EXPRESSION ANALYSIS OF ANTI-SENSE *Os*PPO GENE PROMOTER IN RESPONSE TO PLANT GROWTH HORMONES IN TRANSGENIC MODEL PLANT

Master of Philosophy in

PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

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EXPRESSION ANALYSIS OF ANTI-SENSE *Os*PPO GENE PROMOTER IN RESPONSE TO PLANT GROWTH HORMONES IN TRANSGENIC MODEL PLANT

A Dissertation Submitted in the Partial Fulfillment of the Requirements for the Degree of Master of Philosophy in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

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DECLARATION

This is to certify that the dissertation entitled **"EXPRESSION ANALYSIS OF ANTI-SENSE *Os*PPO GENE PROMOTER IN RESPONSE TO PLANT GROWTH HORMONES IN TRANGENIC MODEL PLANT''** submitted by **Rabia Ubaid** is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of M. Phil in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY.

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Dedicated

to my

beloved parents

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Rabia Ubaid

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LIST OF ABBREVIATIONS

%	Percentage
˚C	Centigrade
ABA	Abscissic acid
BAP	6-Benzyl Amino Purine
bp	Base pairs
CTAB	Cetyl Trimethyl Ammonium Bromide
DEAE	Diethyl Amino Ethyl
DMSO	Dimethyl Sulpho Oxide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Tri Phosphates
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
g/L	Gram per liter
GUS	β-glucuronidase
HCl	Hydro Chloric acid
H_2O_2	Hydrogen per oxide
hrs	Hours
IAA	Indole 3-Acetic acid
IBA	Indole-3-Butyric acid
k Da	Kilo Dalton
kb	Kilo base pairs
Km	Michaelis constant
LB	Lauria Bartini
L-DOPA	L-3, 4-dihydroxyphenylalanine
M	Molar
mg	Milli gram
mg/ L	Milligram per liter
mL	Milli liter

mM	Milli molar
MS	Murashige and Skoog
NAA	α-Naphthaline Acetic Acid
NaCl	Sodium Chloride
ng	Nano gram
o	Ortho
OD	Optical density
*Os*PPO	*Oryza sativa* Polyphenol oxidase
p	Para
PCR	Polymerase Chain Reaction
pM	Pico Molar
PPOs	Polyphenol oxidases
RNA	Ribo Nucleic Acid
ROS	Reactive oxygen species
rpm	Revolution per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
TAE	Tris Acetate Ethylene Diamine Tetra Acetic Acid
TE	Tris Ethylene Diamine Tetra Acetic acid
w/v	Weight by volume
X-Gluc	5-Bromo-4 Chloro-3-Indolyl-β-glucoronicacid
µL	Micro liter

ABSTRACT

Polyphenol oxidases (PPOs) are ubiquitous copper containing enzymes, involved in the oxidation of *ortho*-diphenolics into quinones. They are broadly distributed and extensively studied oxidative enzymes, playing pivotal role against various biotic and abiotic stresses. Purpose of the present study was to examine the activity of antisense *Os*PPO promoter in response to exogenous applications of α-naphthaline acetic acid (NAA), 6-benzyl amino purine (BAP) and abscissic acid (ABA). Tobacco plants were transformed with antisense *Os*PPO promoter, fused with GUS reporter gene using *Agrobacterium* mediated transformation and transformation was confirmed by PCR and histochemical analysis. GUS expression pattern of antisense *Os*PPO promoter was analysed in response to various concentrations of NAA (0.1 µM, 1 µM, 10 µM and 100 µM), BAP (1 µM, 20 µM, 40 µM and 80 µM) and ABA (50 µM, 150 µM, 250 µM and 350 µM). It was found that antisense *Os*PPO promoter was strongly induced in response to different concentrations of these hormones, however induction of promoter was increased with increasing concentration of hormone and timing of treatment upto certain level and then it was decreased. Among different concentrations of these hormones applied, maximum GUS expression was observed at 10 µM of NAA treatment after 12 hours. Moreover promoter was strongly induced in response to 80 µM of BAP application and 350 µM of ABA. Microscopic analysis showed GUS expression in various parts of leaves e.g. guard cells, midrib, and veins while expression was also observed in root hairs. The results revealed that antisense *Os*PPO promoter is responsive towards hormonal stresses and suggested the idea that antisense *Os*PPO gene promoter can be utilized in transformation experiments regarding biotic stresses carries considerable importance in crop biotechnology for generating resistant cultivar against various stresses.

