0.47 $\pm$ 0.03	0.45 $\pm$ 0.01
		WT treated	0.48 $\pm$ 0.02	0.56 $\pm$ 0.03	0.86 $\pm$ 0.01	0.83 $\pm$ 0.02
		Transgenic treated	− 0.22 $\pm$ 0.01*	− 0.48 $\pm$ 0.01*	− 0.87 $\pm$ 0.02*	− 0.83 $\pm$ 0.01**
MeJ	<i>NtPPO1</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.92 $\pm$ 0.23	0.132 $\pm$ 0.02	− 2.41 $\pm$ 0.07	− 0.153 $\pm$ 0.01
		WT treated	2.44 $\pm$ 0.50	8.92 $\pm$ 0.34	5.80 $\pm$ 0.20	10.95 $\pm$ 0.25
		Transgenic treated	− 4.20 $\pm$ 0.06**	4.65 $\pm$ 0.27*	− 4.70 $\pm$ 0.04*	7.13 $\pm$ 0.34*
	<i>NtPPO2</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.25 $\pm$ 0.01	− 0.77 $\pm$ 0.19	− 2.60 $\pm$ 0.01	− 0.48 $\pm$ 0.02
		WT treated	2.80 $\pm$ 0.14	8.27 $\pm$ 0.21	5.93 $\pm$ 0.40	11.33 $\pm$ 0.32
		Transgenic treated	− 3.62 $\pm$ 0.03*	2.87 $\pm$ 0.05**	− 5.37 $\pm$ 0.30**	7.34 $\pm$ 0.25*
	<i>NtPPO3</i>	WT negative	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
		Transgenic negative	0.10 $\pm$ 0.03	0.35 $\pm$ 0.03	− 2.33 $\pm$ 0.05	− 0.70 $\pm$ 0.03
		WT treated	0.95 $\pm$ 0.05	8.91 $\pm$ 0.28	6.11 $\pm$ 0.50	11.21 $\pm$ 0.19
		Transgenic treated	− 4.73 $\pm$ 0.02**	3.72 $\pm$ 0.04*	− 4.59 $\pm$ 0.42*	7.47 $\pm$ 0.21*

WT wild type, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene

Statistically significant difference is indicated by asterisks (* P < 0.05 and ** P < 0.01)

(Asselbergh et al. 2008) that might be involved in activation of other defense responses (Lee and Luan 2012).

Expression analysis in response to JA

MeJ, the volatile form of JA, has widely been used to study jasmonate signaling pathways and mechanisms of plant defense (Zhang et al 2015). In the present study, transgenic tobacco plants along with control were treated at 100 and 200 μ M MeJ for 6 and 12 h. The transcript levels in transgenics along with control were analyzed by qRT-PCR for *NtPPO1*, *NtPPO2* and *NtPPO3*. As shown in Fig. 6a, 100 μ M MeJ after 6 h resulted in a differential expression pattern in both control and transgenic plants. However, there was an up-regulation (2.8-fold) of the target gene in WT plants at 100 μ M MeJ applications. Interestingly, the expressions of *NtPPO1*, *NtPPO2* and *NtPPO3* were significantly

down-regulated (P < 0.05; P < 0.01) up to 4.2-, 3.6- and 4.7-fold, respectively in transgenic plants after 6 h of 100 μ M MeJ application (Fig. 6a; Table 3). The expression levels of *NtPPO1*, *NtPPO2* and *NtPPO3* in transgenics decreased by 4.6-, 2.8- and 3.7-fold, respectively, after 12 h with 100 μ M MeJ, and it was significantly (P < 0.05; P < 0.01) lower than that of WT (Fig. 6b; Table 3). A similar suppression of *NtPPO* activity was also observed at 200 μ M MeJ after 6 and 12 h (Fig. 7a, b; Table 3). However, with an increase in time period from 6 to 12 h, the transcript levels of *NtPPO1*, *NtPPO2* and *NtPPO3* increased in (non-transgenic) control plants following the treatment with 200 μ M MeJ. In contrast, significant down-regulation of *NtPPO* was observed in transgenic plants at 6 and 12 h as compared with the control plants at 200 μ M MeJ application. Overall, these results showed that silencing of *PPO* gene reduced the expression level of *NtPPO* in transgenic plants in response to both

