Effects of Potato Polyphenol Oxidase Gene Silencing in Transgenic Tobacco

Doctor of Philosophy

in

PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

EJAZ AZIZ

DEPARTMENT OF PLANT SCIENCES,
FACULTY OF BIOLOGICAL SCIENCES,
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
2017
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A Dissertation Submitted in the Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

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DEPARTMENT OF PLANT SCIENCES,
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ISLAMABAD, PAKISTAN
2017
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Name: Ejaz Aziz
Dedicated

to

my respected, great, ever-loving parents to whom i belong, who gave me an urge to seek knowledge, who have motivated my thoughts to explore uniqueness in this universe and for their endless support and encouragement
Foreign Examiners

1. **Dr. Xianchun Xia**
   Professor in Applied Genetics and Wheat Breeding
   Institute of Crop Sciences
   National Wheat Improvement Center
   Chinese Academy of Agricultural Sciences (CAAS)
   Zhongguancun South Street 12
   Beijing 100081
   China
   Phone: +86-10-82108610, 13717542298
   Fax: +86-10-82108547
   Email: xiaxianchun@caas.cn; xiaxianchun@yahoo.com

2. **Dr. Kazuo Nakashima**
   Program Director
   Stable Food Production Program
   Japan International Research Center for Agricultural Sciences (JIRCAS)
   1-1 Ohwashi, Tsukuba, Ibaraki, 305-8686 Japan
   Tel: +81-29-838-6600
   Fax: +81-29-838-6316
   Email: kazuid@affrc.go.jp
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Ejaz Aziz
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<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AI</td>
<td>Aliphatic index</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>°C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl trimethyl ammonium bromide</td>
</tr>
<tr>
<td>cTP</td>
<td>Chloroplast transit peptide</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle of threshold</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribo nucleoside triphosphates</td>
</tr>
<tr>
<td>EC</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>FTC</td>
<td>Forest tent caterpillar</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberlic acid</td>
</tr>
<tr>
<td>GRAVY</td>
<td>Grand average of hydropathicity</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<tr>
<td>Hyg</td>
<td>Hygromycin</td>
</tr>
<tr>
<td>IGMAP</td>
<td>Interactive genome map for plants</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>LB</td>
<td>Lauria-Bertani</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MeJ</td>
<td>Methyl jasmonate</td>
</tr>
<tr>
<td>µl</td>
<td>Micro liter</td>
</tr>
<tr>
<td>mg</td>
<td>Milli gram</td>
</tr>
<tr>
<td>ml</td>
<td>Milli liter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mM</td>
<td>milli Molar</td>
</tr>
<tr>
<td>MEGA6</td>
<td>Molecular evolutionary genetic analysis 6</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MSA</td>
<td>Multiple sequence alignment</td>
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<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
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<tr>
<td>NtPPO</td>
<td><em>Nicotiana tabacum</em> polyphenol oxidase</td>
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<tr>
<td>PO</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonia-lyase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPO</td>
<td>Polyphenol oxidase</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<tr>
<td>RGLP2</td>
<td>Root germin like protein-2</td>
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<tr>
<td>SA</td>
<td>Salicylic acid</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-D-galactopyranoside</td>
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<td>WT</td>
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ABSTRACT

Polyphenol oxidases (PPOs) are nuclear encoded copper metalloenzymes that catalyze the oxidation of ortho-diphenols to ortho-quinones. PPO genes play an important role in plant defense mechanisms against biotic and abiotic stresses as well as responsible for undesirable browning in some fruits and vegetables. In the current study, ninety five PPO gene coding sequences from thirty six different plant species were analyzed in silico for multiple sequence alignment and phylogenetic relationships to gain insight into the structure-function relationships within the plant PPO family with special emphasis on potato. The obtained results indicated highly conserved copper binding domain in all the studied sequences. Phylogenetic clustering of monocot and dicot PPO proteins into separate groups reflects the lineage specific gene family expansion and duplication events through evolution. Interestingly, grand average of hydropathicity values proposed that all potato PPO proteins are hydrophilic in nature.

To evaluate the stress induced expression of PPO gene, in vitro grown tobacco plants were subjected to wounding, abscisic acid (ABA) and methyl jasmonate (MeJ) stresses. Transcript levels measured by qRT-PCR for endogenous tobacco PPO gene showed an up-regulation of 14 folds by wounding and 6 folds by MeJ application, while no significant induction was observed with ABA treatment. An up-regulation of endogenous PPO gene in response to wounding and MeJ suggested a possible role of PPO in biotic stresses. Keeping in view the role of tobacco endogenous PPO induction by wounding and MeJ as well as already studied reports of PPO in other plants, Agrobacterium tumefaciens mediated genetic transformation of in vitro grown tobacco plants was carried out. The expression construct containing a copper binding domain segment of PPO gene ligated in anti-sense orientation downstream to a wound inducible OsRGLP2 promoter in p1391Z backbone vector was used for this transformation event.

Expression analysis of transgenic tobacco plants with OsRGLP2 driven anti-sense segment of PPO gene was carried out with qRT-PCR in response to various stresses e.g. wounding, MeJ, ABA and whitefly (Trialeurodes vaporariorum) infestation. Expression analysis of PPO gene in transgenic tobacco showed a significant higher suppression (3.5 folds) of PPO in response to wounding. Similarly, MeJ application down-regulated the PPO expression up to 4.7 folds, while no significant expression of PPO was observed in...
case of ABA treatment. Moreover, considerable level of \textit{PPO} reduction (6 folds) was observed with whitefly feeding assay in transgenic tobacco. These results indicated that expression of potato \textit{PPO} in an anti-sense orientation has down-regulated the \textit{PPO} activity. This down-regulation of \textit{PPO} by wounding, MeJ and whitefly infestation clearly links the role of \textit{PPO} in biotic as well as abiotic stresses. \textit{PPO} gene suppression in transgenic plants by using anti-sense potato \textit{PPO} gene construct suggested that this tool can be used to inhibit the enzymatic browning in fruits and vegetables e.g. potato.
INTRODUCTION

Plants which are exposed to stresses hamper their fitness for survival in the environment. Naturally, plants are equipped with the defensive mechanisms to cope with these problems such as to protect from pathogens (Li and Steffens, 2002). Plant defensive mechanisms triggered by the biotic and abiotic stresses are widely variable. These mechanisms include programmed cell death (Greenberg, 1997; Li and Steffens, 2002), production of reactive oxygen species (Bolwell, 1999), strengthening and cross linking of cell walls (Brisson et al., 1994), initiation of defense related genes (Dixon and Harrison, 1990) and metabolism of phenolic compounds (Nicholson and Hammerschmidt, 1992). These stresses are considered versatile and dictate the plants to attain different phenotypes over the time. Secondary metabolites including defensive proteins and enzymes, particularly enable plants to cope with the stress as a defense mechanism over multiple stresses and interestingly they are inducible (Thipyapong et al., 2007). Polyphenol oxidases (PPOs) are nuclear encoded copper metalloenzymes that are also induced by biotic and abiotic stresses.

Polyphenol oxidases, also known as tyrosinases represent the enzyme that causes the enzymatic oxidation of monophenol or o-diphenol in to o-quinones using molecular oxygen. Series of these reactions leads to the browning in damaged fruits and vegetables which are usually a result of pathogenic infection and wounding. Plant PPO genes are often characterized as defensive proteins due to their herbivore, pathogen and wound inducible expression (Thipyapong et al., 1995; Constabel and Ryan, 1998). The role of PPO genes under various biotic (bacterial, fungal and insects) and abiotic (drought, salinity, cold and heat) stresses in plants has been well demonstrated in several studies (Li and Steffens, 2002; Thipyapong et al., 2004a; Thipyapong et al., 2007).

1.1 Distribution and Localization

PPO is known as a ubiquitous enzyme present in all life forms including prokaryotes and eukaryotes e.g. bacteria, fungi, plants, invertebrates and chordates (Mayer, 2006; Tran et al., 2012). Bacteria e.g. Bacillus species and Marinomonas mediterranea (Bolwell, 1999; Fernández et al., 1999) are also found to contain PPO and
fungal groups e.g. deuteromycetes and basidiomycetes also express PPO activity (Echigo and Ohno, 2001). Perez-Gilabert et al. (2001) isolated latent form of PPO from ascocarp of Terfezia claveryi. Bryophytes are targeted for the presence of PPO e.g. Physcomitrella patens (Richter et al., 2005). Recently, Tran et al. (2012) identified the presence of PPO genes in moss Physcomitrella patens. Among plants, PPO has been reported from both fruits and vegetables in several studies. The critical role of PPO genes in plant defense and food quality has led to their identification and characterization in several plant species including Solanum lycopersicum (Shahar et al., 1992; Thipyapong et al., 1995; Thipyapong and Steffens, 1997), Solanum tuberosum (Hunt et al., 1993), Nicotiana tabacum (Goldman et al., 1998), Malus domestica (Boss et al., 1995), Trifolium pratense (Sullivan et al., 2004), Vitis vinifera (Dry and Robinson, 1994), Prunus armenica (Chevalier et al., 1999), Triticum aestivum (Demeke and Morris, 2002) and Populus trichocarpa (Tran and Constabel, 2011). Robinson (2008) also reported PPO genes from banana, lettuce, tobacco and pineapple.

Moreover, two distinct PPO genes have been characterized in seeds and leaves of coffee (Mazzafera and Robinson, 2000), in the Chinese cabbage (Nagai and Suzuki, 2001) and in dodder (Bar-Nun and Mayer, 1999). The presence of PPO has also been well documented in several other plant species such as Eriobotrya japonica (Selles-Marchart et al., 2006), Cynara cardunculus (Dogan et al., 2005a), Sclerocarya birrea (Mduli, 2005), Uapaca kikiana (Muchuweti et al., 2006), Annona muricata (Bora et al., 2004), Origanum (Dogan et al., 2005b) and Prunus armeniaca (Arslan et al., 1998). Besides these cash crops, medicinal plants have also been exploited for PPO e.g. Melissa officinalis (Dogan et al., 2013) and Ferula species (Erat et al., 2006; Robinson, 2008). This identification and characterization of PPO gene is of prime importance as this could help in the better understanding of the structural and functional differences in multi-gene family.

In studied plants, PPO is localized in almost all parts of plant body e.g. flowers, fruit, leaves, roots and tubers (Thygesen et al., 1995). Most of PPO genes from higher plants have been predicted to be localized in chloroplast (Fortea et al., 2009), whereas in fungi the enzyme seems to be localized in cytosol (Kim et al., 2001; Mayer, 2006). In
earlier study, Arnon (1949) investigated the intracellular location of Beta vulgaris PPO in chloroplasts. However, being localized in thylakoid membrane, its content in tender and juvenile portions of plant was found to be higher than in mature and hard tissues (Gooding et al., 2001). However, PPO being a stable enzyme also exists in dormant form in mature tissues of plants (Bucheli et al., 1996). PPO is a nucleus encoded protein localized in plastids and 80% of PPO is bound to thylakoid membrane while remaining 20% is found in stroma (Lax and Vaughn, 1991). In recent studies, PPO protein have also been indentified which lack chloroplast targeting sequence (Tran et al., 2012) that can localized in vacuole (Ono et al., 2006) and in the cytosol (Nakayama et al., 2001). PPO showed a differential expression of its gene during growth, development and stress which suggests that PPO belongs to a multi-gene family (Newman et al., 1993; Webb et al., 2013).

1.2 Structure and Reaction Mechanism

In general plant PPO proteins are divided in to three domains: an N-terminal chloroplast transit peptide (cTP), a highly conserved type-three copper centre, and a C-terminal domain (Tran et al., 2012). The N-terminal region contains 8-12 kDa bipartite cTP (Bucheli et al., 1996) which is important for regulating the imports in to lumen of thylakoid through Tat (twin arginine-dependent translocation) pathway (Koussevitzky et al., 2008). The mature PPO proteins has a di-copper center having two conserved copper-binding domains called as CuA and CuB, each with three histidine residues that coordinate with copper ion and comprise the catalytic site (Klabunde et al., 1998). The length of each copper binding domain is approximately 50 amino acids (aa) which is separated by 100 aa linker fragment (Van Gelder et al., 1997a). In most plants, domains were found to be highly conserved, however, CuA domain is comparatively more variable than CuB domain and this variation may affect substrate preferences. In some plants broad bean (Vicia faba) and grape berry (Vitis vinifera), the end of C-terminal region was found to be more sensitive to proteolytic cleavage and this proteolytic cleavage of C-terminal region has been linked for enzyme activation (Dry and Robinson, 1994). Analysis of many cloned PPO genes exhibit the presence of multiple PPO genes without an intron. For example, in case of tomato, seven single-exon PPO genes have
been characterized (Newman et al., 1993). On the other hand, five single-exon PPO genes have been identified in potato (Thipyapong et al., 1995). Subsequently, PPO genes with introns were identified from pineapple and wheat (Zhou et al., 2003; Koussevitzky et al., 2008). However, single PPO gene has been identified in Lactuca sativa and Vitis vinifera (Thipyapong et al., 2007). Moreover, little information is available for the presence of nine genes in noneconomic plants, whereas no PPO genes were reported from Arabidopsis genome. The crystal structure of monomeric 39 kDa Ipomoea batatas PPO is found to be ellipsoidal in shape with dimensions of 55x45x45 Å. The secondary structure is shown to be primarily made of α helices with the core of the enzyme formed by a four-helix-bundle composed of α helices α 2, α 3, α 6 and α 7 and there are two copper ions present at active site (Klabunde et al., 1998).

PPO catalyze oxidation of the monophenolic hydroxyl group and dihydric phenol to o-diphenol and o-quinone in the presence of molecular oxygen (Lerner, 2009). These series of oxidation reactions occurred in plant tissue when PPO is released from thylakoid of chloroplasts in response to mechanical injury, herbivory and pathogenic attacks. Self-polymerization or reaction of quinones with amino acids and proteins in the plant body leads to the formation of black or browning of tissue, which is attributed the key source of postharvest browning of fruits and vegetables (Figure 1.1) (Liu et al., 2007; Anderson et al., 2010). The oxidation of phenolic substrates by PPO is the major cause of browning in vegetables and fruits during storage, handling and processing which is responsible for serious loss in food industry.

PPO induced black and/or brown quinine additives is a topic of interest for plant biologists. Several groups has also been studied the involvement of PPO in the postharvest physiology of many vegetable and fruit crops (Mayer and Harel, 1991). Parveen et al. (2010) have reported the potential substrates of PPO based on structural comparison and enzymatic assays from flavanol, flavonol, anthocyanin, flavones, and hydroxyl cinnamic acid and hydroxyl benzoic acid subclasses of phenolic acid, and isoflavonoid subclasses of flavonoid polyphenols. For the activation of PPO activity in plant tissue, suitable substrate should be available in the chloroplast. Typical PPO substrates are o-diphenols due to their readily oxidizeable OH-groups (Martinez and Wh-
Figure 1.1: Schematic representation of mechanism of PPO enzyme action as proposed by Yoruk and Marshall (2003).
itaker, 1995; Parveen et al., 2010). However, among polyphenols, flavonoids and phenolic acids were identified as major PPO substrates (Parveen et al., 2010).