Introduction

Polyphenol oxidases (PPOs) are found in invertebrates, mammals, plants and both eukaryotic and prokaryotic microorganisms. Plants contain different forms of PPO. Parsley, durum wheat, banana, and grapes plants were used for the extraction and purification of PPO. The optimum temperature for PPO is in between 30 ºC – 80 ºC and optimum pH range is 4.0 – 8.5. The affinity of PPO varied with the type of substrate used. In plants PPO have chief affinity for pyrogallol, catechol and 4-methylcatechol.

Polyphenol oxidases are ubiquitous enzymes that are composed of dinuclear copper center, which have ability to insert oxygen at ortho position in hydroxyl group of aromatic ring and then oxidation of diphenol to quinone. This reaction utilizes molecular oxygen. The active site of enzyme is composed of copper which is bound by a cysteine residue and six histidine residues and it is highly conserved structure. This enzyme is universally distributed in plants, animals, bacteria and fungi. Most of its biological functions are unknown in plants and in fungi (Mayer, 2006).

Enzymatic browning in plants became the major reason for the loss in the quality of vegetables and fruits. Polyphenol oxidase acts on the phenolic compounds present in plants and cause browning. Active site of enzyme has copper atoms and these copper atoms plays important role in catalytic activity of PPO (Zawistowski *et al*., 1991). There are two different reactions that are catalyzed by PPO, first is monophenol hydroxylation into *o*-diphenols and second is *o*-diphenols oxidation into *o*-quinones (Mayer, 1986; Nagai and Suzuki, 2001). Zaini *et al*. (2013) reported that there will be a spontaneous polymerization of *o*-quinone which will produce melanins. Melanin is a pigment having higher molecular weight and is darker in colour.

1.1 Extraction, purification and characterization of PPO

Polyphenol oxidases are extracted and purified from different plants. Extraction along with purification of PPO is a multistep process. PPO was purified from leaves of tea plant with the help of Diethyl amino ethyl (DEAE) cellulose column and 3.32 folds of purification were achieved having 5.11 percent recovery (Unal *et al*., 2011). *Cleome gynandra* (spider flower), leaves were used for characterization of PPO by using ammonium sulphate, Sephadex G-75 gel filtration chromatography and DEAE-Sepharose

ion-exchange chromatography resulted in 37.8 folds purification with 44.3 % recovery (Gao *et al*., 2011).

PPO is richly present in *Solanum melongena* (eggplant). PPO causes enzymatic browning of eggplant resulted loss in its quality. Isoform of PPO obtained from eggplant had purification of 259 folds by using standard chromatography (Mishra *et al*., 2012). In another report, durum wheat was used to extract PPO, followed by partial purification through ion - exchange chromatography using DEAE column and 26.33 folds purification was obtained with 24.7 % recovery (Altunkaya and Gokmen, 2012).

1.2 Molecular weight of PPO

Madani *et al*. (2011) reported that PPO extracted from *Hibiscus rosa–sinensis*, when analyzed through Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) discovered bands in natural conditions both on purified and also on crude extracts. The crude extract has shown many bands and 70.795 k Da was the molecular weight of more distinct band among them. Molecular weight of PPO extracted from other species are as follows: banana 62 k Da (Galeazzi and Sgarbieri, 1981), cabbage 65 k Da (Nagai and Suzuki, 2001), bean seed 120 k Da (Paul and Gowda, 2000). PPO extracted from parsley has the molecular weight of 237 k Da (Dogru and Erat, 2012).

Zaini *et al*. (2013) estimated that PPO extracted from snake fruit has the molecular weight of 38 k Da. The molecular size of PPO from *Salacca zalaccake* (snake fruit) is lower than that obtained from other fruit plants such as loquat (Selles-Marchart *et al*., 2006), apples (Tsurutani *et al*., 2002), *Beta vulgaris* (Gandia-Herrero *et al*., 2004), peach (Cabanes *et al*., 2007) with a molecular weight of 59.2, 65, 54 and 60 k Da respectively.