Fig. 5 Relative expression analysis of *NiPPO* gene activity in response to 200 μ M ABA treatment shown on the log₂ scale. **a** After 6 h treatment. **b** After 12 h treatment. WT wild type, *NiPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NiPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NiPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments, and statistically significant difference is indicated by asterisks (* P < 0.05; ** P < 0.01)

concentrations of MeJ (100 μ M and 200 μ M). However, pronounced reduction was observed at 100 μ M MeJ after 12 h.

Mahanil et al. (2008) reported 1.5- to 7.3-fold lower *PPO* activity in anti-sense *PPO* transgenic tomato plants than non-transformed control and 40-fold decreased *PPO* activity was observed in *PPO*-silenced tomato plants than non-transformed control (Thipyapong et al. 2004a). Increased *NiPPO* expression by MeJ in WT tobacco may indicate MeJ defense signal against pathogens. As Constabel et al. (1995) reported, *PPO* activity increased rapidly after exposing wild-type tomato plants to MeJ vapors. Tobacco and hybrid poplar also strongly induced *PPO* in response to MeJ (Constabel and Ryan 1998). However, in case of hybrid poplar, *PPO* genes were found to be expressed differentially during development following MeJ treatment (Wang and Constabel 2004b; Tran and Constabel 2011). All these studies strongly support the involvement of MeJ in up-regulation of *PPO* gene under

Fig. 6 Relative expression analysis of *NiPPO* gene activity in response to 100 μ M MeJ treatment shown on the log₂ scale. **a** After 6 h treatment. **b** After 12 h treatment. WT wild type, *NiPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NiPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NiPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments, and statistically significant difference is indicated by asterisks (* P < 0.05; ** P < 0.01)

stress conditions. Moreover, failure to induce the *PPO* expression using anti-sense technique may help to overcome the browning reactions in important crops.

Expression analysis for whitefly infestation

In response to herbivory, plants have the ability to perceive signals rapidly and accurately against biotic attackers and activate the effective defense system (Mithöfer and Boland 2012). The plant must identify and respond to mechanical and chemical signals triggered upon insect attacks (Felton and Tumlinson 2008). Previously, different defense signaling pathways have been reported that were modulated after pest attack, such as JA and SA signaling pathways (Thaler et al. 2012). *Trialeurodes vaporariorum* is a kind of polyphagous whitefly that has caused serious losses in vegetable, horticultural and ornamentals crops worldwide (Lei et al. 1998).

The transcript level was analyzed using biotic stress caused by the whitefly (*T. vaporariorum*) infestation on the transgenic tobacco plants. Overall, transcript analysis

Fig. 7 Relative expression analysis of *NtPPO* gene activity in response to 200 μ M MeJ treatment shown on the log2 scale. **a** After 6 h treatment, **b** after 12 h treatment. WT wild type, *NtPPO1* qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments, and statistically significant difference is indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

by qPCR revealed a significant down-regulation ($P < 0.05$; $P < 0.01$) of *PPO* gene in transgenic plants after increasing the time period of white fly infestation. qRT-PCR analysis of the proximal coding (*NtPPO1*) region showed a slight up-regulation of 0.75-fold than the control plants after 2 days. However, the expression of *NtPPO1* gene was down-regulated after 5 days of herbivory imposed by whiteflies feeding. This down-regulation was consistently followed and finally reached up to sixfold after 10 days (Fig. 8a; Table 4). Similarly, the *NtPPO2* regions (core) also showed a slight up-regulation after 2 days and then followed by a gradual reduction of 2.7-fold in its expression after 5 days. After 10 days, down-regulation attained a level of fivefold in the *NtPPO2* core region (Fig. 8b; Table 4). The terminal coding region (*NtPPO3*) showed very similar trend to the core coding (*NtPPO2*) region, showing an up-regulation after 2 days and a down-regulation after 5 and 10 days of whiteflies infestation (Fig. 8c; Table 4).