1.3 Role of PPO in Growth and Development

PPO plays a central role in growth and development of several plants as well as in defense against the biotic and abiotic stresses (Bhonwong et al., 2009). In previous studies, it has been shown that PPO gene family is differentially expressed at distinct physiological and developmental conditions in different plant organs. In a study, higher transcript level of PPO was investigated in growing fruit while reduced in mature fruit (Gooding et al., 2001). Likewise, PPO activity has been reported in banana flesh during its growth and ripening periods as higher expression was recorded in unexpanded leaves than mature leaves (Gooding et al., 2001). In tobacco, PPO homologue tobP1 was observed which expressed exclusively in flower tissue suggested possible role of PPO in growth and development (Goldman et al., 1998). PPO was supposed to reduce the plant nutritional quality as a result of alkylation of essential amino acid by PPO-generated quinones. These quinones may produce oxidative stress which have shown toxic effects on herbivore resulted in increased insect resistance (War et al., 2012).

Further, role of PPO in development has been suggested in Fuji apple in which two PPO genes expressed differentially during vegetative and reproductive development as well as under wounding treatment. The expression of MD-PPO2 was detectable in all stages of flower development while APO5 was expressed only at the time of post-anthesis stage. However, transcript level was significantly reduced as the fruit ripened. Interestingly, MD-PPO2 expression was found higher in both mature and immature leaves but APO5 was transcriptionally more active only in immature leaves (Kim et al. 2001). Similarly, seven PPO genes have been reported in tomato that expressed differentially in various vegetative and reproductive organs (Thipyapong et al., 1997). The role of PPO in pigment formation has also been studied (Vaughn and Duke, 1984a). Thygesen et al. (1995) reported PPO as multigene family in potato that has specific temporal and spatial pattern of expression. The higher PPO expression was observed in roots, tubers, stolon, and flowers but lower in stem and leaves. Further investigation revealed that POT32 expressed in all stages of tuber and root development but not in
photosynthetic tissues. Moreover, $POT_{33}$ was expressed in tubers and $POT_{72}$ in roots. In tomato, $PPO$ ($P2$) activity was also examined in floral meristem that is associated with the developmental expression of $PPO$ gene (Shahar et al., 1992). Similarly, wounding induced $PPO$ activity in young leaves of tomato (Thipyapong and Steffens, 1997). Recently, higher $PPO$ activity was detected in leaves and nodules of $Trifolium pretense$ indicating its role in development (Webb et al., 2013). Furthermore, it has been observed that altering $PPO$ activity showed no effect on plant growth and development.

1.4 $PPO$ in Biosynthetic Process

The importance of $PPO$ has been also depicted due to its involvement in the biosynthesis of secondary metabolites and some specialized pigments. In $Larrea tridentata$, $PPO$ showed significant role in the biosynthesis of 8-8’ linked lignans (Cho et al., 2003). Nakayama et al. (2001) reported that specific $PPO$ genes are required for the synthesis of aurone pigment in $Antirrhinum majus$. Similarly, betalin biosynthesis has shown to be linked with $PPO$ in the Caryophyllaceae where $PPO$ acts as hydroxylases (Gandía-Herrero and García-Carmona, 2013). In another study, interaction between biosynthesis of betalin and tyrosianse has been ascribed based on the fact that tyrosinase from $Beta vulgaris$ and $Portulaca grandiflora$ were able to hydroxylate tyrosine to dihydroxyphenylalanine (DOPA) that can be further oxidized to quinone (Strack et al., 2003). Further, Gandía-Herrero et al. (2007) demonstrated in violet flowers of $L. productus$ that biosynthesis of betanidin pigment is dependent on tyrosinase activity. Recently, direct role of $PPO$ in biosynthesis of the hydroxycoumarin esculetin has been investigated in walnut (Araji et al., 2014). Kaintz et al. (2014) found that aurone formation in $Coreopsis grandiflora$ has been regulated by product of $PPO$ transcript.

1.5 Role of $PPO$ in Photosynthesis

The localization of $PPO$ in chloroplast has led to the suggestion that $PPO$ can play potential role in photosynthesis (Boeckx et al., 2015a). Previously different hypothesis was proposed on role of $PPO$ in photosynthesis based on many observations. $PPO$ proteins and plastids association in evolving high level of oxygen, $PPO$ proteins reside in photosynthetic pigments, inactivation of cyclic and non-cyclic
photophosphorylation by phenolic compounds which is prevented by \textit{PPO} through oxidizing these phenolic substrates and regulation of \textit{PPO} activity by different environmental effects such as extreme temperature, drought and time of year signifying the possible role of \textit{PPO} in photosynthesis (Neuman and Drechsler, 1967; Vaughn and Duke, 1984b; Sheptovitsky and Brudvig, 1996; Fothergill and Rees, 2006; Lee et al., 2007; Boeckx et al, 2015b).

\textit{PPO} can directly influence photosynthesis serving as oxygen buffer, or interaction with Mehler-peroxidase, water-water cycle to assist in reactive oxygen scavenging (Tolbert, 1973; Mayer and Harel, 1979) and this modulation of available oxygen is carried out by \textit{PPO} which catalyses phenols to quinones (Steffens and Zabeau, 1994). It has been suggested that \textit{PPO} can increase the rate of photosynthesis by mitigating photooxidative-damage endured during abiotic stresses. This hypothesis was supported by higher \textit{PPO} activity in \textit{Trifolium pratense} during winter seasons, when risk of photoinhibition is high (Fothergill and Rees, 2006; Boeckx et al, 2015b). In another study, transgenic tomato plants with suppressed \textit{PPO} performed better under conditions designed to impose photoinhibition than non-transformed tomato plants (Thipyapong et al., 2004b). Further, Sheptovitsky and Brudvig (1996) reported that \textit{PPO} is more significant for the dark reactions in the thylakoid lumen than those in the light, i.e. when \(O_2\) is short. This is because \textit{PPO} is not activated in acidic condition during light reaction as low pH is responsible for \textit{PPO} activation during dark reaction (Sheptovitsky and Brudvig, 1996; Winters et al., 2003; Schmitz et al., 2008).

\subsection*{1.6 \textit{PPO} Mediated Browning and its Prevention}

\textit{PPO} belongs to class of copper-binding enzyme that catalyzes the oxidation of phenolic compounds to quinones (Thygesen et al., 1995). These compounds further polymerize to form black or brown pigment called melanin (Mayer, 2006). The brown or black adducts as a result of \textit{PPO} catalyzed-oxidations is responsible for more than 50\% of the losses of industrial production of vegetables and fruits such as in apples, potatoes, strawberries, grapes, alfalfa, dandelion and walnuts (Nishimura et al., 2003; Sullivan et al., 2004; Spagna et al., 2005; Chisari et al., 2007; Wahler et al., 2009; Holdebaum et al., 2010). Such enzymatic browning may be reduced or eliminated by using \textit{PPO} inhibitors...
and this process may involve different mechanism of inhibitor action (Roudsari et al., 1981). Since browning reduces nutritional and sensory qualities, several techniques and mechanisms have been developed to control PPO activity. Inactivation of PPO activity can be controlled by number of physical methods such as applying high hydrostatic pressure, heat, radiation and electric field as well as by certain other chemical agents such as oxalic acid, sodium chloride and sulfating agents to suppress browning in vegetables and fruits.

The inhibition of PPO activity was achieved in apple by applying high hydrostatic pressure which affects PPO protein structure (Hendrickx et al., 1998; Plaza et al., 2003). Similarly, high temperature was also found to be responsible for suppression of PPO activity. Valderma et al. (2001) reported suppressed PPO activity in apple at 75 °C for 10 minutes. Likewise, 50 % reduction in PPO expression was recorded in strawberry when heated for 10 minutes at 55 °C while PPO activity was completely eliminated at 65 °C for 10 minutes (Dalmadi et al., 2006). On the other hand, Giner et al. (2002) described the use of pulse electric field for controlling enzymatic browning in pear and peach. The browning potential was reduced up to 70 % in peach by applying electric field of 24.3 kV/cm for 5000 µ seconds while 72 % in pear with 22.3 kV/cm for 6000 µ seconds. Calcium-ascorbate with low dose gamma radiations has been shown to be active as PPO inhibitor (Fan et al., 2005).

In addition, several chemical agents have been identified that can be used in anti-browning of vegetables and fruits linked with PPO activity such as ascorbic acid, citric acid, oxalic acid, kojic acid and ferulic acid have been commonly used PPO inhibitors. These chemical agents either act as PPO enzyme inhibitors or changes pH which leads to suppression or reduction of PPO activity (Rapeanu et al., 2006). The anti-browning ability of ascorbic acid has been well documented in Allium species as well as in Asian pear (Arslan et al., 1998; Arzani et al., 2009). It has been investigated that sodium metabisulphate and amino acid cystein are very effective anti-browning agent (Iyidogan and Bayiindirli, 2004). However, ascorbic acid showed mounting reduction in PPO than cystein but its effects is short lived (Ozoglu and Bayiindirli, 2002). Zheng and Tian (2006) demonstrated that oxalic acid may be applied in litchi to prevent it pericarp
browning. Similarly, anti-browning role of oxalic acid was also confirmed in rice (Peng et al., 2001). Besides acids, certain salt has also been proved as good anti-browning agents as sodium bisulfate was found against Granny smith apple slices (Fan et al., 2005). Furthermore, 62 % reduction in PPO activity was investigated in tomato when treated with 0.1 M NaCl (Spagna et al., 2005). Several findings revealed that above mentioned chemical compounds are effective anti-browning agents and can be used in synergistically to enhance the anti-browning ability in different plants (Ozoglu and Bayiindirli, 2002; Iyidogan and Bayiindirli, 2004; Mosneaguta et al., 2012). However, the limitations of some of the above mentioned anti-browning agents due to their toxic effects and pressure from regulatory agents has been resulted in the development of alternative technologies for the prevention of enzymatic browning. Such cost effective as well as safe methods will be discussed in next section.

1.7 Use of Sense and Anti-sense PPO in Plants

Genetic engineering provides a fast and precise alternative method for reducing the browning process in important crops. Several reports have described reduced PPO-catalyzed enzymatic browning in different cash crops by down-regulation of PPO gene expression using transgenic transformation with PPO gene fragments in orientation such as anti-sense, sense or double-stranded RNA (Bachem et al., 1994; Coetzer et al., 2001; Murata et al., 2001; Thipyapong et al., 2004a; Wahler et al., 2009; Zhao et al., 2009; Richter et al., 2012). These methods functioned on the principle of gene silencing mechanism or RNA interference (Wesley et al., 2001). Modern biotechnological approach of using sense and anti-sense technology for controlling enzymatic browning in potato provides a platform to monitor PPO activity in important cash crops (Coetzer et al., 2001). The use of anti-sense technology emerged as a tool to evaluate the role of PPO in plant defense as it did not affect growth, development and flowering of plants (Thipyapong et al., 2004b).

In potato, enzymatic browning has been successfully reduced by suppression of PPO using sense or anti-sense technology with tomato or potato PPO genes (Bachem et al., 1994; Coetzer et al., 2001). Similarly, targeted region of PPO gene was also transformed into apple in an anti-sense orientation to produce transgenic apple with
reduced *PPO* activity that results in lower browning potential in fruits and shoots when bruised, sliced or bitten (Murata et al., 2001; Cao et al., 2004). Interestingly, *PPO* gene silencing in *T. pratense* didn’t showed any alteration in leaf nitrogen contents and growth (Webb et al., 2013). Down-regulation of *PPO* gene resulted in reduced *PPO* activity to many folds which caused more vulnerability to pathogen attacks of *P. syringae* in tomato (Thipyapong et al., 2004a). Similarly *PPO* suppressed tomato lines with anti-sense *PPO* gene showed 1.5-7.3 folds lower *PPO* activity than *PPO* over-expressing lines. *PPO* suppressed lines showed more susceptibility to insect. Growth rates, leaf area consumed by feeding larva on *PPO* suppressed lines was more along with their lower mortality rates as compared to *PPO* over-expressing tomato lines (Mahanil et al., 2008). Furthermore, *PPO* over-expression enabled with less growth, less area damage by insects and more mortality in transgenic tomato lines (Bhonwong et al., 2009).

On the other hand, reduction in *PPO* activity as well as browning of potato was also reported using artificial microRNAs techniques (Chi et al., 2014). Similarly, invasiveness of *P. infestans* was reduced and accumulation of defensive phenolics compounds was found in transgenic potato with down-regulated *PPO* genes (Llorente et al., 2014). *PPO* activity was reported to be lowered in transgenic Yali pear using anti-sense expression vector than control plant (Li et al., 2011). Likewise, browning of potato tubers due to bruising was reduced by anti-sense suppression of *PPO* gene (Steffens and Zabeau, 1994). However, *PPO* silencing causes changes of secondary metabolites and also suggested a fundamental role of *PPO* in secondary metabolism by acting as an indirect regulator of cell death in walnut (Araji et al., 2014). Overall, silencing of *PPO* genes proved to be effective method in controlling enzymatic browning, although possible side effects on plant resistance to abiotic and biotic stresses need to be further ascertained.

**1.8 PPO Induction and its Role in Disease Resistance**

The natural resistance of plants to diseases is based not only on preformed defenses, but also on induced mechanisms. Besides many of the negative features of *PPO* proteins, *PPO* is considered playing a defensive role to pathogen attack. High levels of *PPO* have been attributed to be helpful to alleviate the pathogen attacks (Raj et al., 2006).
**PPO** catalyzed phenolic oxidation also involved in the resistance to the disease development. Interestingly, **PPO** gene family in tomato display wide variation in temporal and spatial expression and induction by biotic and abiotic factors (Thipyapong et al., 1997). In earlier studies, **PPO** was found to be significantly induced in regions of lesion and away from lesion which may indicate resistance responses (Bashan, 1986; Khirbat and Jalali, 1998). However, control of **PPO** expression in some plants underscored the role of **PPO** in plant defense responses (Thipyapong and Steffens, 1997; Haruta et al., 2001).

**PPO** induction in response to attack of *Pseudomonas syringae* and *Alternaria solani* in tomato depicted the role of **PPO** in disease resistance (Thipyapong and Steffens, 1997). Similarly, overexpression of **PPO** considerably improved resistance to *P. syringae* in transgenic tomato lines (Li and Steffens, 2002). It was found that the transgenic tomato plants which were overexpressing **PPO** oxidize more efficiently the phenolic substrates as compared to the untransformed plants. Transgenic lines were found possessing with over 100-fold less bacterial population on infected leaves indicating that **PPO**-mediated phenolic oxidation results in restricting plant disease development (Li and Steffens, 2002). However, using anti-sense technology **PPO** down-regulation in transgenic tomato lines revealed that susceptibility to *P. syringae* dramatically increased in **PPO** suppressed transgenic tomato lines (Thipyapong et al., 2004a). Recently, role of **PPO** in imparting resistance to potato soft rot infection was confirmed by elevated concentration of phenolics in inoculated tubers (Ngadze et al., 2012).