1.3 Stability and optimum temperature

The optimal temperature is essential for the proper functioning of enzyme. For inhibiting the activity of enzyme, proper temperature of reaction should be avoided (Liu *et al*., 2013). Palma-Orozco *et al*. (2011) suggested that an optimal temperature for PPO is dependent upon the environment where the fruit develop, for example higher temperature is required for achieving maximum activity of enzyme in tropical regions.

Madani *et al*. (2011) examined the temperature effects, 4-methylcatechol was used (as substrate) from 25 °C – 70 °C. It was found that the activity of PPO from

Hibiscus increased with rise in temperature. The highest activity was attainted on 45 °C. Optimum temperature values of PPO for aubergine is 30 °C (Dogan *et al*., 2002), for *Rosa canina* (dog rose) it is 20 °C (Sakiroglu *et al*., 1996), for that of Amasya apple it is 56 °C (Oktay *et al*., 1995) and 25 ºC for *Lonicera japonica* (Liu *et al*., 2013).

Dogru and Erat (2012) described that optimal temperature for PPO was dependent on substrate molecule and activities of enzyme were analysed by using six different substrates. According to them optimum temperature for 4-methyl catechol and catechol is 40 °C, 45 °C and 35 °C for dopamine, L-3, 4-dihydroxyphenylalanine (L-DOPA) and progallol respectively. Enzymatic activity of PPO increased as the temperature was increased from 10 ºC to 45 ºC but above 60 ºC its activity was decreased.

1.4 Stability and optimum pH

There are different optimal pH values for PPO obtained from different plant species. Optimum pH has been found in between 4.0 - 8.5. While PPO from fruits and vegetable show their maximum catalytic activity at neutral values of pH and these pH values differs slightly with PPO source, and type of substrate molecule used (Ionita, 2013).

The optimum pH values depend upon the purity of PPO and the kind of buffer used (Wesche-Ebeling and Montgomery, 1990; Zhou and Feng, 1991; Erat *et al.*, 2010). Unal *et al*. (2011) reported that PPO extracted from tea leaf has a pH that varies from 4.03 to 7.0. Diverse values of optimal pH for PPO have been reported in literature for example 6.8 for cocoa beans (Lee *et al*., 1991), 7.0 of Amasya apple (Siddiq *et al*., 1992) and 5.5 of strawberry (Wesche-Ebeling and Montgomery, 1990).

It was investigated by Doğru and Erat (2012) that the optimal pH values of PPO from parsley differs with types of substrates used. The pH was 4.0 for 4-methyl catechol, catechol and catechin, 8.0 for dopamine, 4.5 for L-DOPA and 8.5 for pyrogallol. PPO extracted and purified from the leaves of *Cleome gynandra* was analysed for enzymatic activity, at different values of pH by using L- DOPA as substrate. PPO was active between 6.5 - 10.0 pH, and showed its maximum enzymatic activity at pH 8.5. Maximal activity reduces to half at pH 6.8 while less than 22 % of activity was shown by enzyme when pH was decreased below 6.0 (Gao *et al*., 2011).

1.5 Substrate specificity of PPO

The affinity of PPO varied with the type of substrate used. PPO from tea had more affinity for 4-methylcatechol. Many substrates were used to test PPO activity out of which 4-methylecatechol was the best. PPO had shown no activity against gallic acid (Daungmal and Apentene, 1999). *Km* values for the PPO of potato are 1.5 mM for pyrogallol, 6.8 for catechol and 1.1 mM for 4-methylcatechol (Madani *et al*., 2011).

Numerous phenolic compounds (chlorogenic acid, catechol, caffeic acid and 4-methyl catechol) were used as substrate specificity for durum wheat PPO. It is reported that durum wheat PPO had more affinity for catechol and has least activity for 4-methylcatechol (Altunkaya and Gokmen, 2012). Several compounds i.e. *o*-dianisidine, tetramethyl benzidine, *p*-phenylenediamine, guaiacol and diaminobenzidine were used to test the substrate specificity of PPO from *Mangifera indica* in the presence of hydrogen peroxide. *O*-dianisidine (1 mM) showed best affinity with PPO followed by *p*-phenylenediamine (John *et al*., 2011).