However, comparatively greater down-regulation of *PPO* was shown by *NtPPO1* (proximal coding region of *PPO*) which was ~sixfold, indicating that the *PPO* gene is an important component of defense mechanism with the inducible nature of the *PPO* gene which was expressed in response to pathogen defense. *PPO* is found to be linked with the protection of plants against pathogens and herbivores. *NtPPO* suppression is consistent with tomato *PPO* anti-sense down-regulation up to 40-fold by *P. syringae* infestation without affecting the plant phenotype (Thipyapong et al. 2004a). Bhonwong et al. (2009) reported 1.5- to 2.9-fold reduction in *PPO* expression against cotton bollworm in *PPO*-suppressed tomato plants. Richter et al. (2012) also observed that *PPO* suppression enhanced the susceptibility to pathogens in dandelion. Ye et al. (2012) studied that phloem-feeding herbivorous insects can trigger transcript accumulation of *PPO* and *POD* genes that deter further insect damage in rice. Recently, induction of *PPO* activity was found in whitefly herbivory in wild-type tobacco plants that can be correlated with *NtPPO* regulation of the present results (Zhao et al. 2015) and *PPO* activity was recorded to be higher in whitefly-infested pepper genotypes than in non-infested genotypes (Latournerie-Moreno et al. 2015). Earlier, *PPO* expression was found to be reduced in cotton by silencing of the *PPO* gene in response to beet armyworm, which implicated *PPO* as a component of defensive system against insects (Bhonwong et al. 2009).

Similarly, *PPO* over-expression was found to enhance resistance against insect attack (Thipyapong et al. 2004a; Mahanil et al. 2008), and the anti-herbivory role of *PPO* was established in many transgenic plants (Wang and Constabel 2004b). *PPO* up-regulation engineered insect resistance in transgenic poplar against forest tent caterpillars (Wang and Constabel 2004a). Aspen *PPO* transcripts were also reported in elevations after forest tent caterpillar feeding (Haruta et al. 2001). The present inducible *PPO* gene suppression can indirectly be linked to the defensive role against herbivores as reported in several crops such as cotton (Kranthi et al. 2003), tomato (Thaler 2002) and soybean (Bi and Felton 1995). Moreover, the defensive role of *PPO* against insect attack was also confirmed in tomato due to the presence of herbivore-inducible signal systemin that was involved in *PPO* induction in response to herbivores (Constable et al. 1995).

Overall, the modification of the *PPO* gene in transgenic tobacco showed altered transcript levels of herbivore-induced *PPO* expression which is in accordance with a previous study on transgenic tobacco (Ren and Lu 2006). Herbivore-induced down-regulation of *PPO* gene in *PPO*-silenced plants confirmed the vital role of *PPO* in insect defense. Anti-sense down-regulation of *PPO* in transgenic tomato suggested enhanced vulnerability to Colorado potato

Fig. 8 Relative expression analysis of the *NtPPO* gene activity in response to whitefly infestation shown on the log2 scale. **a** Expression analysis targeting the proximal coding region of the endogenous tobacco *PPO* gene. **b** Expression analysis targeting the core region of the endogenous tobacco *PPO* gene. **c** Expression analysis target-

ing the terminal coding region of the endogenous tobacco *PPO* gene. 18S and actin were used as reference genes. The data are the mean of three independent experiments, and statistically significant difference is indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

Table 4 Difference in the expression level of *PPO* gene in response to insect herbivory in transgenic plants