Besides tomato, **PPO** has also been found to exhibit resistance to the disease development in other plant species. These reports correlate the **PPO** activity with disease resistance or link the **PPO** overexpression or induction to the pathogenic attack. In bean, it was found that anthracnose caused by *Colletotrichum lindemuthianum* also increased the activities of **PPO** and levels of phenolics. This pathogen also faced a higher resistance in disease development in various varieties of beans (Campos et al., 2004). The similar behavior was also seen in the cereals crops when they were infected with various fungal species. **PPO** activity was found to be raised in earlier reports when cereal crops were infected by fungal strains that results in improved disease resistance as seen in the pearl
millet-Sclerospora graminicola and wheat-Alternaria triticina interactions (Campos et al., 2004; Raj et al., 2006). In banana, Mycosphaerella fijiensis caused leaf-spot disease which was positively correlated with the activities of PPO, ascorbic acid, phenylalanine ammonia-lyase (PAL) and catalase (Krishnamoorthy et al., 2004). Likewise, isogenic Indian mustard lines when attacked by Albugo candida revealed higher PPO and peroxidase (PO) activities and induced a resistance in the development of white rust (Banga et al., 2004). Phytophthora infestans infected potato tubers were induced by PPO activities (Tomiyama and Stahmann, 1964). The increased PPO activity was also reported due to fungal infection (Fusarium graminium and Erwinia amylovora) in wheat and pear (Mohammadi and Kazemi, 2002; Honty et al., 2005). PPO plays a complex role in plant-microbe interactions. Symbiotic mycorrhizae were also found inducing PPO activity in Moringa concanensis and Zizphus xylopyrus. This higher expression of PPO by symbiotic partner can be helpful to infected plants by imparting resistance from pathogenic attacks (PanWar and Vyas, 2002).

The activation of PPO activity in response to abiotic stresses may be beneficial to plant host by conferring resistance to pathogen infection. Ethylene-induced resistance to Ceratocystis fimbriata infection over sweet potato root part was seen by Stahmann et al. (1966) with an increase in PPO and PO activities. An application of silicon to the Podosphaera xanthii-inoculated cucumber also boosted PPO and PO activity and retard infection caused by the same pathogen (Liang et al., 2005). In the same way, beans treated with Trichoderma also resulted in an increased PPO and PO induction and conferred resistance to the P. syringae infection (Gailīte et al., 2005).

Hydrogen peroxide (H₂O₂) is regarded as an inducer of PPO and many other genes which are involved in plant defensive mechanisms (War et al., 2011). Transgenic crops e.g. cotton, tobacco and potato with an elevated expression of H₂O₂, showed decreased invasiveness of several fungal infection caused by Alternaria, Phytophthora, Rhizoctonia and Verticilium species (Punja, 2001). Expression of dandelion PPO gene in Arabidopsis thaliana (a plant having no PPO gene) resulted in active PPO protein formation. The extracts of transgenic Arabidopsis plants exhibited substrate-dependent antibacterial activity against P. syringae pv. tomato indicating a strong contribution of a
specific single PPO isoform to disease resistance. Therefore, the specific PPO isoenzymes can be proposed as a new family of pathogenesis-related (PR) proteins (Richter et al., 2012). Overall, these reports depicted that PPO plays pivotal role against disease resistance in several plant species.

1.9 PPO in Insect Resistance

Role of PPO is also well-known in defense as it is regulated and induced in response to insect pests attack. For example, Beet armyworm (Spodoptera exigua) and cotton bollworm (Helicoverpa armigera) are one of the devastating polyphagous pests causing serious damages to important cash crops including tomato. Control measures for beet armyworm and cotton bollworm were expensive and unsuccessful as these insects developed resistance against insecticides. Use of transgenic crop provided a good tool to control insect attacks (Godfrey and Kaffka, 2003). In an earlier study, reduction of forest tent caterpillar (FTC) attacks in Populus was achieved by overexpression of PPO gene (Wang and Constabel, 2004a). There are evidences that suggest the role of PPO in plant defense against arthropod herbivory especially in the disease incidence. So far, some reports also correlate the role of PPO activity to the resistance in insect herbivory (Ramiro et al., 2006). Moreover, it was evident that an inhibition of PPO activity leads to higher growth rates as well as insect feeding (Felton et al., 1992). PPO was also found reducing the growth rate of Lepidopteran larvae when incorporated in artificial diet (Felton et al., 1989). Interestingly, PPO activity can be induced in many plants by wounding and arthropod herbivory and an induction of PPO activity empowered those plants with increased resistance to insect herbivores.

In a previous study, systemic induction of PPO gene against herbivory, methyl jasmonate and wounding treatments were reported in trembling aspen (Haruta et al., 2001). The systemic induction of PPO due to insect attack is also reported in tobacco, hybrid poplar and potato (Steffens and Zabeau, 1994; Thipyapong et al., 1995; Constabel et al., 2000). It was found that the stressed plants of tomato due to the attack of insects or wounding, when treated with JA (both known to induce PPO activity) developed a broad-spectrum increase in resistance including other insects, mites, aphids and even a bacterial pathogen (Stout et al., 1998). PPO modified expression in transgenic provides a powerful
tool to find the role of PPO against arthropods specifically significant to estimate the role of PPO in induced resistance. Another study by Barbehenn et al. (2007) showed slight reductions in growth rates of two caterpillars (Lymantria dispar and Orgyia leucostigma) feeding on poplar plant with overexpressing PPO. Mahanil et al. (2008) performed insect assays using leaves of non-transformed, PPO overexpressing and PPO suppressing tomato plants. Growth of attacking larva was 60% lower on leaves of PPO overexpressing plants than on leaves of non-transformed lines. While in PPO down-regulated tomato plants, the larval growth was 37% higher than on non-transformed control plants. Further, elevated PPO levels in overexpressing lines resulted in higher mortality of insect larva (Mahanil et al., 2008). This result provided clue for PPO in insect defense.

Recently, to evaluate PPO expression in resistance to two major lepidopteran insects, the common cutworm (Spodoptera litura) and the cotton bollworm (Heliothis armigera), transgenic tomato lines with PPO over expressing (sense) and PPO down-regulated (anti-sense) were used. PPO over expressing plants showed increased resistance in terms of lower growth rates of cutworms and less consumed foliage indicating a vital role of PPO in insect resistance (Thipyapong et al., 2004b). Populus plants overexpressing PPO showed a little effect on insect herbivory on contrary (Barbehenn et al., 2007). In another study, larvae feeding on PPO overexpressing tomato showed reduced weight gain and high mortality indicating role of PPO in plant defense against insects (Bhonwong et al., 2009).

1.10 PPO in Water and Salt Stress

PPOs also take part in salt and drought stresses. In previous report, total phenolic contents were found higher in osmotic stressed rice seedlings than normal rice plants. In shoot 93% phenolics were observed under osmotic stress that affects to reduce in activity of PPO. Cell membrane fluidity is believed to be adjusted by PPO content alteration to adjust cells in stress (Lee et al., 2007). Amplified level of phenolic contents in barley stem accompanied by lowered PPO activity was thought to adjust osmotic stress (Ali and Abbas, 2003). PPO down-regulated plants showed a better drought adaptation showing plant adjustments with leaf wilting, curling and yellowing as compared with non-
transformed and *PPO* overexpressing lines in water stress. Hence, down-regulation of *PPO* indicated a water stress adapting behavior. Further, non-transformed plants induced *PPO* in mature leaves and *PPO* overexpressing lines developed abscission in order to cope with drought and bound nutrients movement. Such a differential expression of *PPO* correlates *PPO* genes in various plants parts especially in tissues to settle in different stresses (Thipyapong et al., 2004b). In another work, Coconut *PPO* activity was reported to be increased in water stress (Shivishankar, 1988). Recently, wheat *PPO* activity was also found elevated in drought and salt (Kaur and Zhawar, 2015).

Salt and UV radiation can also induce *PPO* activity as growing *Trigonella* calli on medium supplemented with sodium chloride (NaCl) was observed to have an increased *PPO* levels (Niknam et al., 2006). On contrary *PPO* activity decreased in tomato under UV environment to reduce stress injury (Balakumar et al., 1997). *PPO* activity was reported to be increased using 100 mM NaCl and phenyl urea resulting the decreased phenolics, flavonoids and peroxidase in shoot. Ultimately phenolics and indole acetic acid decreased in roots which cause an increase in *PPO*, peroxidases and flavonoids, which finally decrease the growth of salt stressed plant than non-saline control plants (Ali (Ali and Abbas, 2003). Recently, strawberry *PPO* gene was observed to be regulated by salt stress (Jia et al., 2016).

**1.11 *PPO* in Wounding**

Wound inducibility of *PPO* makes it important in plant defense. *PPO* expression was observed in hybrid poplar by mechanical injury which simulates insect attacks. A considerably high *PPO* activity developed in wounded and unwounded leaves on treated plants. On treatments of MeJ and attack of forest tent caterpillar induced *PPO* expression showing *PPO* role in insect defense and MeJ signals to *PPO*. Wound and herbivore induction of *PPO* in hybrid poplar supports the defensive role of *PPO* protein against insects (Constabel et al., 2000). Potato with down-regulated *PPO* expression obtained using *Agrobacterium* transformation. Reduced *PPO* activity resulted in a considerable lowered browning after wounding as compared to non-transformed plants. These results highlight the importance of *PPO* mediated phenolic oxidation in browning of potato (Arican and Gozukirmizi, 2003). GUS derived expression of pineapple *PPO* revealed that this *PPO* promoter possesses chilling and wound inducible activity (Zhou et al., 2003).
Similarly, high level of \textit{PPO} induction due to wounding is also reported previously in pineapple (Stewart et al., 2001). \textit{PPO} activity revealed a direct link to latex fluidity and its coagulation indicating importance of \textit{PPO} latex coagulation and wound sealing in dandelion (Wahler et al., 2009).

Expression profile analysis of \textit{PPO} gene in injured artichoke showed that \textit{PPO} was much induced after 48 hours despite the browning discoloration was in progress before. To find out the participation of \textit{PPO} gene in reaction to wounding injury, \textit{PPO} mRNA transcript levels were analyzed in injured artichoke heads by qRT-PCR experiments after different time intervals after wounding. Data showed that \textit{PPO} transcript levels started increasing gradually shortly after wounding, attaining maximum levels of inductions after 48 hours and then decreasing after 72 hours. Such wound induced expression of \textit{PPO} gene correlates \textit{PPO} for its possible role in plant defense (Quarta et al., 2013). This notion of \textit{PPO} in plant defense is supported by experimental data obtained in tomato with altered \textit{PPO} expression (Thipyapong et al., 2004b). In another report, rubber tree \textit{PPO} mRNA transcripts were found to be regulated by wounding stress, plant hormone ethylene, H$_2$O$_2$, and methyl jasmonate treatments (Li et al., 2014).

In a recent study, potato (\textit{Solanum tuberosum} L. cv. Estima) was transformed to reduce \textit{PPO} activity by down-regulation of \textit{PPO} gene. Potato tubers with reduced \textit{PPO} activity did not go through browning after tuber cutting. Levels of fifteen metabolites out of 134 were differed in wild and transgenic tubers at the time of harvest. Following in T1 and T2 lines a considerably 63 metabolites changed after 48 hours after injury. During harvesting wild types of potato affected more by injury and had large impact on metabolic profile and high browning than \textit{PPO} down-regulated potato lines. Overall, mechanical damage to potato tubers has a considerably greater impact on the metabolite profile than any potential unintended effects resulting from the down-regulation of \textit{PPO} gene expression (Shepherd et al., 2015).
1.12 *PPO* Induction and Elicitors

Role of plants hormones in defense is vital such as ethylene, jasmonic acid (JA), abscisic acid (ABA), gibberllic acid (GA) and salicylic acid (SA). These hormones act as signals for any stress conditions and interact mutually to overcome pathogen or insect attack (Pieterse and Dicke, 2007; Spoel et al., 2007; Pieterse et al., 2009). *PPO* has been signaled by various signaling molecules such as ethylene (Newman et al., 2011), ABA (Chai et al., 2013; Kaur and Zhawar, 2015; Jia et al., 2016), GA (Zhou et al., 2003), JA and SA (Shetty et al., 2012; Jia et al., 2016) indicating role of *PPO* in plant defensive. Histochemical analyses of transgenic tomato lines for GUS activity in various vegetative and reproductive tissues was carried out to evaluate *PPOB* responsiveness to ethylene application. It was revealed that *PPOB* expression and induction was tissue specific and developmentally regulated. Further, ethylene was induced and localized mainly to mitotic or apoptotic tissues (Newman et al., 2011).

Methyl jasmonate (MeJ) is an essential hormone in the immune system of plants which can trigger production of defense related protein such as PPO under herbivorous attacks (Pozo and Azcón-Aguilar, 2007; Pieterse et al., 2009). The MeJ linked induced resistance against insects also activated other defense enzymes that showed anti-herbivory effects such as protease inhibitors and peroxidase along with PPO (Constabel and Ryan, 1998; Moore et al., 2003). In an earlier study, *PPO* activity was found to be enhanced by exogenous application of JA in *Solanum lycopersicum* that restraint the growth of caterpillar *Manduca sexta* (Minton et al., 2016). Six *PPO* genes of eggplant were induced by MeJ (wound signal) and JA in transgenic tobacco showing potential of *PPO* in such stresses in plants (Shetty et al., 2012). Koussevitzky et al. (2004) reported elevated level of *PPO* activity induced by MeJ and wounding. Further, the authors showed that pretreatment of tomato with MeJ enhanced the import and processing of *PPO* into chloroplast. Likewise, higher *PPO* gene expression and transcript level in tomato has been investigated by exogenous JA treatment (Thipyapong and Steffens, 1997). In *Datura wrightii* trichome, MeJ exposure induced the *PPO* activity by more than three folds (Hare and Walling, 2006).
On the contrary, combined treatment of hot air and MeJ resulted in reduced PPO expression in peach fruit (Jin et al., 2009). In another study, increased PPO expression was also found in tomato seeds by exposure to MeJ specifically in micropylar end of the endosperm (Maki and Morohashi, 2006). However, exogenous MeJ has been shown to increase the activity of other enzymes beside PPO such as in catalase, superoxide dismutase and ascorbate peroxidase in harvested loquat fruit (Cao et al., 2009). Most recently, Asghari and Hasanlooe (2016) demonstrated the enhanced PPO and some other defensive enzymatic activities in strawberry fruit by exposure to MeJ. Similarly, the application of MeJ significantly enhanced the expression of PAL, PPO and cinnamyl alcohol dehydrogenase in two Brassica napus cultivars (Farooq et al., 2016). Collectively, these data suggest that increase in PPO activity can be mediated by exogenous application of MeJ in several plant species. Subsequently, interaction of PPO and MeJ has good potential to be used in establishing pathogen resistance, enhancing shelf life of fruits and to increase antioxidant capacity in plants.

1.13 Aims and Objectives

Keeping in view the role of PPO in plants, a research project was designed to express anti-sense potato PPO gene under the control of wound inducible rice OsRGLP2 promoter. The present study is focused on the following objectives:

- The identification and retrieval of PPO genes from diverse group of available plant species and analyses of their conserved motifs and phylogenetic relationships
- To characterize the endogenous tobacco (Nicotiana tabacum) PPO gene expression in wounding stress, MeJ and ABA application
- To develop transgenic tobacco with expression vector harboring anti-sense PPO gene ligated downstream to a wound inducible promoter
- Expression profiling of transgenics in response to mechanical wounding, elicitors and biotic assay
Chapter 2

MATERIALS AND METHODS

This chapter is further divided into five main sections; in the first section, computational analyses of PPO gene from different plant species are described. In the second section, the effect of wounding, MeJ and ABA on untransformed tissue cultured tobacco plants were discussed in relation to the induction of PPO. In the third section, anti-sense PPO gene (AsPPO) containing construct was prepared and transformed into Agrobacterium tumefaciens. While in the fourth section, Agrobacterium mediated genetic transformation of AsPPO of tobacco plants was carried out. Finally, in the fifth section, the treatment of the transgenic plants with elicitors (MeJ and ABA), wounding and whitefly was described in relation to the induction of the AsPPO gene under the control of wound inducible OsRGLP2 promoter.

2.1 Computational Analyses

Computational analyses of PPO gene from different plant species was performed using various bioinformatics tools.