1.6 Effects of inhibitor on PPO activity

Literature has reported the effects of inhibitors on the activity of PPO. Inhibitors like ascorbic acid were used by researchers to prevent browning of plants. The actions of ascorbic acid, cysteine and sodium metabisulphite on PPO from tea were examined using catechol. High degree of inhibition was obtained with sodium metasulphite and ascorbic acid (Unal *et al*., 2011). Ethylene diamine tetra acetic acid (EDTA), iron chloride and ascorbic acid were used to study the effects of inhibition on PPO from *Hibiscus*. Ascorbic acid was potent inhibitor than EDTA, while there is no effect of iron chloride on *Hibiscus* PPO activity (Madani *et al*., 2011).

Queiroz *et al*. (2011) studied the effects of several inhibitors i.e. sodium metasulphite, sodium sulphite, ascorbic acid and citric acid on PPO activity. These inhibitors were involved in decreasing PPO activity. Among inhibitors like citric acid, oxalic acid, cysteine, and ascorbic acid, ascorbic acid was most effective inhibiting agent of PPO activity (Altunkaya and Gokmen, 2012). The extremely efficient inhibitors for PPO from memey were sodium metabisulphite, ascorbic acid, tropolone and succinic acid. When 10 mM of each of these inhibitors was used, they completely inhibit the PPO

activity while potassium sorbate, benzoic acid and citric acid activated enzyme rather than inhibition (Palma-Orozco *et al*., 2011).

1.7 Role of PPO in resistance against diseases

PPO has been commonly involved in providing resistance to plants against various pathogens, since quinones. Quinones are the primary products of PPO activity and they are very reactive electrophiles. They can go through intricate secondary metabolic pathways. Reactive oxygen species (ROS) may be generated during the reverse disproportionation of quinones to semiquinones. Polyphenol oxidases generate quinones and ROS which play variety of defensive functions in plants (Thipyapong *et al*., 2004).

The function of PPO against *Pseudomonas syringae* was further verified when trangenic tomato having 5 to 10 folds enhanced PPO activity level showed 100 folds decrease in *P. syrinagae* growth and 15 fold fewer lesions in comparison with non-transgenic control tomato plants at a large inoculum concentration (Li and Steffens, 2002). The effectiveness of defense mechanism depends upon the nature and virulence of pathogen. Interpretations of transgenic and non-transgenic tomato genotype proposed a positive association between PPO action and phases of foliar resistance to *A. solani*, the causative agent of early blight.

PPO generates H_2O_2 which functions in cell signaling by acting as diffusible activator of protective genes in cell, biosynthesis of phytoalexin, ethylene and salicylic acid production and initiate cell death which results in the formation of restricted lesions bordered from neighboring healthy tissues. Reactive oxygen species may functions to stimulate plant immunity and plant defensive genes (Thipyapong *et al*., 2004).

1.8 Role of PPO in defense against insects

Felton *et al*. (1992) suggested the idea that PPO provide defense against leaf eating insects. PPO activity in tomato leaves was induced by methyl jasmonate and systemin, which are herbivores defense inducing signal molecules. Major plant defense signal molecules are methyl jasmonate and oligo galacturonic acid and they strongly induce PPO activity (Constabel *et al*., 1995). The induction of PPO activity in tomato by various signals occurs in corresponding with a group of additional anti-herbivore proteins containing many types of anti-nutritive enzymes such as threonine deaminase, arginase and protease inhibitors (Bergey *et al*., 1996; Chen *et al*., 2005).

PPO was found to perform similar defensive function against insects in tobacco. It is found that protease inhibitors and PPO are upregulated by methyl jasmonate and systemin in tobacco (Constabel and Ryan, 1998; Ren and Lu, 2006). Induction of PPO by strong herbivores wound and methyl jasmonate was showed by leaves of poplar species (Constabel *et al*., 2000; Haruta *et al*., 2001). PPO induction in tomato by both methyl jasmonate and insects has been simulated in both field studies and in laboratory (Stout *et al*., 1997; Thaler *et al*., 1996 and 2002).

Induction of PPO by methyl jasmonate treatment and by wounding has been confirmed in trees and herbaceous crops (Constabel and Ryan, 1998). An increased resistance has shown by potato genotype which has high PPO activity towards Colorado potato beetle (Castanera *et al*., 1996). Melo *et al*. (2006) reported that high level of PPO did not affect the resistance to coffee leaves miner. Negative correlation was found for growth rate of *Helicoverpa zea* caterpillars with leaves of tomato and PPO levels of tomato fruit (Felton *et al*., 1989). Greater performance has shown by *Manduca quinquemaculata* caterpillars on young tobacco leaves having higher levels of PPO (Kessler and Baldwin, 2002).