Stress treatment	Target genes used	Expression level (mean $\pm$ SE) (2 days)	Expression level (mean $\pm$ SE) (5 days)	Expression level (mean $\pm$ SE) (10 days)
Insect herbivory	<i>NtPPO1</i>	0.74	- 2.60*	- 6.0**
	<i>NtPPO2</i>	0.90	- 2.70*	- 5.04**
	<i>NtPPO3</i>	0.32	- 1.02*	- 5.67**

NtPPO1 qRT-PCR primers targeting the proximal coding region of the endogenous tobacco *PPO* gene, *NtPPO2* qRT-PCR primers targeting the core region of the endogenous tobacco *PPO* gene, *NtPPO3* qRT-PCR primers targeting the terminal region of the endogenous tobacco *PPO* gene

Statistically significant difference is indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$)

beetle than control plants (Thipyapong et al. 2007). The previous works on over-expression or under-expression of *PPO* in transgenic plants are directly or indirectly in accordance with our results demonstrating that the *PPO* gene plays a significant role in insect resistance through herbivore-inducible mechanisms along with other defensive genes (Chakraborty and Chakraborty 2005).

The present study clearly demonstrates that whitefly infestation on *PPO*-silenced tobacco plants repressed the *PPO* transcript level which might be induced in the initial stage of feeding. This down-regulation of *PPO* under herbivorous attack depicted the positive role of *PPO* during biotic stress. However, it is evident that only alteration in *PPO* gene expression is not sufficient for all plants against insect

attacks due to complex insect resistance phenomenon. Anti-sense down-regulation of *PPO* in several important crops may provide insight into insect pest management programs, because over-expression of *PPO* in tomato and cotton was proved as a weak management strategy against lepidopterans (Hoover et al. 1998). On the other hand, insect resistance may be reduced in different plant–pest interactions if *PPO* activity is suppressed. Further, reduction in expression of *PPO* is found against insect herbivory through anti-sense technology using conserved copper-binding domain as a transgene under the control of wound-inducible promoter may be helpful in controlling browning in potato and apple (Coetzer et al. 2001; Thipyapong et al. 2004b). However, further detailed investigation will be required to assess the effects of *PPO* suppression and over-expression against pest–plant interactions.

Conclusion

In conclusion, our results have shown that the *PPO* gene was responsive to the applied stresses of wounding, MeJ and whitefly assay, but not to ABA. In response to mechanical wounding, transgenic tobacco harboring anti-sense potato *PPO* showed significant down-regulation of endogenous *PPO* after 24 h, depicting its potential role in plants during mechanical injury or biotic stress. However, ABA treatment showed no significant induction and down-regulation of *NtPPO* gene in wild-type and transgenic plants, respectively. Moreover, suppression of *PPO* gene has significantly reduced the *NtPPO* transcript level in response to 100 μ M and 200 μ M of MeJ treatments in transgenic tobacco plants that is linked with *PPO* induction in defense responses. Furthermore, whitefly infestation also showed down-regulation of the *PPO* gene in transgenic tobacco plants which indicates the critical role of *PPO* in biotic stress. These results indicate that the expression of potato *PPO* in anti-sense orientation inhibits *PPO* activity. Overall, *PPO* induction in the current study by biotic and abiotic stresses clearly links *PPO* to plant defense mechanism. Suppression of tobacco *PPO* by using the present construct harboring the anti-sense conserved copper-binding domain of potato *PPO* gene under the control of a plant origin wound-inducible promoter suggests that it can be a valuable tool for future analysis such as in potato to prevent enzymatic browning.

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Author contributions EA, TM and PLG conceived and designed the research. EA carried out the vector construction, plant transformation and expression analysis, performed data analyses and drafted the manuscript. RB, WA and TM contributed reagents and materials,

participated in study design and coordination, and drafted the manuscript. SR and PLG contributed to data analyses and drafted the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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