2.1.1 Sequence retrieval and multiple sequence alignment

The full length coding sequences of ninety five PPO genes from thirty six different plant species were retrieved from Sol Genomics Network (https://solgenomics.net/organism) and National Center of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Moreover, EMBOSS Transeq program (http://www.ebi.ac.uk/Tools/st/emboss_transeq) was used to translate the nucleotide sequences into amino acid sequences. Beside it, multiple sequence alignment (MSA) of all PPO amino acid sequences were executed using ClustalW (Thompson et al., 1994). The MSA was confirmed and curated manually by observing at the superposed structures in order to examine the conserved functional motifs.

2.1.2 Phylogenetic analysis

The neighbor-joining approach implemented in the Molecular Evolution Genetic Analysis version 6.0 (MEGA6) (Tamura et al., 2013) was employed for constructing phylogenetic relationship among different plant species. Based on PPO sequences, the
reliability of the phylogenetic tree was assessed with 1000 bootstrap replicates. Further, percentage amino acid composition was calculated using MEGA6 (Tamura et al., 2013).

2.1.3 Chromosomal distribution of PPO genes

The location of potato PPO genes on chromosome was predicted using online data base Interactive Genome Map for Plant (IGMAP; http://nipgr.res.in/igmap.html).

2.1.4 Physiochemical characterization

The physicochemical analyses of selected potato PPO proteins were calculated by ProtParam tool (http://web.expasy.org/protparam/), including isoelectric point (IP), molecular weight (MW), total number of negative (-R) and positive (+R) residues, aliphatic index (AI), grand average of hydropathicity (GRAVY), instability index (II) and extinction coefficient (EC). Besides these parameters, the subcellular localization studies of PPO genes were implemented using WoLF PSORT and CELLO server v.2.5 respectively (Yu et al. 2006). Phosphorylation potential for potato PPO genes was predicted using NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) while NetNGlyc 1.0 server was used to identify N-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.1.5 Structure prediction

For the elucidation of structure of PPO, conserved domain motifs in PPO genes were discovered using Multiple EM for Motif Elicitation server (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Bailey et al., 2015). Furthermore, the discovered motifs were blast in NCBI. Depending upon the acquired information, the modeling of the 3D structure of the potato PPO proteins was performed by using Swiss Model server (http://swissmodel.expasy.org/interactive) (Biasini et al., 2014). The quality and validation of the predicted models were analyzed by Rampage Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/) and structure visualization was performed using pymol software (https://www.pymol.org/).
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2.2 Tobacco Tissue Culture and Induction of Endogenous PPO Gene

2.2.1 Plant material

Tobacco plants (Nicotiana tabacum cv. Xanthi) were used as a tool to study the expression of PPO gene under different stress conditions in un-transformed as well as in transgenic plants. The seeds of N. tabacum were obtained from National Agriculture Research Centre (NARC), Pakistan.

2.2.2 Seed sterilization, germination and plant growth

Seeds of tobacco were washed with distilled water for 5 times in a petri dish. Further, seeds were sterilized in 70 % ethanol for 5-8 minutes and again rinsed with distilled water immediately. Afterwards, seeds were treated with 20 % clorox followed by washing with distilled water for 3-5 times. Seeds were dried by blotting on sterilized filter paper and placed on simple MS media (Table 2.1) for germination. These plates were maintained in growth chamber under control conditions of 16:8 dark and light cycle at 27 °C. Small seedlings at 2-3 leaves stage were shifted to large jars containing simple MS media.

2.2.3 Wounding of un-transformed plants

For induction of the expression of endogenous PPO genes, the leaves of un-transformed plants were mechanically damaged (with the help of forceps) and placed for 12, 24, 36 and 48 hours. After mechanical injury, the wounded leaves were collected, frozen in liquid nitrogen and stored at -80 °C for further experiments.

2.2.4 Treatment of un-transformed plants with MeJ and ABA

To examine the expression in response to elicitors, non-transgenic tobacco plants were treated with the 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. To analyze the expression of PPO gene endogenously, seven days old non-transgenetic tobacco plants were sprayed with 100 μM, 200 μM, 300 μM, 400 μM and 500 μM ABA and MeJ solutions respectively and placed in growth room for 24 hours. C-
Table 2.1: MS medium composition for seeds germination.

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>4.43 g/L</td>
</tr>
<tr>
<td>2</td>
<td>Sucrose</td>
<td>30 g/L</td>
</tr>
<tr>
<td>3</td>
<td>Gelatin</td>
<td>2 g/L</td>
</tr>
</tbody>
</table>
Control plants were sprayed with double distilled water. Samples treated with MeJ and ABA were harvested after 24 hours. The harvested samples were immediately frozen in liquid nitrogen and stored at -80 °C till further use.

2.2.5 RNA isolation

Total RNA was isolated from frozen samples, harvested from mechanical wounding, ABA and MeJ treatments using Spectrum™ Plant Total RNA isolation kit (Sigma, USA). Briefly, ~100 mg of frozen plant material was homogenized in a FastPrep FP120 instrument (MP Biomedicals, Solon, OH, USA) at 6.0 m/s for 40 seconds. The homogenized plant sample was lysed by adding 500 μL of lysis buffer along with 5 μL of 2-mercaptoethanol (2-ME) and vigorously vortexing the reactants for 30 seconds. This mixture was incubated for 3-5 minutes at 56 °C and centrifuged for 3 minutes 15000 rpm to pellet down cellular debris. In filtration step, lysate supernatant was shifted into a new collection tube with a blue filter column and centrifuged for 1 minute at maximum speed. In next step, 300 μL of binding solution was added to the clarified lysate for binding of RNA to the silica of column. Thereafter, the binding solution and lysate were mixed by pipetting for 5 times and the solution was shifted to binding column followed by centrifugation for 1 minute at 15000 rpm.

The tube was dried on clean absorbent paper after decanting the flow-through liquid from binding column to a waste container. Further in first washing step, 300 μL of wash solution 1 was added directly into the binding column and centrifuged for 1 minute at 15000 rpm. After first washing step flow-through was discarded and 500 μL of wash solution 2 was transferred into the column and centrifuged at 15000 rpm for 1 minute. The third column washing step was same as described in second washing step. After removing the washing solutions, the column was dried by centrifugation for 1 minute at 15000 rpm and binding column was placed into a fresh collection tube. Finally in elution step, 50 μL of elution buffer was directly applied into the center of binding matrix inside the column and incubated for one minute at room temperature followed by centrifugation for one minute at 15000 rpm. The eluted RNA was stored at -80 °C until further processing.
2.2.6 DNase treatment

To avoid genomic DNA, freshly isolated RNA was treated with DNase. The reaction mixture consist of 2.5 μL DNase, 2.5 μL RNAsin, 10 μL RDD buffer, 50 μL eluted RNA and 35 μL of double distilled water. The samples were vortexed, spin down and incubated for 20 minutes at room temperature. After DNase treatment, samples were cleaned with a Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) as described in manufacturer’s protocol.

2.2.7 Assessment of quality and quantity of RNA

The concentration of purified RNA was measured at 260/280 nm using 1 μL of RNA sample through Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Furthermore, the quality of RNA was also assessed by 2% agarose gel electrophoresis.

2.2.8 cDNA synthesis

The first strand cDNA was obtained from 3 μg of RNA with nonamer and random oligonucleotide primer (2.5 μM) in a volume of 18.4 μL. The reactants were incubated for 5 minutes at 65 °C followed by incubation at room temperature for ten minutes. To complete the recipe of reaction mixture, 40 U RNAsin (Promega), 25 mM dNTPs, 200 U Superscript II reverse transcriptase (Invitrogen), 10 μM dichlorodiphenyltrichloroethane (DTT) and 5X first-strand (FS) buffer were added to it and volume reached to 30 μL. This reaction mixture was incubated in a water bath at 42 °C for 1 hour and further incubated at 70 °C for 10 minutes to inactivate superscript enzyme. The reactants were spin down for short while and volume was raised to 100 μL with nano-pure water. Freshly prepared cDNA was stored at -20 °C till further use.

2.2.9 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. The primer set (NiPPO2) was designed from core region of endogenous tobacco PPO for qRT-PCR
(Table 2.2). 18s rRNA and actin were selected as reference genes for the normalization of PPO gene expression. qRT-PCR for all primers was started with denaturation at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds and 75.5 °C for 15 seconds. Melting curve analysis consisting of 95 °C for 15 seconds, 55 °C for 1 minute and 95 °C for 15 seconds. 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 and Bertone, 2009) and Limma (Gentleman et al., 2006) add-on packages were used for the manipulation and analysis of the Ct values.

2.3 Antisense Polyphenol Oxidase Gene Vector Construction (AsPPO)

The targeted PPO gene ligated in antisense orientation downstream to wound inducible OsRGLP2 promoter in pCAMBIA1391Z_OsRGLP2 vector which was used for transformation in tobacco.

2.3.1 Construct designing

Based on computational analysis, 655 bp of potato PPO di-copper binding domain region was selected and amplified with following gene specific primers which were tailed with EcoRI and AvrII restriction enzymes sites.

\[
PPO1: 5'-CCT AGG GAT GAT CCA ACT TTC GC-3'
\]

\[
PPO2: 5'-GAA TTC TTG AAG TTA CGC CAT GG-3'
\]

PCR conditions used for amplification were pre-PCR denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 47 °C for 30 seconds and extension at 72 °C for 1 minute. Final cycle was same except extension at 72 °C for 20 minutes was done to make sure that any remaining single-stranded DNA became fully extended. Then a final hold step at 4 °C was employed for short term storage for reaction in a gradient MultiGene Thermal Cycler (Labnet). For cloning, the ligation of amplicon of interest was carried out using T/A cloning vector (pTZ57R/T; Fermentas). For ligation, total reaction mixture was prepared in 30 μL containing PCR product (4 μL), PEG 4000 (3 μL), T4 DNA ligase (1 μL), 10X ligation bu buffer (3 μL), vector (1 μL) and value was made up to 30 μL with autoclaved nanopu-
Table 2.2: List of primers used in qRT-PCR for expression analysis of endogenous *NtPPO* gene in un-transformed tobacco plants.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>5’-GGTGAGCGATTTGTCTGGT-3’&lt;br&gt;5’-CAGGCTAGGTCTCGTTG-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>5’-CCTGAGGTCTTTTCCAACCA-3’&lt;br&gt;5’-GGATTCCGGCAGCTTCCATT-3’</td>
</tr>
<tr>
<td><em>NtPPO2</em></td>
<td>5’-AACCCGGTCCGTGTAAGTCC-3’&lt;br&gt;5’-CTTCGATTACGCAACCAGCCA-3’</td>
</tr>
</tbody>
</table>

**Key:** 18S; 18S rRNA, *NtPPO*; *Nicotiana tabacum* polyphenol oxidase
Chapter 2

re water. The ligation reaction was carried out at 22 °C for 16 hours in a water bath.

2.3.2 Preparation of electro competent cells

The single colony of *E. coli* strain DH5α was cultured in 3 mL liquid LB media at 37 °C with continuous shaking of 250 rpm for overnight. This starter culture was shifted into liquid LB media and incubated with continuous shaking at 37 °C until it attained O.D<sub>600</sub> of 0.5. Bacterial culture was harvested by centrifuging (4000 rpm) for 15 minutes at 4 °C and washed with ice cold autoclaved 15 % glycerol. The resultant pellet was washed and resuspended in ice cold 15 % glycerol. The washing step was repeated for three times. After final washing the 15 % glycerol was decanted and pellet was resuspended in residual 15 % glycerol. The harvested bacterial suspension was shifted into 50-100 μL aliquots and stored at -80 °C for further use. The viability of cells was checked by spreading different dilutions of competent cells on LB media and incubated at 37 °C overnight.

2.3.3 Electroporation

The competent cells (50 μL) mixed with 2 μL ligated mixture, was transformed into DH5α by using Bio-Rad Micropulser<sup>TM</sup> (Cat# 165-2100) at a recommended voltage. Transformed mixture was again mixed with 1 mL liquid LB and shaked for 1 hour at 37 °C. Confirmation of electroporation into *E. coli* cells was carried out by spreading transformed mixture on LB plates supplemented with IPTG/XGal and ampicillin (50 mg/L) and incubated at 37 °C for 16 hours. White and blue colonies were screened after 20 hours of incubation.

2.3.4 Plasmid isolation and confirmation of transformation by PCR

White colonies were picked and cultured in liquid LB media supplemented with ampicillin (50 mg/L) and incubated for 12 hours at 37 °C with constant shaking of 250 rpm. Plasmid isolation was carried out from growing culture (Appendix 1) and successful cloning was confirmed via PCR using specific pair of primers *PPO1* and *PPO2* by taking isolated plasmid as template DNA. The amplified product was checked on 1.5 % agarose gel which confirms the cloning of *PPO* gene targeted region.
2.3.5 Sequencing of targeted region of PPO gene

The confirmed clone containing targeted region of PPO gene was purified and sent to Macrogen (Korea) for sequencing. The obtained sequence data was analyzed with the sequences already reported in NCBI database to check out the similarity.

2.3.6 Restriction digestion

The cloned PPO gene from T/A cloning system and expression backbone p1391z_OsRGLP2 were restricted with EcoRI and AvrII restriction enzymes at 37 °C for 3 hours. The following reaction mixture was used for digestion of p1391z_OsRGLP2 vector and cloned PPO gene segment;

1. EcoRI 0.5 μL (20 units/μL)
2. AvrII 0.5 μL (5 units/μL)
3. NEBuffer 3.1 1 μL
4. Plasmid 4 μL
5. Nano pure water 4 μL

The digested products were run on low melting agarose gel and finally eluted by Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. The integrity and quality of restricted products were confirmed by running on 1.5 % agarose gel.

2.3.7 Ligation of PPO gene segment in expression vector p1391Z_OsRGLP2

The recombinant plasmids were made by ligating the digested p1391Z_OsRGLP2 plasmid and eluted PPO gene specific insert downstream to OsRGLP2 promoter in antisense orientation. Ligation reaction was carried out at 22 °C for overnight using eluted PPO gene specific insert (6 μL), digested p1391Z_OsRGLP2 (2 μL), T4 DNA ligase (5 units/1μL) and 10X ligation buffer (1 μL). The expression cassette containing PPO gene targeted region ligated downstream to OsRGLP2 in antisense direction in p1391Z_OsRGLP2 was named as AsPPO.
2.3.8 Transformation of AsPPO into Agrobacterium tumefaciens

Transformation of expression vector (AsPPO) into Agrobacterium tumefaciens (EHA101) was carried out by electroporation. The procedure used for the preparation of A. tumefaciens (EHA101) competent cells was same as done for DH5α strain of E. coli (Section 2.3.3) except the incubation temperature, which was 28 °C for 24-48 hours. Electroporation was carried out by using 2 μL of AsPPO and 50 μL of A. tumefaciens strain EHA101 electrocompetent cells at voltage of 2.2 kV/0.1 cm with Bio-Rad Micropulser™ (cat# 165-2100) following its immediate suspension in 1 mL of liquid LB media. Electrocompetent cells (without plasmid) were used as a negative control during electroporation. The resultant electroporated mixture was incubated at 28 °C for 2 hours with continuous shaking at 250 rpm. Screening of transformed A. tumefaciens was carried out by spreading the cells over solid LB plate supplemented with kanamycin (50 mg/L) after incubation at 28 °C for 24-48 hours. The resultant colonies were subjected to plasmid isolation and transformation was confirmed with the help of PCR as described in section 2.3.1 and with hygromycin (HygF and HygR) gene primers. The amplification of hygromycin gene region was carried out in a total volume of 25 µL containing 16.2 µl of nano pure water, 2.5 µl of 10 X PCR buffer, 1.5 µl of 2 mM dNTPs, 1.5 µl of 25 mM MgCl₂, 1 µl of each primer, 0.3 µl of Taq polymerase (5 U) and 1 µl of plasmid using gradient Multigene Thermal Cycler (Labnet). The PCR cycling profile was as follows: 5 minutes at 94 °C, 35 cycles of 40 seconds at 94 °C, 40 seconds at 55 °C, 45 seconds at 72 °C and final extension of 20 minutes at 72 °C. The sequences of hygromycin primers are given below;

HygF: 5’-GCTCCATACAAGCCAACCAC-3’

HygR: 5’-CGAAAGTTTCGACAGCGTCTC-3’

The plasmid was further confirmed by restriction digestion with AvrII and EcoRI enzymes as described previously in section 2.3.6.