Transgenic poplar plants having overexpressing PPO genes facilitates defense against tree feeding caterpillars but mixed type of results were provided by these studies. First-instar *Malacosoma disstria* caterpillars had reduced growth rates on higher levels of poplar PPO, only when all the experimental work was done in fall (Wang and Constabel, 2004). Similarly, fourth-instar *Lymantria dispar* caterpillars had showed a decrease in growth rates on higher PPO levels of poplar, when examine in winters (Barbehenn *et al*., 2007).

1.9 Role of PPO during water stress

Thipyapong *et al*. (2004) reported the response shown by A14-6 (transgenic) plants having 20 – 40 fold lessened PPO activity besides S-28 (transgenic) plants having 2 – 6 fold elevated PPO activity towards drought. In short, it was examined that A14-6 began displaying symptoms like leaf curling, wilting and also yellowing in older leaves far ahead than those of S-28.

Expression studies revealed that the induction of PPO in reaction to water stress was higher in mature leaves plus abscission regions, which can assist death of cells.

Abscission of mature leaves can characterize adaptive approach during water stress, because this possibly will reduce additional loss of water and will allow enough nutrients distribution to the growing tissues (Thipyapong *et al*., 2004). Literature has reported PPO induction during H_2O stress in transgenic tomatoes (English-Loeb *et al*., 1997). In another plant for example coconut, higher induction of PPO was detected under H_2O stress (Shivishankar, 1988). However, more investigation is required for understanding the physiological functions of PPO during H_2O stress.

1.10 PPO activity and hormonal stress

PPO activity was found to be regulated during various hormonal stresses in many plants. Plants can be treated with jasmonic acid to induce defense against herbivores. In tomato, stress of jasmonic acid resulted in the induction of PPO which caused the decrease in abundance, performance and preference of several herbivores including aphids, thrips, flea beetles and caterpillars (Thaler *et al*., 1996).

Thipyapong and Steffens (1997) studied the response of PPO F promoter towards jasmonic acid, salicylic acid and methylene treatment in tomato. It was found that the uptake of jasmonic acid by shoots caused 2.2-fold induction of PPO F activity at leaf nodes after 48 hours of treatment. Salicylic acid treatment induced PPO F activity in both apical and mature tissues. Ethylene treatment induced PPO F activity in older leaves.

Rout (2006) examined the activity of PPO during auxin stress in tea plant. An increase in PPO activity was found during Indole-3-butyric acid (IBA) stress. Later on Ling *et al*. (2013) also studied the change in activity of PPO during IBA stress in tetraploid locust. PPO activity was increased when different IBA treatments were applied.

1.11 Regulation of PPO activity by sense and antisense orientation

Thipyapong *et al*. (2007) generated two forms of transgenic tomato plants, sense PPO plants with overexpressing PPO activity and antisense PPO plants having suppressed activity of PPO. Two characteristic antisense PPO and sense PPO transgenic lines (A19-3 and A14-6, S-28 and S-18) were additionally utilized for PPO analysis. It was examined that A19-3 and A14-6 showed 2-40 fold reduction in PPO activity, along with low intensities of immunologically noticeable PPO in homogenates of leaf, while S-

28 and S-18 have 2-10 fold induction of PPO activity and increased immunologically noticeable PPO, and 30 fold increase in PPO transcripts (Thipyapong *et al*., 2004).

The change of PPO activity had no influence on growth and development of plant. Growth rates, germination percentages, total area of leaf, dry weights of roots and shoots and amount of seeds for each fruit were statistically not different between SP, OP transgenic and control tomato plants. The transgenic tomato plants displayed same morphology and also set seeds normally, suggested that under the control growth conditions of plant growth chamber and greenhouse, PPO did not show critical function in plant growth, development and metabolism (Steffens *et al*., 1994; Li and Steffens, 2002; Thipyapong *et al*., 2004).

1.12 Some other potential roles of PPO

Sherman *et al*. (1991) suggested that PPO may have potential to develop simultaneously through adaptation towards an oxygenated climate where PPO role is important in photosynthetic machinery of land-dwelling plants. Meanwhile PPO genes might progress to perform various roles in different species of plants. PPO role in formation of pigments was proposed in *Antirrhinum* (snapdragon flowers) for the synthesis of yellow pigments commonly known as aurones, and in order *Caryophyllales* including carnations, cacti, amaranths, beet, ice plants etc. for the synthesis of red or yellow pigments known as betalains (Nakayama *et al*., 2000).

