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RESPONSE OF TOBACCO POLYPHENOL OXIDASE GENE TO WOUNDING, ABSCISIC ACID (ABA) AND METHYL JASMONATE (MeJ)

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Abstract

Polyphenol oxidases (*PPO*s) are ubiquitous enzymes in plant kingdom and catalyze the oxidation of phenols to highly reactive quinones. *PPO* genes are induced by both biotic and abiotic stresses and implicated in several physiological processes including plant defense against pathogen and insects. Here, effect of wounding, abscisic acid (ABA) and methyl jasmonate (MeJ) applications on *PPO* induction in *Nicotiana tabacum* (*NtPPO*) was characterized by RT-PCR. In response to MeJ treatment at different concentrations, maximum expression of *NtPPO* genes was observed with six folds induction at 500 µM concentration, while target transcript level was relatively low (0.2-0.9 fold) with different concentrations of ABA. Upon wounding, *NtPPO* gene was strongly induced (up to 14 folds) after 36 hours treatment. Thus, wound and MeJ inducibility of *NtPPO* gene is a strong indicative of its role in plant defense mechanism against biotic and abiotic stresses.

Key words: Polyphenol oxidase, Wounding, Tobacco, Gene expression.

Introduction

Plants in nature are continuously exposed to environmental stresses such as drought, salinity, high temperature, which negatively affect the growth and productivity (Shinwari et al. 1998; Mahajan & Tuteja, 2005; Munns, 2005). In response, plants perceive signals that lead to the synthesis of transcription factors and subsequently regulate genes for synthesis of proteins which help to cope with stress (Shinozaki & Yamaguchi-Shinozaki, 2000).The role of polyphenol oxidases (*PPO*s) in plant defense has been implied due to its up-regulation by pathogens (Thipyapong *et al.*, 1997; Mayer, 2006; Raj *et al.*, 2006).

Defensive role of *PPO* has been proposed based on activities of *PPO* reaction products upon wounding, pathogen infection, insect attacks and induction of *PPO* by abiotic, biotic environmental constrains and signaling molecules (Thipyapong *et al.*, 1995; Thipyapong *et al.*, 1997; Maki & Morohashi, 2006; Mahmood *et al.*, 2015; Akhtar & Mahmood, 2017). The role of *PPO* in inhibition of postharvest proteolysis further made it significant (Sullivan *et al.*, 2004; Sullivan & Hatfield, 2006). The *PPO* activity ultimately results quinones and reactive oxygen species to cope with stresses (Thipyapong *et al.*, 2007), and there are few studies on function of *PPO* for abiotic stress tolerance. Stresses signal transduction pathway such as abscisic acid (ABA), methyl jasmonate (MeJ), ethylene and systemin signals can trigger *PPO* expression. Chewing insects amplify wounding signals which is thought to be mediated by jasmonic acid (JA) and generation of reactive oxygen species (ROS) and is responsible for restriction of pathogen spread to the other parts of plant (Torres et al., 2002). C:\Users\Rehan\TOBACCO\Tobacco PPO paper.docx - _ENREF_17*PPO* genes are induced by wounding and/or MeJ (Constabel *et al.*, 2000; Zhou *et al.*, 2003; Wang & Constabel, 2004; Raimbault *et al.*, 2010; Shetty *et al.*, 2011) as well as during wound sealing (Wahler *et al.*, 2009).

In *Solanaceae* family, potato, tomato, eggplant and lulo , *PPO* genes were characterized in abiotic and biotic stresses (Li & Steffens, 2002; Thipyapong *et al.*, 2004; Mahanil *et al.*, 2008; Shetty *et al.*, 2011; Arias *et al.*, 2012; Chi *et al.*, 2014). To understand the role of *PPO* in plant defense and to various stresses e.g. wounding, MeJ and ABA application, a study was designed to characterize the endogenous tobacco (*Nicotiana tabacum*) *PPO* gene expression.

Materials and Methods

Plant growth: Tobacco seeds (*N. tabacum* var. *Xanthi*) were grown on half strength Murashige and Skoog (MS) medium (Phyto Technology Laboratories) (Murashige & Skoog, 1962). Seedlings were grown in growth room at 25ºC, 50% humidity and 16/8 light cycle. Seven days old plants were used for wounding treatment, MeJ and ABA applications.

Wound induction: Seedlings of *N. tabacum* were subjected to mechanical wounding by forceps. Leaves on each plant were injured carefully. Wounded plants were kept on MS media for 12, 24, 36 and 48 hours respectively along with controlled unwounded plants.

ABA and MeJ treatments: Solutions of ABA (Sigma-Aldrich) and MeJ (Sigma-Aldrich) were prepared from 1000 folds 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. Seven days old tobacco plants were sprayed with 100 µM, 200 µM, 300 µM, 400 µM and 500 µM ABA and MeJ solutions and placed in growth room for 24 hours. Control plants were sprayed with control solution.