2.4 Tobacco Transformation with AsPPO Gene

For the transformation of tobacco plant with AsPPO gene, already tissue cultured plant material was used (as described in section 2.2.2).
2.4.1 Explant preparation for transformation

Small leaf discs were prepared by cutting young leaves into small pieces and were placed upside down on solidified MS media previously used for seed germination (Table 2.1; Section 2.2.2). Nearly 10-15 leaf discs were placed on single plate, sealed with parafilm and incubated for two days in growth chamber under controlled conditions (as described in section 2.2.2.).

2.4.2 Transformation procedure

For transformation experiment, recombinant A. tumefaciens strain EHA101 was first streaked over LB plates having 50 mg/L kanamycin. A resistant colony was carefully picked with the help of autoclaved loop. This resistant colony was grown in liquid LB with the same antibiotic selection in incubator shaker at 28 °C for 36-48 hours with constant shaking of 250 rpm. Sterilized explants (leaf discs) were placed in bacterial cultures having OD$_{600}$ of 0.1 to 0.2 in petri dishes with constant shaking for 5-10 minutes. The infected explants were washed with cefotaxime (500 mg/L) to inhibit the overgrowth of Agrobacterium.

2.4.3 Co-cultivation

The infected leaf discs were dried on a sterile autoclaved blotting paper and were shifted to the co-cultivation media (Table 2.1). Plates were sealed and incubated for two days at 28 °C in dark.

2.4.4 Selection of transformants

Infected leaves after co-cultivation were shifted to selection media (Table 2.3) having hygromycin (50 mg/L) and cefotaxime (250 mg/L). The explants which were not transformed got pale yellow and died. The transformants that survived on selection medium were shifted to fresh selection media (Table 2.3).

2.4.5 Shoot induction and regeneration

After 15-28 days regeneration of putative transgenic explants was started in the form of small calli from the cut edges of explants. These calli were separated individually
Table 2.3: Composition of MS selection medium.

<table>
<thead>
<tr>
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<td>30 g/L</td>
</tr>
<tr>
<td>3</td>
<td>BAP</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>4</td>
<td>IAA</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td>5</td>
<td>Gelatin</td>
<td>2 g/L</td>
</tr>
<tr>
<td>6</td>
<td>Hygromycin</td>
<td>50 mg/L</td>
</tr>
<tr>
<td>7</td>
<td>Cefotaxime</td>
<td>250 mg/L</td>
</tr>
</tbody>
</table>
and shifted to jars for shoot induction. As the selection medium is containing phytohormones, several shoots were emerged from the putative transgenic calli within 3 weeks. Shooting initiated in the form of small shoots after three weeks on same selection media. These shoots were later removed individually from each callus and were shifted to jars at the same growth conditions.

2.4.6 Root induction and hardening of transgenics

The regenerated shoots having two to three internodes were cut off and transferred to simple half strength MS medium for root induction. These plants were later shifted to soil and maintained in green house to obtain fully mature transgenic plants.

2.4.7 Affirmation of tobacco transformation

Transgenic plants were confirmed via PCR. For PCR, gDNA from transgenic plants and control untransformed plants were extracted following the CTAB method (Richards et al., 1997). PCR was performed using PPO gene specific primers (PPO1 and PPO2) and hygromycin primers (HygF and HygR). The primer sequences and reaction conditions for PCR were same as described in previous sections (2.3.1 and 2.3.8).

2.5 Expression Analysis

To analyze the transgene expression, qRT-PCR was carried out after treating transgenic plants with both biotic and abiotic stresses. Each experiment was repeated thrice.

2.5.1 Wounding induction and treatment of transgenic plants with elicitors

To study the effect of wounding on the induction of PPO, the leaves of control and transgenic plants were damaged mechanically (with the help of forceps) and placed for 12 and 24 hours on solid MS medium. Similarly, the induction of the expression of PPO gene in transgenic plants was also studied by applying ABA and MeJ (100 µM and 200 µM). Samples treated with MeJ and ABA were harvested after 6 and 12 hours. The rest of the procedures for stressed plants were carried out as described previously in section 2.2.3 to 2.2.9. However, three different sets of primer were designed from
tobacco *PPO* cDNA (Accession No. Y12501.1) sequences for qRT-PCR analysis. As an internal control 18s rRNA and actin were also used (Table 2.4).

### 2.5.2 Biotic stress

The expression of *PPO* gene was also studied in response to insect infestation by using adult whiteflies (*Trialeurodes vaporariorum*) in transgenic plants. The whiteflies were collected by aspiration which had been maintained on tobacco plants growing in controlled conditions (26±2 °C and relative humidity 60-80 %) at Department of Molecular Biology and Genetics, Aarhus University, Denmark. 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 independent and by their choice. For RNA isolation, leaves were harvested and stored at -80 °C after 2, 3 and 5 days of infestation. Further steps from isolation of RNA to qRT-PCR analysis were carried out as discussed previously in section 2.2.5 to 2.2.9 and the same primers sets (Table 2.4) were used for qRT-PCR as described in section 2.5.1.
Table 2.4: List of primers used in qRT-PCR for expression analysis of endogenous *NtPPO* gene in transgenic tobacco plants harboring antisense *PPO*.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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</table>
| 18S         | 5’-GGTGGAGCGATTTGTCTGGT-3’  
              | 5’-CAGGCTGAGGTCTCGTTCGT-3’ |
| Actin       | 5’-CCTGAGGTCTTTTTCCAAACCA-3’  
              | 5’-GGATTCCGGCAGCTCCATT-3’ |
| *NtPPO1*    | 5’-ATGGACAGCGTTCCCTATTACA-3’  
              | 5’-GGAGTACATTGCTAAATACCAGTTAGC-3’ |
| *NtPPO2*    | 5’-AACCCGTTCCGTGTGAAAGTCC-3’  
              | 5’-CTTCGATTACGCAACCAGTCCA-3’ |
| *NtPPO3*    | 5’-TCTCAAAGCTGGACAGAGCC-3’  
              | 5’-CCATCTTCGTCAAGGACCCA-3’ |

**Key:** 18S; 18S rRNA, *NtPPO*; *Nicotiana tabacum* polyphenol oxidase
RESULTS AND DISCUSSION

3.1 Computational Analyses

Computational analyses of PPO gene from different plant species was performed using various bioinformatics tools. The detailed of the computational analyses are given below;

3.1.1 Mining of PPO protein sequences

Ninety five PPO gene coding sequences were randomly selected from thirty six different plant species which were retrieved from NCBI, Sol Genomics Network and translated to amino acids. The accessions numbers, gene identification numbers, length of amino acids and name/abbreviations for PPO genes along with species name are given in Table 3.1. Majority of the reported sequences belongs to family Solanaceae (38), Rosaceae (13), Salicaceae (6), Asteraceae (4), Amaranthaceae (3), Poaceae (17), Fabaceae (4), Euphorbiaceae (1), Theaceae (1) and Rhamanaceae (1). The size of the all PPO genes lies between 495 to 2036 bp while peptide sequences range from 164 to 639 amino acids. Most of the sequences are transcribed from single exon. IGMAP database predicted the position of 12 potato PPO genes on chromosomal DNA which were shown to be scattered on two different chromosomes. Among those 12 potato PPO genes, eleven gene copies appeared as a cluster at two different loci on chromosome 8 while single gene copy (PGSC0003DMG400022430) was observed on chromosome 2 (Figure 3.1). The clustering of majority of potato PPO genes on the same chromosomal location is an indication of common ancestry from which they have evolved by a series of duplication events. Duplication of PPO gene is a common phenomenon reported in many species such as poplar, monkey flower and soybean (Tran et al., 2012).

3.1.2 Physicochemical properties of potato PPO proteins

The physicochemical analysis of potato PPO proteins showed significant variation in size and molecular weight (MW) (Table 3.2). The MW of PPO proteins ranges from 18.088 to 70.326 kDa, which is an indication of variation in the size of polypeptides. However, another report describes the MW of mature plant PPO proteins to
Table 3.1: Description of the PPO genes.

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Figure 3.1: Chromosomal position of potato *PPO* genes based on available data in IGMAP (Interactive Genome Map for Plants).
Table 3.2: List of potato *PPO* genes along with different computed parameters of potato PPO proteins.

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<th>II</th>
<th>Al</th>
<th>GRAVY</th>
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(MW: molecular weight; pl: isoelectric point; +R: positive charged residues; -R: negatively charged residues; EC: extinction coefficient; II: instability index; Al: Aliphatic index values; GRAVY: Grand average of hydropathicity; N-glyco: N-glycosylation; P-site: Phosphorylation site).
be ~ 52-62 kDa (Chevalier et al., 1999). The gene located on chromosome 2 expressed highest MW (70.326 kDa). The values of isoelectric point (pI) ranged from 5.79 to 8.79, which is consistent with the report of Dirks-Hofmeister et al. (2014), who reported pI values for dandelion PPO genes from 5.5 to 7.4. The extinction coefficient (EC) of potato PPO proteins at 280 nm ranged from 14440-94200 cm. The EC values generally depends upon the presence of tyrosine, phenylalanine and tryptophan residues in a protein (Adeloye and Ajibade, 2011). The instability index (II) values of potato PPO proteins ranged from 35.08 to 68.36. Forty percent of these proteins are unstable in in vitro conditions because their instability index values were higher than 40.

Aliphatic index of protein measure the relative volume occupied by aliphatic side chains of the amino acids: leucine, isoleucine, alanine and valine. The protein with higher AI value is considered to be involved in stabilizing the internal conditions of plant during a rise in temperature (Atsushi, 1980). The aliphatic index values for PPO proteins ranged from 67.68 to 76.86. The higher AI values suggest that PPO proteins and the plant harboring these genes can withstand a higher temperature. The most abundant amino acid found was leucine (8 %), whereas the least were tryptophan and methionine (2 %). The average total number of negatively charged (Asp and Glu) residues was found higher than the positively charged (Arg and Lys) residues in studied potato PPO proteins. Besides these parameters, GRAVY values ranged from -0.272 to -0.603 which showed all potato PPO proteins are hydrophilic in nature.

3.1.3 Subcellular localizations of potato PPO proteins

The prediction of subcellular localization of any protein is important to understand the protein function. The subcellular localization of PPO protein was predicted using CELLO v.2.5 which showed that 83 % of the studied potato PPO proteins are localized in chloroplast (Table 3.2). These results are in accordance with the earlier findings regarding eleven dandelion PPO genes (Dirks-Hofmeister et al., 2014). Plant PPO genes possess N-terminal transit peptide that direct the PPO proteins to the lumen of thylakoid membrane (Marusek et al., 2006). The substrates for PPO are located in the vacuole whereas PPO genes are sequestered as latent enzyme in chloroplast. Destruction of cellular compartmentalization is necessary for PPO enzyme-substrate interactions (Bo-
However, the proteins of *StuPPO1* and *StuPPO7* are found to be nuclear as well as extracellular. Moreover, only the protein of PGSC0003DMG400018919 is observed in the cytoplasm and nucleus. Fungal PPO genes were reported to be localized in cytoplasm and have no transit peptide (Rast et al., 2003). The subcellular localization of PPO gene (XP_015159896.1) using PSORT was confirmed to be localized in peroxisome. However, some PPO genes are found deficient in N-terminal transit peptide, and identified to be localized in the vacuole and cytosol (Nakayama et al., 2001; Ono et al., 2006; Tran et al., 2012).

### 3.1.4 Prediction of N-glycosylation and phosphorylation sites

A variable number (2-7) of N-glycosylation sites in potato PPO protein was predicted using NetNGlyc 1.0 server (Table 3.2). The highest number of N-glycosylation sites (7) was detected in XM_015304411.1 while no N-glycosylation site was identified in PGSC0003DMG400018919. N-glycosylation sites are likely important for proper folding, stability and functioning of PPO proteins. In *Solanum melongena* PPO gene three N-glycosylation sites were reported (Shetty et al., 2011). Expression of *SmePPO2* which is restricted only to the root was reported to be non-glycosylated and it was suggested to have some specific function in root tissues (Shetty et al., 2011). Glycosylation is predicted at conserved serine/threonine residues in N-terminal of plant and fungal PPO genes (Marusek et al., 2006). The activity of mammalian tyrosinase and its folding was also found dependent on N-glycosylation at a number of sites (Branza-Nichita et al., 2000; Olivares et al., 2003).

Variable number (7-40) of phosphorylation sites were also observed in protein sequences. Highest number (40) of p-sites was identified in XP_006347083.2. Nine threonine, ten tyrosine and thirteen serine residues were already reported to be phosphorylated in eggplant PPO (Mishra et al., 2013; Mishra and Gautam, 2016). In another study, a conserved serine was identified in linker region that is predicted to be phosphorylated in all plant PPO genes (Marusek et al., 2006). Phosphorylation of proteins can affect the functions and activities of protein, its subcellular location, half-life, docking with other proteins and intrinsic biological activity (Cohen, 2000).
3.1.5 Multiple sequence alignment and conserved motifs analysis

The amino acid sequence of all the studied PPO proteins showed the presence of some conserved regions. All the analyzed species possessed six histidine residues which are responsible to ligate two copper ions of the active site (Marusek et al., 2006; Malviya et al., 2011). The first three histidine residues encompass a region termed the CuA binding domain, and the following three encompass the CuB binding domain (Figure 3.2) (Marusek et al., 2006). In the CuA region, first histidine residue is located at the beginning of HXXXX motif and the most common is HCAYC (Klabunde et al., 1998). The second conserved histidine of the CuA domain is predicted to form thioether bond with cysteine. The active-site histidines and thioether bridges have been reported widely from bacteria to mammals (Van Gelder et al., 1997b; Klabunde et al., 1998; Eicken et al., 1999; Gerdemann et al., 2002). All the plant species comprised of two cysteine, two tyrosine, one tryptophan, two phenylalanine and one arginine residues in Cu binding A site. In the CuB domain, highly conserved histidine residues were located within the HXXXH motif and valine was observed usually at the fourth position of this motif (Tran et al., 2012). Besides cysteine, tryptophan, glutamic acid, methionine, glycine, aspartic acid, proline and phenylalanine were highly conserved residues in CuB region. These all conserved residues are found to be involved in folding of PPO genes (García-Borrón and Solano, 2002).