Expression of chalcone 4'-*o*-glucosyltransferase along with aureusidin synthase has permitted the accumulation of the aureusidin 6-*o* glucoside, due to which yellow colored pigments in flowers of transgenic *Torenia hybrid* (torenia) normally lacks the pathway of endogenous biosynthesis (Ono *et al*., 2006). Cho *et al*. (2003) studied that in *Larrea tridentate* (creosote bush), PPO is involved in synthesis of creosote bush lignans. Lignans are metabolites having strong antioxidant, anti-cancer and antiviral properties. Capability of PPO to transform monophenols into *o*-diphenols, PPO has been supposed to perform functions in hydroxylation of the *p*-coumaric acid into caffeic acid during phenyl propanoid pathway (Vaughn and Duke, 1984; Kojima and Takeuchi, 1989).

No obvious variances in leaf caffeic acid and *p*-coumaric acid pools in between non transgenic control and A14-6 tomato plants were set up, signifying that PPO is not involved in hydroxylation of coumarate 3 in leaves (Thipyapong *et al*., 2004). While in

certain circumstances, PPO over expression might be valuable than that of down regulation of PPO e.g. during heterologous expressing genes of PPO from red clover in alfafa has decreased post-harvest breakdown of proteases during the initial phases of ensiling, therefore decreasing the damage of nutritive value (Sullivan *et al*., 2004).

1.13 Objectives

The main objective of the present study was the transformation *of Nicotiana tabacum* cv. Samsun plants with antisense *Os*PPO promoter fused with GUS gene, then assessment of GUS expression induced by antisense *Os*PPO promoter in response to different treatments of plant growth hormones (NAA, BAP and ABA) in transgenic tobacco plants.

MATERIALS AND METHODS

2.1 Surface sterilization of seeds

Nicotiana tabacum cv. Samsun seeds were washed with autoclaved distilled water for 2 to 3 times and seeds floating on surface of water were removed. Remaining seeds were treated with 70 % ethanol for 2 to 3 minutes. Ethanol was discarded and seeds were rinsed with autoclaved distilled water at least three to four times. Then 30 % bleach was added for 2 minutes and washing was performed with distilled H_2O and seeds were dried on sterile filter paper.

2.2 Propagation of plantlet as a source of leaf disks

Seeds were subsequently put into glass bottles having solid MS (Murashige and Skoog) media with pH 5.8 (Table 2.1). Glass bottles were sealed with parafilm then kept in growth chamber at 25 °C at 16/8 light and dark condition. Germination of seeds was started within 2 weeks. Seedlings approximately 5 to 7 cm tall were then used to prepare leaf disks. Fresh leaves from tobacco plant were utilized for the preparation of leaf disks. Leaf disks were cut down and shifted to petri plates containing solid MS (Murashige and Skoog) media (Table 2.1) having pH 5.8. Arrangement of leaf disks on media was done in such a way that their adaxial side touched the media. Around 10 to 15 leaf discs were kept in each petri plate. Plates were wrapped with parafilm and placed at 25 °C at 16/8 light and dark period for 48 hours.

Table 2.1: Composition of MS Media.

Sr. No.	Compounds	Concentration (g/L)
1	MS salt	4.443
2	Sucrose	30
3	Phytagel	2

Key: MS: Murashige and Skoog

2.3 Agrobacterium culture preparation

Agrobacterium strain EHA101 transformed with expression vector (pCAMBIA 1391z) containing antisense *Os*PPO promoter fused with GUS gene from the stock of

glycerol culture was streaked on LB (Lauria Broth) media (Table 2.2). Kanamycin (50 mg/ L) was added to medium and pH was adjusted up to 7. Petri plates were then kept in incubator for 48 hours at 28 °C. These bacterial colonies were utilized for preparation of starter culture. Bacterial colonies started to appear on media after 36 hours. Two days later single bacterial colony was selected from petri plate and inoculated into 100 mL liquid LB media (Lauria Broth) containing 50 mg/L of kanamycin with continuous shaking at 250 rpm for 48 hours at 28 °C in the dark.

Table 2.2: Composition of solid LB media.