Primer designing: Primers were designed from coding region of *PPO* gene (A27686.1) sequence of *N. tabacum* by ApE (A plasmid Editor) software. Sequence of primers is given as:

*NtPPO*2F: AACCCGTTCCGTGTGAAAGTCC
*NtPPO*2R: CTTCGATTACGCACCGATGCCA

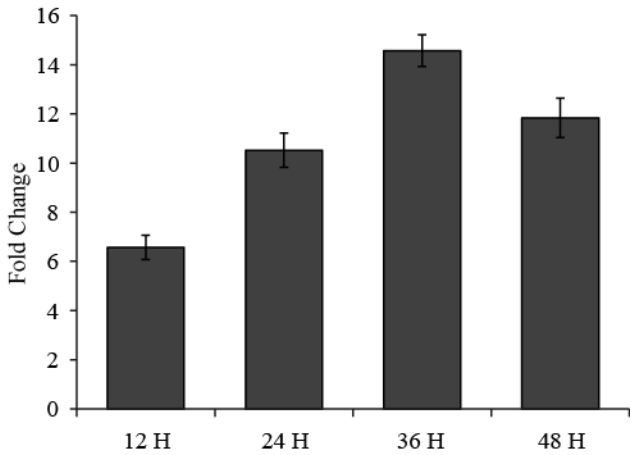

Fig. 1. Quantification of *NtPPO* mRNA level induced by wounding. Seven days old tobacco plants were subjected to mechanical injury growing on MS media and RT-PCR was performed after intervals of 12, 24, 36 and 48 hours to detect wound induced response of *NtPPO* gene.

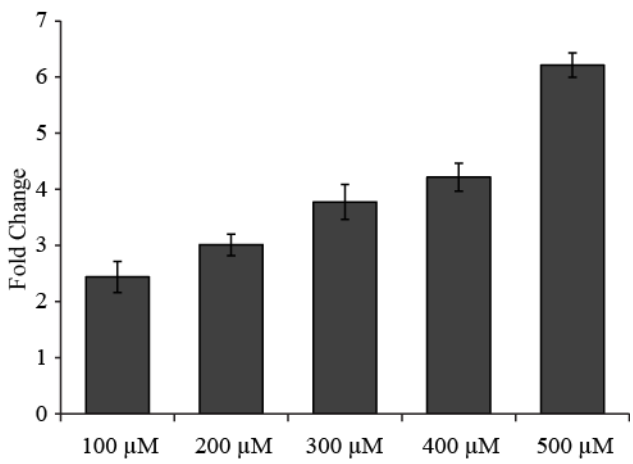

Fig. 2. RT-PCR analysis of *NtPPO* gene activity by MeJ applications. Quantitative RT- PCR was carried out to detect *NtPPO* transcripts level in seven days old tobacco plants by sprays of MeJ solutions (100 µM, 200 µM, 300 µM, 400 µM and 500 µM) growing on MS media.

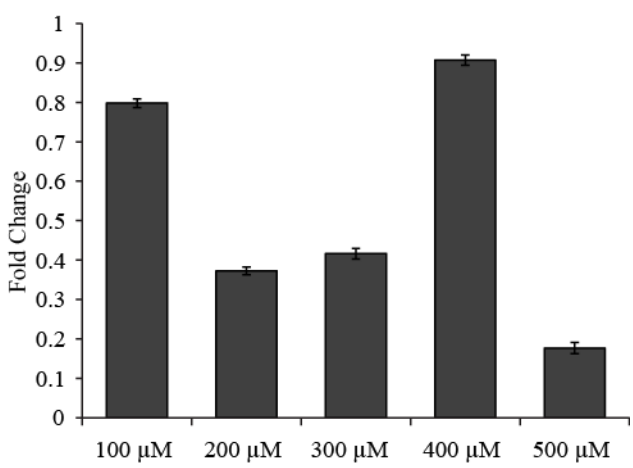

Fig. 3. RT-PCR analysis of *NtPPO* gene activity by ABA applications. Quantitative RT- PCR was carried out to detect *NtPPO* transcripts level in seven days old tobacco plants by sprays of ABA solutions (100 µM, 200 µM, 300 µM, 400 µM and 500 µM) growing on MS media.

RNA isolation and cDNA synthesis: Total RNA was extracted from the ABA, MeJ and wound stressed plants along with control plants using Spectrum ™ Plant Total RNA kit (Sigma). Reverse transcription was carried out using 3 µg of total RNA to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen).

***NtPPO* gene expression analysis:** Quantification of *PPO* transcripts was performed in ViiA™7 Real-Time PCR system with the Power SYBR® Green master mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. *PPO* gene was used as target and 18S gene was used as internal control. After successful amplification of targets, the cycle threshold (Ct) 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 & Bertone, 2009) and Limma (Gentleman *et al.*, 2006) add-on packages were used for the manipulation and analysis of the Ct values.

Results

Induction of *NtPPO* by wounding: Effect of wounding was monitored in seven days old tobacco plants growing on half strength MS medium. Young leaves were mechanically wounded which simulates the attack of insect pests. To analyze the expression of *NtPPO* gene in response to wounding; transcript levels of *NtPPO* endogenous gene was in increasing trend with the passage of time after wounding. Transcript level was found to increase after 12 hours (6 folds) reaching to the maximum level of 14 folds after 36 hours of wounding treatment (Fig. 1), which started to decline after 48 hours.