Copper binding site B is relatively less conserved than the CuA domain. Copper binding region A represented more substitutions than deletions, while CuB exhibited deletion event excessively. The MSA of 18 potato PPO protein sequences revealed the stretches of conserved protein sequences from residues 226 to 241, 265 to 289, 424 to 439 and 466 to 484 (Figure 3.2) between tyrosinase domain. Downstream to CuB region, a highly conserved aspartate was located four residues after the third histidine of the CuB domain that is responsible for hydrogen bonding with tyrosine side chains. The C-terminal region of most of the studied PPO genes showed conserved signature domain KFDV that is similar to the C-terminal domain of hemocyanin (Marusek et al., 2006). Tran et al. (2012) also reported KFDV motif in three Selaginella PPO genes. However, the exact role of these entire conserved motifs still needs to be discovered.
Figure 3.2: Multiple sequence alignment of the studied PPO amino acid sequences. A: Conserved copper binding domain A is boxed, conserved histidine is highlighted in pink while cysteine in brown. B: Conserved copper binding domain B is boxed, conserved histidine is highlighted in pink while aspartate in red.
3.1.6 Prediction of conserved motifs

Conserved motifs were predicted from potato PPO proteins using MEME software. Total five motifs were predicted with their width and sites (Table 3.3). A stretch of 31 amino acids (ERILGKLIDPTFALPYWNWDHPKGMRLPPM) was conserved in all the potato PPO sequences that belong to tyrosinase superfamily. Other motifs (as given in table 3.3) are also associated with PPO_KFDV superfamily and PPO_DWL superfamily. Tyrosinase superfamily is a part of hemocyanins and PPO proteins (Malviya et al., 2011). Tyrosinase is assumed to bind CuA and CuB, both of which are bound by three conserved histidine residues. The presence of these copper binding domains is in accordance with multiple alignment results. The identification of PPO middle domain (PPO_DWL domain) of 50 amino acids has been reported in prokaryotes as well as in eukaryotes (Malviya et al., 2011). The function of PPO_KFDV domain that is located in the C-terminal region of PPO gene is still not clear (Li et al., 2014). The role of PPO in different physiological processes such as in browning reaction and defense mechanisms has been reported in several studies.

3.1.7 3D structural analysis and its validation

Three-dimensional structures (3D) of protein contribute to understanding protein, interactions, functions and active sites. Nuclear Magnetic Resonance spectroscopy and X-ray crystallography are difficult and costly than in silico methods (Jaroszewski, 2009). To determine the structure of unknown proteins, it is possible to compare it with the structure of already known protein present in protein data bank (Fiser et al., 2000; Webb and Sali, 2014). 3D structures for eighteen potato PPO genes were modeled using Swiss-Modeler program (one representative is shown in Figure 3.3). For further analysis, the best model was selected from three models based on Z-score (Benkert et al., 2011).

The accuracy and stereochemical quality of the predicted model were evaluated using the Ramachandran plot calculation with RAMPAGE server (one representative is shown in Figure 3.4). In Ramachandran plot, the residues were classified according to their region in the quadrangle. The 3D structure of potato PPO genes showed the four helical bundles and di-nuclear copper center (consisting of six conserved histidine residu-
### Table 3.3: Motifs found in the potato PPO protein.

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<tr>
<td>4</td>
<td>LDKAISFSINRPASSRTQQEKNAAQEMLTNFNEIKYDNRDYIRFDVFLNVD</td>
<td>50</td>
<td>PPO_KFDV</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>EVDTPQLQIMTNNLTLMYRQMVTNPCPSQFFGAAYPLG</td>
<td>39</td>
<td>N/A</td>
<td>15</td>
</tr>
</tbody>
</table>
**Figure 3.3:** The three-dimensional structure of predicted potato PPO protein in ribbon display mode showing N-terminal domains in violet, C-terminal domain in blue and linker region in red.
Figure 3.4: RAMPAGE values indicating the number of residues in favored, allowed and outlier region.
Similar structural organization in Dandelion \textit{PPO} genes has been reported by Dirks-Hofmeister et al. (2014). Moreover, 83\% of the protein models (15 out of 18) showed similar structural organization. The results of RAMPAGE analysis revealed that on an average, only 2.4\% of the residues were outliers while 94.4\% were in favored region and 3.2\% were in the allowed region. Ramachandran plot analysis was used in several studies to determine the quality and reliability of PPO proteins structures (Nokthai et al., 2010; Fernandes et al., 2012). It is being claimed that the predicted 3D model for potato \textit{PPO} genes had good quality and reliability (as supported by various parameters).

### 3.1.8 Phylogenetic analysis

A neighbor-joining phylogenetic construction from multiple sequence alignment of protein sequence of thirty six plant species was drawn (Figure 3.5). Phylogenetic tree of the studied sequences bifurcated into two distinct clades. Most of the dicots are arranged in clade I while monocots clustered in clade II. The clade I is further subdivided into two clusters (Cluster I and II) which mostly consist of species from family \textit{Solanaceae}. The cluster I of clade I consist of six members and all of them belongs to family \textit{Solanaceae} (\textit{S. tuberosum}, \textit{S. melongena}, \textit{S. lycopersicum}, \textit{N. tabacum}, \textit{N. benthamiana} and \textit{N. sylvestris}), while cluster II contains three species (\textit{S. tuberosum}, \textit{S. lycopersicum} and \textit{T. officinale}).

Among the \textit{S. melongena} seven \textit{PPO} (\textit{V1-V3}, \textit{V5-V7} and \textit{SmePPO2}) genes clustered in one group with bootstrap value of 61 while GQ246219.1 and ACT22523.1 showed higher homology and a bootstrap value of 97. The grouping of \textit{S. melongena} \textit{PPO} (\textit{SmePPO2}), \textit{S. tuberosum} \textit{PPO} (\textit{POT33}) and \textit{S. lycopersicum} (\textit{tomA}) was also reported in previous studies (Shetty et al., 2011; Mishra et al., 2013). The similarity of \textit{SmePPO2} and \textit{tomA} might be due to specific expression pattern of \textit{PPO} genes i.e. in root tissues (Shetty et al., 2011). However, significant resemblance between eggplant, potato, tomato and tobacco PPO proteins is an indication of occurrence of duplication events which is supported by the lack of intron in the \textit{PPO} genes of these species (Shahar et al., 1992; Thygesen et al., 1995; Goldman et al., 1998; Shetty et al., 2011).

Potato \textit{PPO} genes also showed higher resemblance with each other as \textit{StuPPO1},
Figure 3.5: Phylogenetic comparison of potato PPO proteins with other plant PPO proteins. The studied plants name with accession numbers are as follows; *S. oleracea*
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StuPPO1, StuPPO2, StuPPO3 grouped alone. On the other hand, potA, potB and StuPPO4 clustered with tomE, tomF and P2 of tomato PPO genes. These findings are in accordance with previous reports in which potA and potB shared significant homology. In clade I, three of S. oleracea PPO genes showed 100 % similarity with each other. Thus, monophyletic clustering of all the species of Solanaceae family likely indicates the similar structural and functional homology of PPO genes.

Clade II consists of 25 species which is also subdivided into two main clusters (Cluster I and II). Cluster I of clade II mainly consists of the members of Poaceae family. The cluster I of clade II contains eleven species which were further divided into two sub clusters (sub clusters I and II). Subcluster I contains seven species (Oryza sativa, Oryza officinalis, Saccharum spp., Triticum monococcum, T. dicoccoides, T. aestivum and Phytolacca americana). Malviya et al. (2011) reported the clustering of O. sativa, T. monococcum and T. aestivum in a single group. However, in the present study it was found that two T. aestivum PPO genes (AF507945.1, AB254806.1) clustered separately with 100 % similarity. Moreover, five PPO genes from T. aestivum (PPO-A2c, PPO-B2c, PPO-D2a, PPO-D2b and PPOB) formed distinct group in sub cluster I (Clade II and Cluster I). Beecher and Skinner (2011) justified the sequence conservation of PPO-D2a, PPO-D2b and PPO-B2c genes to be due to expression of PPO activity in wheat kernels and specific localization. The members of the family Salicaceae (AAK53414.1, AF368291.1 and AF263611.1) grouped together in sub cluster II is also supported by bootstrap value (100). A higher similarity of these popular species is due to duplication event during the course of evolution (Tran et al., 2012).

Cluster II of clade II contains seventeen species and is divided into two sub clusters (sub clusters I and II). Sub cluster I (which consists of MdPPO and PaPPO genes) acted as outgroup while member of PPO genes of Asteraceae family (PPO-1, PPO-2 and PPO-3) showed higher resemblance with each other. Likewise, three PPO genes (AY017302.1, AY017303.1 and AY017304.1) from T. pratense (Fabaceae) also clustered together. PtdPPO3 and PePPO showed similarity with each other supported by bootstrap value of 72. Overall, all the six Populus PPO genes grouped in a single clade II. Our findings are supported by the previous study (Tran et al., 2012) in which seven
Populus PPO genes formed monophyletic group. These findings suggest that Populus PPO genes shared a common ancestry but they were evolved independently. However, the clustering of Populus PPO gene (AY665681.1) with V. vinifera (P43311.1) was also reported in previous study (Tran et al., 2012).

In sub cluster II of second clade five species from the family Rosaceae including Malus domestica (MdPPO1, MdPPO2, MdPPO3 and MdPPO4), Eriobotrya japonica (EjPPO), Pyrus bretschneideri (PbPPO), P. pyrifolia (PpPPO1) and Fragaria pentaphylla (FpPPO1 and FpPPO1) showed maximum homology with bootstrap value of 94. Clustering of those PPO sequences which showed homology depends upon the homogeneity of duplication events and may depend upon their source organism. Moreover, the distribution of PPO genes in different clusters showed the pattern of lineage-specific gene family expansion as well as deletion or insertion events during the course of evolution.

3.2 Induction of Endogenous PPO in Un-transformed Nicotiana tabacum (NtPPO)

3.2.1 Induction of NtPPO by wounding in tobacco plants

PPOs are generally related to plant defense (Mayer, 2006; Constabel and Barbehenn, 2008). Disease susceptibility and herbivory were also strongly linked to PPO activities (Thipyapong et al., 2007). Wounding was induced in seven days old un-transformed 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 showed increasing trend with the passage of time after wounding. As shown in Figure 3.6, wounding increased NtPPO endogenous transcript level of gene up to 6-14 folds as compared to control (unwounded). After 12 hours, the transcript level increased up to 6 folds and reached maximum level of up to 14 folds after 36 hours of wounding. Among the different time interval treatments, 36 hours resulted in an induction of maximum 14 folds transcript level of mRNA (Figure 3.6), which started declining after 48 hours. Depending upon the results, it can easily be concluded that mRNA level of NtPPO was up-regulated by wounding. In comparison with our findings,
Figure 3.6: Quantification of *NtPPO* mRNA level induced by wounding. Seven days old tobacco plants were subjected to mechanical injury growing on MS media and qRT-PCR was performed after intervals of 12, 24, 36 and 48 hours to detect wound induced response of *NtPPO* gene. The data is the mean of three independent experiments. P values were calculated by t-test. (*p*<0.05; **p*<0.01; ***p*<0.001).
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PPO mRNA accumulation increased in response to wounding and defense related hormones SA and MeJ in poplar (Mayer, 2006; Flurkey and Inlow, 2008). Based on the previous reports, PPOs are part of innate immunity in plants (Fuerst et al., 2014).

Some of plant defenses are triggered through tissue damage and/or mechanical injury (Howe and Jander, 2008). In tobacco, it was found that an endogenous induction of PPO gene by wounding is suggesting 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). Our results are also in accordance with the localized wounding of lateral roots (Thipyapong et al., 1997), wounding stress (Quarta et al., 2013) and chilling injury in pineapple which 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 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). Strong up-regulation of PPO gene in rubber plant by wounding may correlate its probable role in plants defense (Li et al., 2014).

3.2.2 Induction of NtPPO by elicitors in tobacco plants

Plant defense against chewing insects is signaled by MeJ (Stotz et al., 2000). It is well known that MeJ induced PPO activity along with other responses which are defense responses of plant (Thaler et al., 2002). Plants were sprayed with various concentrations of ABA and MeJ and were left for 24 hours in growth chamber. Transcript level of NtPPO was up-regulated from 2-6 folds in response to MeJ treatment (Figure 3.7). An increase in NtPPO transcript level was found with increasing MeJ concentrations. The mRNA level increased up to 2.4 folds upon 100 µM MeJ and maximum induction of transcript level was observed on 500 µM MeJ.
Figure 3.7: 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. The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Unlike MeJ, ABA application could not significantly induced expression of *NtPPO* (Figure 3.8). 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). 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 JA and its over-expression 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. These findings are inconsistent with banana *PPO* up-regulation by wounding and MeJ (Sreedharan et al., 2012). It has been reported that MeJ induced six *PPO* genes in eggplant and in transgenic tobacco (Shetty et al., 2012). Recent reports showed that red Swiss chard *PPO* promoter was regulated by MeJ in transgenic *Arabidopsis* (Yu et al., 2015). *PPO* of many plants was regulated by MeJ which indicated role of *PPO* in plant defense such as in hybrid poplar (Constabel et al., 2000), trembling aspen (Haruta et al., 2001), tomato (Li and Steffens, 2002), walnut (Escobar et al., 2008), rubber tree (Li et al., 2014) and strawberry (Jia et al., 2016).

### 3.3 Anti-sense Polyphenol Oxidase Gene Construct (AsPPO) Designing

#### 3.3.1 Cloning of the *PPO* gene amplified region into *E. coli*

PCR amplified 655 bp *PPO* region from potato was ligated directly into T/A cloning vector pTZ57R/T (MBI, Fermentas), harboring ampicillin resistant gene and *lacZ* complementary system for screening by X-Gal and IPTG (Figure 3.9). The ligation product was transformed into *E. coli* (DH5α) through electroporation; these *E. coli* cells were grown on liquid LB and streaked over LB agar plates with XGal/IPTG and ampicillin (50 mg/L). After 16-20 hours incubation at 37 °C overnight, white and blue colonies were observed.
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Figure 3.8: 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, 400 μM and 500 μM) growing on MS media. The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).

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Figure 3.9: Diagrammatic representation of T/A cloning vector along with targeted *PPO* gene insert.
3.3.2 Confirmation of cloning

White colonies were picked up and grown in LB liquid medium containing ampicillin (50 mg/L) and incubated for 12 hours at 37 °C with 250 rpm shaking. Plasmid was isolated from cultures and confirmed by PCR using specific pair of primers *PPO1* and *PPO2* (Section 2.2.1). A product of ~ 655 bp (Figure 3.10) was observed on 1.5 % agarose gel which confirms the cloning of *PPO* gene targeted region. However, plasmid was further confirmed by restriction digestion using *AvrII* and *EcoRI* restriction enzymes that generated ~ 655 bp *PPO* gene fragment.

3.3.3 Sequencing of *AsPPO* clone

Sequencing with gene specific primers confirmed the presence of ~ 655 bp conserved copper binding domain of potato *PPO* gene. Results showed 98 % similarity with already reported *PPO* gene of *Solanum tuberosum* (U22922.1) which also confirmed the cloned core region of potato *PPO* gene.

3.3.4 Construction of recombinant expression vector

Anti-sense *PPO* (*AsPPO*) construct was designed to examine the role of *PPO* gene in model plant. For this, targeted region of *PPO* gene insert from T/A cloning vector and p1391Z_OsRGLP2 (Mahmood et al., 2013) were digested by *AvrII* and *EcoRI* restriction enzymes. After elution from gel, recombinant plasmids were made by ligating the digested and eluted *PPO* gene specific insert downstream to *OsRGLP2* promoter in anti-sense orientation (Figure 3.11) which was already available in p1391Z_ OsRGLP2 vector. The expression cassette containing *PPO* gene targeted region ligated downstream to *OsRGLP2* promoter in an anti-sense direction was given a name of *AsPPO*.