Sr. No.	Compounds	Concentration
1	Tryptone	10 g/L
2	Yeast extract	5 g/L
3	NaCl	10 g/L
4	Kanamycin	50 mg/L
5	Agar	15 g/L

Key: NaCl: Sodium chloride

2.4 Infection of tobacco leaf disks

The OD (optical density) of starter culture (EHA101 strain containing *Os*PPO promoter fused with GUS) was set on 0.155. Starter culture was transferred to beaker and leaf disks were wounded artificially and then dipped in culture for 10-15 minutes with mild shaking under sterile conditions. Leaf disks were gently taken out from culture and put on sterile filter paper for drying.

2.5 Co-cultivation of tobacco leaf disks

Leaf disks were shifted to petri plates containing co-cultivation media (Table 2.3). The pH of co-cultivation medium was also set to 5.8. Petri plates were then wrapped with parafilm under sterile conditions. Petri plates were put in incubator at 28 °C for 48 hours.

Table 2.3: Composition of co-cultivation media.

Sr. No.	Compounds	Concentration
1	MS salt	4.43 g/L
2	Sucrose	30 g/L
3	IAA	0.1 mg/L
4	BAP	1 mg/L
5	Phytagel	2 g/L

Key: MS: Murashige and Skoog, IAA: Indole 3-Acetic acid, BAP: 6-Benzyl Amino Purine

2.6 Shifting of leaf disks to selection and regeneration media

Leaf disks from co-cultivation medium were taken and poured into beaker containing cefotaxime (250 mg/L). Washing of leaf disks with cefotaxime has helped to remove surplus growth of bacterial cells. After washing dried them on sterilized filter paper and shifted to petri plates containing selection media (Table 2.4) having pH 5.8. Petri plates were sealed with parafilm and kept in growth chamber at 25 °C at 16/8 light and dark period. After every one week leaf disks were shifted to new selection media. Callus formation was noticed on leaf disks after three weeks and calli were then shifted to fresh selection medium. When callus started to increases in size, it was transferred to glass bottles.

Table 2.4: Composition of selection and regeneration media.

Sr. No.	Compounds	Concentration
1	MS salt	4.43 g/L
2	Sucrose	30 g/L
3	BAP	1 mg/L
4	IAA	0.1 mg/L
5	Cefotaxime	500 mg/L
6	Hygromycin	50 mg/L
7	Phytagel	2 g/L

Key: MS: Murashige and Skoog, BAP: 6-Benzyl Amino Purine, IAA: Indole 3-Acetic Acid

2.7 Shifting of callus to shooting media

Callus was transferred to glass bottles containing shooting media having pH 5.8 (Table 2.5). Glass bottles were sealed with parafilm and kept in growth chamber under control conditions (25 °C at 16/8 light and dark period). Shooting was started after 3-4 weeks and when large number of shoots developed they were shifted to fresh selection and regeneration media in separate glass bottles for enhanced growth.

Table 2.5: Composition of shooting media.

Sr. No.	Compounds	Concentration
1	MS salt	4.43 g/L
2	Sucrose	30 g/L
3	IAA	0.1 mg/L
4	BAP	2 mg/L
5	Cefotaxime	500 mg/L
6	Hygromycin	50 mg/L
7	Phytagel	2 g/L

2.8 Shifting to rooting medium

When shoots extended up to 2-3 nodal stage they were shifted to MS rooting media (Table 2.6) for induction of roots. The pH of media was retained at 5.8. Rooting was developed after 15 days.

Table 2.6: Composition of rooting media.

Sr. No.	Compounds	Concentration
1	MS salt	4.43 g/L
2	Sucrose	3 g/L
3	IAA	0.1 mg/L
4	Hygromycin	25 mg/L
5	Cefotaxime	250 mg/L
6	Phytagel	2 g/L

2.9 Control plants

Same procedure for propagation of plantlets was followed for producing control plants except that they were not treated with Agrobacterium. Control tobacco

plants were also multiplied on MS medium and kept in growth chamber under control conditions.

2.10 Confirmation of transformed tobacco plants by PCR

PCR amplification and histochemical analysis was done for the confirmation of antisense *Os*PPO promoter in transgenic plant genome.