Induction of *NtPPO* transcript in response to ABA and MeJ: Plants were sprayed with various concentrations of ABA and MeJ, left for 24 hours in growth chamber. Transcript level of *NtPPO* was up-regulated from 2-6 folds in response to MeJ treatment (Fig. 2). An increase in *NtPPO* transcript level was found with increasing MeJ concentrations. It was seen that mRNA level increased up to 2 folds upon 100 µM MeJ and maximum induction of transcript level was observed at 500 µM MeJ. Unlike MeJ, lower level of *NtPPO* expression was observed with ABA application (Fig. 3).

Discussion

*PPO*s are generally related to plant defense (Mayer, 2006; Constabel & Barbehenn, 2008). In comparisons with our findings, *PPO* mRNA accumulation increased in response to wounding and defense related hormones SA and MeJ in poplar(Mayer, 2006; Flurkey & Inlow, 2008). Disease susceptibility and herbivory were also strongly linked to *PPO* activities (Thipyapong *et al.*, 2007). Based on the previous reports, it is well known that *PPO*s are part of innate immunity in plants (Fuerst *et al.*, 2014).

Induction of *NtPPO* gene by wounding: Some of plant defenses are triggered through tissue damage mechanical injury (Howe & Jander, 2008). In tobacco, our data has

indicated that an endogenous induction of *PPO* gene by wounding suggests a possible role of *NtPPO* gene in plant defense. Accumulation of *NtPPO* mRNA up to 14 fold by wounding is in agreement to high induction of potato *PPO* activity (Thipyapong *et al.*, 1995). In earlier reports, localized wounding of lateral roots (Thipyapong *et al.*, 1997), wounding stress (Quarta *et al.*, 2013) and chilling injury in pineapple resulted in *PPO* gene activation (Raimbault *et al.*, 2010).

Role of *PPO* has been evaluated in anti-herbivory. *NtPPO* induction by wounding is probably the first possible indication of its defensive role which is in consistent with hybrid poplar *PPO* transcripts activated by wounding, as well as real insect herbivory (Constabel *et al.*, 2000). In aspen, forest tent caterpillar strongly induced *PPO* expression as did by mechanical wounding (Haruta *et al.*, 2001). *PPO* has a strong correlation in latex coagulation and wound sealing in dandelions (Wahler *et al.*, 2009). The pattern of *NtPPO* induction by wounding could be explained by responsiveness to insect herbivory as stimulation and *PPO* mRNA accumulated mainly in eggplant stem and fruits (Shetty *et al.*, 2011). In another report, strong up-regulation of *PPO* gene in rubber plant by wounding may correlate its probable role in plants defense (Li *et al.*, 2014).

Induction of *NtPPO* transcript in response to ABA and MeJ: *PPO* has been signaled by various signaling molecules such as ethylene (Newman *et al.*, 1993), ABA (Chai *et al.*, 2013; Kaur & Zhawar, 2015; Jia *et al.*, 2016) and JA (Adeloye & Ajibade, 2011; Jia *et al.*, 2016) indicating role of *PPO* in plant defense. It is well known that MeJ induced *PPO* activity along with other defense related responses (Thaler *et al.*, 2002). Jasmonates take part in well-timed induction of plant defense responses against insects as activation of plant defense depends upon jasmonate pathway (Erb *et al.*, 2012; Bosch *et al.*, 2014). It has been revealed that treatment by JA and wounding by insects induce *PPO* activity triggering other responses which result in broad spectrum resistance against pests and microbes (Cooper *et al.*, 2004).

PPO up-regulation by jasmonic acid and its overexpression improved resistance against armyworm (*Spodoptera exigua*) defense in tomato (Bosch *et al.*, 2014) which can be correlated to *NtPPO* gene up-regulation by MeJ. An induction of up-regulation of *PPO* mRNA in tobacco by mechanical injury and MeJ might be a possible clue of *NtPPO* participation in biotic stresses and these findings are in consistent with banana *PPO* up-regulation by wounding and MeJ (Sreedharan *et al.*, 2012). It was reported that MeJ induced six *PPO* genes in eggplant and in transgenic tobacco (Shetty *et al.*, 2012). In another report, it was shown that red Swiss chard *PPO* promoter was regulated by MeJ in transgenic *Arabidopsis* (Yu *et al.*, 2015). Many plant's *PPO*s were regulated by MeJ treatment indicating the role of *PPO* in plant defense such as in hybrid poplar (Constabel *et al.*, 2000), trembling aspen (Haruta *et al.*, 2001), tomato (Li & Steffens, 2002), walnut (Escobar *et al.*, 2008), rubber tree (Li *et al.*, 2014) and strawberry (Jia *et al.*, 2016).

Conclusion

From the data of wounding, ABA and MeJ treatments, it was revealed that *NtPPO* might have role in plant protection against both biotic and abiotic stresses. These results are helpful in potential understanding of plant responses to such stresses. Conclusively, *NtPPO* is a potential candidate gene for developing transgenic crops tolerant to both biotic and abiotic stresses.

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