3.3.5 Transformation of *AsPPO* into *Agrobacterium tumefaciens*

*AsPPO* expression cassette was transformed into *Agrobacterium tumefaciens* strain EHA101 through electroporation. *Agrobacterium* mediated transformation was confirmed by colony PCR using *PPO* gene specific primers as well as hygromycin gene primers that results in desired amplification (Figure 3.12). Further, *EcoRI* and *AvrII* restriction digestion of isolated plasmid from clones confirmed the correct transformation
Figure 3.10: PCR amplified products using primers PPO1 and PPO2. Lane 1: Ladder (1 kb, Fermentas), Lane 2, 3, 4, 5: amplified products of ~ 655 bp.

Figure 3.11: A Schematic representation of p1391Z_OsRGLP2 promoter construct A: p1391Z_OsRGLP2 vector without insert B: p1391Z_OsRGLP2 vector with antisense targeted region of potato PPO gene (under the control of OsRGLP2 promoter).
**Figure 3.12:** PCR amplification of hygromycin gene. Lane 1: Ladder (1 kb, Fermentas), lane 2 to 6: products of hygromycin gene amplified from isolated plasmid from clones.
of AsPPO in *Agrobacterium*.

### 3.4 Agrobacterium Mediated Transformation of Tobacco with AsPPO

Young growing leaves from tobacco plants were cut into small discs and immersed in *Agrobacterium* culture harboring AsPPO gene construct for 10 minutes. Afterwards, the explants were incubated on co-cultivation medium and then shifted to selection media (Table 2.2) in petri plates for regeneration in growth chamber under control conditions of 16:8 dark and light cycles at 27 °C. After one week, small bud like calli appeared on cut edges which increased in mass up to 14 days (Figure 3.13). While continuously transferring regenerating bodies after every 3-4 days to fresh media, the calli started regeneration in the form of small shootlets. After 28 days the shootlets developed in such a size that they were cut individually from callus and shifted to the simple MS media in jars for rooting. Forty five days old young shoots completely developed roots and transformed into mature individual plants. These mature plants were shifted to the soil in small pots in green house under control conditions of 16:8 dark and light cycle at 27 °C.

#### 3.4.1 Confirmation of transgenic plants

The transgenic plants were confirmed by PCR amplification of *PPO* gene specific primers as well as hygromycin gene primers. Both confirmations were successful as *PPO* and hygromycin genes were amplified from transgenic plants DNA.

### 3.5 Expression Analysis of Transgenics

#### 3.5.1 Expression analysis in wounding

Plants are regularly affected by their environmental conditions in the form of several biotic and abiotic stresses due to sessile existence. Among many abiotic stresses, wounding is a common injury in plants that occurs as a result of insect herbivory, wind, hail and rain (Wang et al., 2014). In plants, different classes of defense related genes are induced by wounding, pathogen or predator invasion resulting in extensive alterations in transcript levels and protein synthesis (Gulbitti-Onarici et al., 2009). *PPO* is also considered as defense related gene which was induced strongly upon wounding.
Figure 3.13: 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 greenhouse.
In the present study, transgenic plants were wounded with forceps and expression analysis of anti-sense \( PPO \) gene was conducted after 12 and 24 hours through qRT-PCR. The expression analysis was carried out by targeting proximal coding, core and terminal coding regions of \( NtPPO \) gene by three sets of primers (\( NtPPO1 \), \( NtPPO2 \), and \( NtPPO3 \)) for wild type and transgenic tobacco plants as given in materials and methods (Table 2.3). Mechanical wounding of transgenic tobacco plants, containing anti-sense potato \( PPO \) gene at different time intervals showed reduction in the expression of endogenous tobacco \( PPO \) gene than control plants. Transcript level of endogenous \( PPO \) showed reduction of expression up to 1.4 folds after 12 hours (Figure 3.14). Among the different studied regions of \( PPO \) gene, \( NtPPO1 \) was less down-regulated than core and terminal coding regions. While the control plants showed an up-regulation of endogenous \( PPO \) gene by mechanical injury. However, after 24 hours of wounding, \( NtPPO2 \) was more down-regulated (3.5 folds) than \( NtPPO1 \) and \( NtPPO3 \) (Figure 3.15). Overall, 24 hours of wounding resulted in more down-regulation of endogenous \( PPO \) gene than 12 hours wounding.

\( PPO \) is present in all land plants and is an important component of plant defensive mechanism (Aniszewski et al., 2008). In fact \( PPO \) induced expression is well known by several environmental abiotic and biotic factors or stresses (Constabel et al., 2000; Quarta et al., 2013). Peroxidases over-expressing tobacco showed \( PPO \) activity upon wounding and also browning (Lagrimini, 1991). In another study transgenic tomato expressing systemin gene exhibited enhanced \( PPO \) upon wounding (Constabel et al., 1995). These early findings portrayed an indirect involvement of \( PPO \) in wounding.

Mechanical wounding in transgenic tobacco over-expressing peroxidase (\( POD \)) and transgenic tomato over-expressing systemin gene showed up-regulation of \( PPO \) up to 70 folds (Lagrimini, 1991; Constabel et al., 1995). Hybrid poplar \( PPO \) induction and regulation by wounding (Constabel et al., 2000) can be correlated to \( NtPPO \) gene down-regulation in the present study. \( PPO \) wound responses and regulation were also found in other plants which are related to anti-sense \( NiPPO \) down-regulation such as pineapple \( PPO \) regulation by wounding (Strewat et al., 2001). Transcript level analysis in rubber plant revealed that its \( PPO \) is coupled with mechanical stresses especially wounding (Li
Figure 3.14: Relative expression analysis of *NtPPO* gene activity induced by wounding after 12 hours (Key: WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.15: Relative expression analysis of *NtPPO* gene activity induced by wounding after 24 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
In another report, PPO anti-sense down-regulated walnut plants markedly got leaf lesion necrosis, showing PPO mediated cell death in walnut (Araji et al., 2014). NtPPO down-regulation was noticed more than 2 folds after 24 hours showing a little bit late response to injuries which was reported in Populus trichocarpa PPO wound response after 36 hours (Harutta et al., 2001) and was too late in the case of pineapple which showed response after 2 days (Zhou et al., 2003). Similarly, artichoke PPO gene was up-regulated by wounding after 48 hours (Quarta et al., 2013).

Wound induced NtPPO expression points a probable role of PPO in defense mechanisms in plants. PPO induction by mechanical injuries might have a possible role of PPO involvement in insect and pathogen resistance (Haruta et al., 2001). This NtPPO expression by injury from our results can be correlated to transgenic tomato where PPO over-expression enhanced disease tolerance against Pseudomonas syringae (Li and Stiffen, 2002) and PPO over-expression in transgenic Populus trichocarpa enhanced resistance to insects (Wang and Constabel, 2004a). Moreover, dandelion PPO gene was induced by Botrytis cinerea and the same gene in transgenic Arabidopsis exhibited biological activity against P. syringae (Richter et al., 2012). PPO over-expression in transgenic strawberry postponed the fungal attack during fruit development (Jia et al., 2016). Soybean PPO over-expression analysis showed that it promotes early responses to Phytophthora sojae (Chai et al., 2013). Tran et al. (2012) showed that PPO is a part of defense in plants due to wound and pathogen induced activities.

Down-regulation of PPO gene in transgenic plants showed enhanced susceptibility to diseases and pathogen attacks. Reduced PPO expression by anti-sense technology in transgenic tomato amplified vulnerability to P. syringae (Thipyapong et al., 2004a). Similarly PPO down-regulation in dandelion increased P. syringae attacks (Richter et al., 2012). Silenced PPO expression (less than 5 %) by knockdown increased necrosis lesion on walnut leaves despite pathogen absence as compared to wild walnut plants (Araji et al., 2014). NtPPO gene regulation by mechanical injures of different time intervals in transgenic tobacco can be linked to defense activities of NtPPO gene to avoid pathogens attacks (Wang et al., 2004a; Raj et al., 2006; Barbehenn et al., 2007; Thipyapong et al., 2007; Bhonwong et al., 2009). NtPPO gene might have possible
defense on injury points as pathogens activities of *Botrytis* and *Pseudomonas* grow and develop at lesion sites in *Taraxacum officinale* which induced *PPO* activity (Richter et al., 2012).

Wound induced *PPO* expression pattern can be linked to the insects feeding *PPO* induced expression as this pattern of expression indicates *NtPPO* role against insect feeding. Aspen *PPO* over-expressing lines showed *PPO* elevated expression by wounding as was evident in real herbivory by forest tent caterpillars (Haruta et al., 2001). Hybrid poplar *PPO* was induced by insect infestation as well as by wounding treatments (Constabel et al., 2000). Similarly, *PPO* over-expression in populus improved the confrontation against insect like forest tent caterpillar (Wang and Constabel, 2004a). Over-expression of *PPO* in different studies exhibited induction of resistance in plants against insects that affirm the *PPO* role in anti-herbivory. *PPO* over-expression found to be fatal for tree feeding insects (Constabel et al., 2000; Barbehenn et al., 2007), cotton and cutworms (Thipyapong et al., 2007; Mahanil et al., 2008) and armyworms and bollworms (Bhonwong et al., 2009).

Down-regulated *PPO* expression revealed increased susceptibility to diseases and pathogens. Down-regulated *PPO* expression in tomato noticeably increased vulnerability (Thipyapong et al., 2004a; Richter et al., 2012; Araji et al., 2014). *NtPPO* down-regulation in transgenic tobacco by wounding might indicate defensive role as in case of other plants *PPO* (Wang et al., 2004a; Raj et al., 2006; Barbehenn et al., 2007; Thipyapong et al., 2007; Bhonwong et al., 2009; Richter et al., 2012). These findings point out the possible role of *NtPPO* in plant defense mechanism which will be discussed in white fly feeding assay on transgenic tobacco.

### 3.5.2 Expression analysis in ABA

Abscisic acid is an important signaling molecule that can regulate different defense related mechanisms to cope with biotic and abiotic stresses (Kumar et al., 2008; Raghavendra et al., 2010). To elucidate the activity of suppressed *PPO* gene in response to exogenous ABA application, transgenic tobacco plants with anti-sense potato *PPO* gene along with wild type plants were treated with 100 and 200 μm of ABA for 6 and 12
hours. The qRT-PCR analysis was carried out by targeting proximal coding, core and terminal coding regions of *NtPPO* gene by three sets of primers (*NtPPO1, NtPPO2*, and *NtPPO3*) for wild type and transgenic tobacco plants. Transcript level showed a little bit up-regulation of 0.5 fold in wild type control plants while slight down-regulation of 0.3 fold in transgenic treated plant with 100 μm after 6 hours (Figure 3.16). Twelve hours after using similar ABA concentration, there was no obvious regulation of *PPO* gene as wild type treated plants showed an accumulation of *PPO* mRNA up to 0.6 fold and reduction in expression of *PPO* gene in transgenic treated plants up to 0.5 fold (Figure 3.17).

However at 200 μM ABA after 6 hours, *NtPPO* gene showed transcript elevation up to 0.8 fold in wild type and down-regulation of 0.9 fold in transgenic plants (Figure 3.18). Again after 12 hours of treatment at 200 μM, *PPO* gene regulation remained approximately same as after 6 hours (Figure 3.19). Overall, all *PPO* gene regions showed similar expression pattern in response to ABA. Obviously, there is no significant induction and down-regulation of *NtPPO* gene in wild type and transgenic plants respectively. The multifaceted role of ABA has been extensively reviewed in different plants (Mauch-Mani and Mauch, 2005; Bari and Jones, 2009; Wang et al., 2013). The role of ABA in relation to biotic stress is not clear and differential effects can be observed depending on plant and biotic factors (Grant and Jones, 2009). Mounting evidence suggests that ABA plays variable role to compete with adverse stresses in plants (Wang et al., 2013).

Mauch-Mani and Mauch (2005) reported positive role of ABA in plant immunity against certain pathogens. On the other hand, ABA has been supposed to enhance disease susceptibility in several plant-pathogen interactions depicting its antagonistic regulatory mechanisms to cope with biotic stress (Anderson et al., 2004). The expression of *PPO* in transgenic tobacco was not significantly affected by exogenous ABA application which is in accordance with earlier reports. In a previous study, Song et al. (2011) treated tomato plants with exogenous ABA and recorded no significant *PPO* activity difference between wild type and transgenic tomato that supports our results. The antagonistic interaction between ABA and JA or salicylic acid (SA) showed resistance in *Arabidopsis* against *F.*
Figure 3.16: Relative expression analysis of \textit{NtPPO} gene activity in response to 100 μM ABA treatment after 6 hours (Key; WT: wild type, \textit{NtPPO1}: qRT-PCR primers targeted from proximal coding region of tobacco endogenous \textit{PPO} gene, \textit{NtPPO2}: qRT-PCR primers targeted from core region of tobacco endogenous \textit{PPO} gene, \textit{NtPPO3}: qRT-PCR primers targeted from terminal region of tobacco endogenous \textit{PPO} gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.17: Relative expression analysis of *NtPPO* gene activity in response to 100 μM ABA treatment after 12 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.18: Relative expression analysis of *NtPPO* gene activity in response to 200 μM ABA treatment after 6 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.19: Relative expression analysis of *NtPPO* gene activity in response to 200 μM ABA treatment after 12 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
oxysporum which are in agreement with our study (Anderson et al., 2004). Similarly, elevated level 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 in tomato showed induction of defense related gene such as POD, phenylalanine ammonia-lyase (PAL) and PPO that results in enhanced 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 (Asselbergh et al., 2008). It is predicted that induction or accumulation of ABA is negatively linked with different stressors that might be involved in activation of other defense responses (Lee and Luan, 2012; Xu et al., 2013). It has been reported that modulation of ABA signaling during biotic and abiotic stresses primarily affect level of gene expression (Chinnusamy et al., 2008). Recently, Kaur and Zhawr (2015) examined higher level of phenolics in two wheat cultivars by application of exogenous ABA that suggests the ABA-regulation of phenolics in plants.

Chai et al. (2013) investigated 2.4 folds up-regulation of Glycine max PPO gene by addition of exogenous ABA while reduction in PPO expression was linked with SA, JA and ethephon applications. ABA plays important role in several physiological processes including induction of seed dormancy as well as in regulation of stomatal aperture, drought and salt stress (Finkelston et al., 2002; Nambara and Marion-Poll, 2005; Parent et al., 2009; Lim et al., 2015). It has been well documented that ABA signal cascade was linked with regulation of many stress-responsive genes (Siddiqui et al., 1998; Sanchez et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). The complex regulatory process of ABA is supported by its diverse role in plant kingdom as well as by the existence of multiple receptors and signal transduction pathways (Chinnusamy et al., 2008). However, it has been found that besides genetic regulation, epigenetic regulation plays significant role in ABA-mediated plant processes (Chinnusamy et al., 2008). Further studies will require a more detailed analysis, to elucidate the antagonistic and
synergistic signaling pathways linked with PPO gene expression and other elicitors for stress adaptations.

### 3.5.3 Expression analysis in MeJ

Methyl jasmonate, the volatile form of JA, has been widely used to study jasmonate signaling pathways and mechanisms of plant defense (Zhang et al. 2015). To determine the activity of anti-sense PPO gene in response to MeJ application, the transgenic tobacco plants along with control were treated with two different concentrations of MeJ (100 µM and 200 µM) at different experimental time periods (6 hours and 12 hours). The transcript levels in transgenics along with control were measured by qRT-PCR for NtPPO1, NtPPO2 and NtPPO3. As shown in Figure 3.20, treatment with 100 µM MeJ after 6 hours resulted in differential expression pattern in both control and transgenic plants. For example, under the normal conditions (no treatment), no or very low expression for NtPPO1, NtPPO2 and NtPPO3 was observed after 6 hours in WT plants and transgenic plants respectively. However, there was up-regulation (2.8 folds) of target gene in WT plants treated with 100 µM MeJ applications at 6 hours (Figure 3.20). Interestingly, the expressions of NtPPO1, NtPPO2 and NtPPO3 showed significant down-regulation of tobacco PPO gene 4.2, 3.6 and 4.7 folds respectively in transgenic plants after 6 hours of exposure to 100 µM MeJ treatments (Figure 3.20). After treatment with 100 µM MeJ for 12 hours, expression levels of NtPPO1, NtPPO2 and NtPPO3 in transgenics decreased by 4.25, 5.4 and 5.1 folds respectively, this was significantly lower than the WT (Figure 3.21).

Therefore, upon 100 µM MeJ treatment, the expressions of NtPPO1, NtPPO2 and NtPPO3 were clearly down-regulated in transgenics for 6 hours and 12 hours post-treatment of MeJ. Similar suppression of NtPPO activity was also exhibited by transgenic plants with 200 µM MeJ for 6 and 12 hours (Figure 3.22 and Figure 3.23). However, as time passes from 6 to 12 hours, transcript levels of NtPPO1, NtPPO2 and NtPPO3 increased in control plants following treatment with 200 µM MeJ. In contrast, significant down-regulation in activity of NtPPO was observed in transgenic plants at 6 hours and 12 hours as compared to control plants after 200 µM MeJ application. However, after 6 hou-
Figure 3.20: Relative expression analysis of *NtPPO* gene activity in response to 100 μM MeJ treatment after 6 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. *P* values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.21: Relative expression analysis of *NtPPO* gene activity in response to 100 μM MeJ treatment after 12 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.22: Relative expression analysis of \textit{NtPPO} gene activity in response to 200 μM MeJ treatment after 6 hours (Key; WT: wild type, \textit{NtPPO1}: qRT-PCR primers targeted from proximal coding region of tobacco endogenous \textit{PPO} gene, \textit{NtPPO2}: qRT-PCR primers targeted from core region of tobacco endogenous \textit{PPO} gene, \textit{NtPPO3}: qRT-PCR primers targeted from terminal region of tobacco endogenous \textit{PPO} gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
Figure 3.23: Relative expression analysis of *NtPPO* gene activity in response to 200 μM MeJ treatment after 12 hours (Key; WT: wild type, *NtPPO1*: qRT-PCR primers targeted from proximal coding region of tobacco endogenous *PPO* gene, *NtPPO2*: qRT-PCR primers targeted from core region of tobacco endogenous *PPO* gene, *NtPPO3*: qRT-PCR primers targeted from terminal region of tobacco endogenous *PPO* gene). The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
rs of treatments, \textit{NtPPO} suppression was comparatively higher than 12 hours of treatment. Over all, these results showed that silencing of \textit{PPO} gene has reduced the expression level of \textit{NtPPO} in transgenic plants in response to both concentrations of MeJ (100 \mu M and 200 \mu M).

However, pronounced reduction was observed with 100 \mu M MeJ for 12 hours. Several reports depicted the silencing of \textit{PPO} gene expression using anti-sense down-regulation technique. This strategy proved successful in suppressing the overall \textit{PPO} activities and reduction of \textit{PPO}-mediated browning reactions (Chi et al. 2014). For example, Mahanil et al. (2008) reported 1.5-7.3 folds lower \textit{PPO} activity in anti-sense \textit{PPO} transgenic tomato plants when compared to non-transformed control. Similarly, in another study, up to 40-folds decreased in \textit{PPO} activity was observed in \textit{PPO}-silenced transgenic plants than non-transformed control (Thipyapong et al. 2004b). Araji et al. (2014) also generated transgenic walnut lines with greatly reduced \textit{PPO} activity through silencing of \textit{JrPPO1} gene. In the present results, \textit{PPO} activity was highly suppressed in transgenic plants expressing the anti-sense \textit{PPO} gene which correlate well with previous reports. Moreover, the inducibility of \textit{PPO} genes by MeJ has been reported in many plants, including both herbaceous crops and trees (Constabel and Ryan, 1998; Escobar et al. 2008). In the present study, MeJ treatment has increased the expression level of \textit{PPO} gene in WT plants which may indicate the role of MeJ in plant defense against pathogens. In an earlier study, Constabel et al. (1995) reported that \textit{PPO} activity increases rapidly after exposing wild type tomato plants to MeJ vapors. In addition to tomato, other plants like tobacco and hybrid poplar also showed strong induction of \textit{PPO} in response to MeJ treatment (Constabel and Ryan, 1998).

However, in case of poplar hybrid, \textit{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 the up-regulation of \textit{PPO} gene under stress conditions. Moreover, failure to induce the \textit{PPO} expression using anti-sense technique may help to overcome the browning reactions in important crops.
3.5.4 Expression analysis for whitefly infestation

Plants frequently expose to herbivorous insect attack in the natural environment. In response to herbivory, plants have the ability to perceive rapidly and accurately their biotic attackers and activate the effective defense system (Mithöfer and Boland, 2012). Plant must identify and respond to the mechanical and chemical signals that accompany insect attack (Felton and Tumlinson, 2008). Previously, different defense signaling pathways have been reported that are modulated after pest attack such as JA and SA signaling pathways (Walling, 2009; Wu and Baldwin, 2009; Thaler et al., 2012). *Trialeurodes vaporariorum* is a kind of polyphagous whitefly that has caused serious losses in vegetables, horticultural and ornamentals crops worldwide (Lei et al., 1998; Martin and Mound, 2007).

In the present study, to elucidate the role of *PPO* in insects induced defense responses in tobacco plants; *NtPPO* gene was silenced using anti-sense copper binding domain region of potato *PPO* ligated downstream to the wound inducible OsRGLP2 promoter. The transcript level regulation was analyzed using biotic stress which was induced by the whitefly (*Trialeurodes vaporariorum*) infestation on the transgenic tobacco plants. To investigate transcript response of *PPO* against insect feeding, qRT-PCR analysis was carried out. In this study, a functional gene (*NtPPO*) was taken in consideration and three sets of primers were used (Table 2.3). The proximal coding (*NtPPO1*) region showed a slight up-regulation of 0.75 fold higher than the control plants after 2 days. However, the expression of *NtPPO1* gene was down-regulated after 5 days of herbivory by whiteflies feeding. This down-regulation was consistently followed by the transgenic plants finally reaching up to 6 folds after 10 days (Figure 3.24). Similarly, *NtPPO2* regions (core) also showed a little bit up-regulation after 2 days and then followed gradual reduction of 2.7 folds in its expression after 5 days. After 10 days down-regulation attained a level of 5 folds in *NtPPO2* core region (Figure 3.24). The terminal coding region (*NtPPO3*) showed very similar trend to core coding (*NtPPO2*) region which showed an up-regulation after 2 days and down-regulation after 5 and 10 days of whiteflies attack (Figure 3.24).
Figure 3.24: Relative expression analysis of *NtPPO* gene activity in response to whitefly infestation from targeted proximal (*NtPPO1*), core (*NtPPO2*) and terminal coding (*NtPPO3*) regions of endogenous tobacco *PPO* gene. The data is the mean of three independent experiments. P values were calculated by t-test. (*p<0.05; **p<0.01; ***p<0.001).
However, the comparatively more down-regulation of \textit{PPO} was shown by \textit{NtPPO1} (proximal coding region of \textit{PPO}) which was \(~ 6\) folds. These results suggest \textit{PPO} gene is an important component in defense mechanism. Further this down-regulation also confirmed the inducible nature of \textit{PPO} gene that expressed in response to pathogen defense mechanism. \textit{PPO} is found to be linked with the protection of plants against pathogens and herbivores (Shabaz et al., 2009). \textit{NtPPO} suppression is consistent with tomato \textit{PPO} anti-sense down-regulation up to 40 folds by \textit{P. syringae} infestation without effecting plant growth and development in green house (Thipyapong et al., 2004a). Bhonwong et al. (2009) reported 1.5 to 2.9 folds reduction in \textit{PPO} expression against cotton bollworm in suppressed tomato plants that are consistent with our findings. Richter et al. (2012) also reported that dandelion plants showed resistance against biotic attack as a result of \textit{PPO} expression and its \textit{PPO} suppression enhanced susceptibility to biotic pathogens. These results are in accordance with the role of \textit{PPO} in biotic resistance. Ye et al. (2012) studied that phloem-feeding herbivorous insects can trigger production of \textit{PPO} and \textit{POD} genes that deter further insect damage in rice.

Further, silencing of \textit{Col1} in rice showed reduced \textit{POD} and \textit{PPO} expression up to \(27.2\) % and \(48.5\) % respectively. Recently, \textit{PPO} activity was found to enhance against whitefly anti-herbivory in wild type tobacco plants that can be negatively correlated with our results (Zhao et al., 2015). Similarly, activity of \textit{PPO} was recorded higher in whitefly infested pepper genotypes than non-infested (Latournerie-Moreno et al., 2015). Earlier, \textit{PPO} expression was found to be reduced in cotton by silencing of \textit{PPO} gene in response to beet armyworm which implicated \textit{PPO} as a component of defensive system against insects (Bhonwong et al., 2009).

Similarly, \textit{PPO} over-expression was found to enhance resistance against insect’s attack (Thipyapong et al., 2004b; Mahanil et al., 2008). Likewise, anti-herbivory role of \textit{PPO} was established in many transgenic plants (Constabel et al., 2000; Wang and Constabel, 2004a). \textit{PPO} up-regulation generated insect resistance in transgenic poplar against forest tent caterpillars (Wang and Constabel, 2004a). Similarly, \textit{PPO} induction was linked to forest tent caterpillars attacks in hybrid poplar suggesting \textit{PPO} as defensive component in plants (Constabel et al., 2000). Aspen \textit{PPO} transcripts were also reported
in elevations after forest tent caterpillars feeding (Haruta et al., 2001). This inducible
PPO gene suppression that has been observed is indirectly consistent with defensive role
of PPO against herbivores previously reported in several crops such as cotton (Kranthi et
al., 2003), tomato (Thaler, 2002) and soyabeen (Bi and Felton, 1995). Moreover,
defensive role of PPO against insect attack was also confirmed in tomato due to the
presence of herbivore-inducible signal systemin that are involved in induction of PPO
expression in response to herbivores (Constable et al., 1995).

Overall, the modification of PPO gene in transgenic tobacco showed altered level
of herbivore-inducible PPO expression which is in accordance with previous study on
transgenic tobacco (Ren and Lu, 2006). Herbivore-inducible down-regulation of PPO
gene in PPO silenced plants confirmed the vital role of PPO in insect defense. The use of
transgenic plants with modified PPO activity by over-expression or down-regulation
provides a basis to determine the role of PPO against herbivory (Li and Steffens, 2002;
Wang et al., 2004a). Anti-sense down-regulation of PPO in transgenic tomato suggested
enhanced vulnerability to Colorado potato 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 that PPO gene plays major
role in insect resistance through herbivore-inducible mechanisms along with other
defensive genes (Ni et al., 2001; Kruzmane et al., 2002; Chakraborty and Chakraborthy,
2005). These different defensive genes encode anti-herbivore proteins along with PPO
proteins such as protease inhibitors (PI) (Arimura et al., 2005). Systemic induction of
PPO and PI were found elevated by MeJ and systemin in tobacco (Constabel and Ryan,
1998; Ren and Lu, 2006).

The present study, clearly demonstrate that whitefly infestation on PPO silenced
tobacco plants repressed the PPO transcript level which might be induced in 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 in insect pest management programs because over-expression of
PPO in tomato and cotton was proved as weak management strategies 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 as found against insects 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.

3.6 Conclusion

The present study revealed considerable genetic diversity in plant PPO proteins as well as some conserved sequences. Phylogenetic grouping of monocots and dicots PPO proteins separately reflects the lineage specific gene family expansion and duplication events through evolution. The wound inducible promoter provides the ability to investigate the expression of PPO gene under certain stress conditions by down-regulation of target gene in transgenic tobacco plants. Our results have shown that PPO gene was responsive to the applied stresses of wounding, MeJ and whitefly assay but not by ABA. Transcriptional activity of un-transformed tobacco PPO revealed that NtPPO gene activated up to 14 folds by wounding and 6 folds by MeJ. In response to mechanical wounding, transgenic tobacco harboring anti-sense potato PPO showed significant down-regulation of endogenous PPO after 24 hours 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 µM and 200 µM of MeJ treatments in transgenic tobacco plants that is linked with PPO induction in defense responses. Furthermore, whitefly infestation also showed down-regulation of PPO gene in transgenic tobacco plants which indicates critical role of PPO in biotic stress. These results indicate that expression of potato PPO in anti-sense orientation inhibits PPO activity. Overall, PPO induction in current study by biotic and abiotic stresses clearly links PPO in plant defense
mechanism. Suppression of tobacco $PPO$ by using present construct harboring 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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Appendix 1

Plasmid isolation from bacterial culture

1. A single bacterial colony was inoculated into 3 ml of LB medium containing the appropriate antibiotic in a test tube. The medium was incubated overnight at 37°C in case of *E. coli* and for 36-48 hours at 28 °C in case of *A. tumefaciens* with vigorous shaking.

2. The culture was poured into an eppendorf tube and was pellet down by centrifugation at 12000 rpm for 60 seconds at 4 °C in a microfuge. The supernatant was poured out.

3. Bacterial pellet was re-suspended in 100 μl of ice-cold solution I by vigorous vortexing.
   Solution I
   50 mM Glucose
   25 mM Tris HCL (pH 8.0)
   10 mM EDTA (pH 8.0)
   Solution I was prepared in batches of approximately 50 ml, autoclaved and then stored at 4°C.

4. Then 200 μl of solution II was added. The cap of the eppendorf was tightly closed and the contents were mixed by inverting the tube rapidly for five minutes. Then eppendorf was stored on ice.
   Solution II
   0.2 N NaOH (freshly diluted from a 10 N stock)
   1% SDS

5. Then 150 μl of solution III was added. The eppendorf was again tightly closed and was vortexed in an inverted position for 10 seconds to disperse solution III through the viscous bacterial lysate. The eppendorf was again stored on ice for 3-5 minutes.
   Solution III
   Potassium Acetate 5M 60 ml
Glacial Acetic Acid 11.5 ml
H₂O 28.5 ml

6. The contents were centrifuged at 14000 rpm for 5 minutes at 4 °C in a microfuge. The supernatant was transferred to a new eppendorf.

7. An equal volume of phenol:chloroform was added in to the supernatant and contents were vortexed. Then eppendorf was centrifuged at 14000 rpm for 2 minutes at 4 °C in a microfuge. The supernatant was again transferred to new eppendorf.

8. The double stranded DNA was precipitated by adding an equal volume of isopropanol. The eppendorf was kept at room temperatures for 10 minutes and then centrifuged at 14000 rpm for 5 minutes at 4 °C.

9. The supernatant was removed by carefully inverting the eppendorf and the DNA in pellet form was washed with 70 % ethanol.

10. The pellet was air dried and was suspended in nanopure water followed by treatment with RNase. The dissolved pellet of DNA was stored at 20°C till further use.
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