2.10.1 Isolation of genomic DNA

DNA was isolated from transgenic tobacco plants by Cetyl trimethyl Ethyl Ammonium Bromide (CTAB) method. About 0.3g of plant material (4 to 5 leaves of tobacco) was washed with distilled H_2O. Plant material was finely crushed into paste using pestle and mortar with the help of 2 mL CTAB buffer, pre-heated at 65 °C. CTAB buffer was prepared by adding 20 mL 1M EDTA (ethylene diamine tetra acetic acid) having pH 8.0, 100 mL 1 M Tris HCl with pH 8.0, 1.4 M Sodium chloride (NaCl) and 2 % w/v CTAB. 1 % β-Merceptoethanol was also added to CTAB before grinding of plant material. After grinding the mixture was poured into 1.5 mL sterilized eppendorf tubes. The eppendorf tubes were then incubated for 45 minutes on heat block at 65 °C. After incubation the tubes were centrifuged for 10 minutes at 10,000 rpm and the supernatant transferred to new eppendorf tubes while pallet was discarded. Then the chloroform/isoamyl alcohol (24:1) was added in the supernatant in an equal volume and tubes were inverted for 4-5 times for mixing. The mixture was centrifuged for 10 minutes at 10,000 rpm. Supernatant was again collected and transferred to new eppendorf tube and this step was repeated 3-4 times. An equal volume of isopropanol and 45 µL of 3 M Sodium acetate were added to precipitate out DNA. Eppendorf tubes were inverted slowly for 4-5 times and then centrifuged at 12,000 rpm for 12 minutes, DNA precipitated in white form at the bottom of tubes and aqueous phase was discarded. The pellet was washed with 70 % ethanol and then dried in air at room temperature. The pellet was rehydrated in 30-40 µL of 0.1 X TE (Tris EDTA) buffer containing 2 µL of RNase. The DNA was stored at -20 °C.

2.10.2 PCR amplification of antisense *Os*PPO promoter

DNA extracted from leaves of transgenic plants was then processed for PCR amplification by using 25 µL PCR reaction mixture. PCR reaction mixture was composed of 12.5 µL of PCR master mixture, 1 µL of 50 pM of forward and reverse primer and 10 µL of nuclease free water with 50 ng of genomic DNA. Following steps were followed for PCR amplification.

1. For pre denaturation the mixture was heated up to 94 °C for 5 minutes followed by 35 cycles under 95 °C for 30 seconds.
2. For annealing the mixture was cooled for 50 seconds at 55 °C.
3. After annealing temperature was increased up to 72 °C for 1 minute for extension. Final extension was done at 72 °C for 20 minutes.

Following set of primers was utilized.

Forward: 5’ CTGGTCATAACTAGATTAAGAT 3’

Reverse: 5’ GTCGTAGATTAGATTACCGAT 3’

The amplified antisense *Os*PPO promoter along with 1 kbp ladder was loaded with bromophenol blue and run on 1 % agarose gel prepared in TAE buffer and then stained with ethidium bromide and visualized by UV transilluminator.

2.11 Confirmation of transgenic tobacco by GUS staining

Transgenic plants were also confirmed by histochemical staining. The leaf was detached from the transformed plant and kept in 1 mL of histochemical reagent (5 µL of X-Gluc in 1 mL of GUS Buffer) in falcon tube and then kept in incubator at 37 °C for 24 hours. Then the leaf was kept in methanol for removal of chlorophyll.

2.12 Auxin stress on transgenic plants

NAA treatment was applied to almost 2 months old seedlings having dense roots about 2 to 3 cm in length kept in growth chamber in control conditions (16 hours light and 8 hours dark period at 25 °C). For NAA treatment seedlings were immersed in MS

liquid medium (MS 4.43 g/L, sucrose 30 g/L) having pH 5.8, containing various concentrations of NAA (α-Naphthaline acetic acid) i.e., 100 µM, 10 µM, 1 µM and 0.1 µM (Table 2.7) as reported by Wu *et al*. (2009). Leaves and roots were excised after 0, 3, 6, 12, 24 and 48 hours of NAA treatment and put into X-Gluc solution for histochemical staining. Control plants were also treated under similar conditions as described for transgenic plants.

Table 2.7: Different solutions of NAA.

Sr. No.	Solutions	Quantity
1	100 µM	1862 µL/100 mL
2	10 µM	186.2 µL/100 mL
3	1 µM	18.62 µL/100 mL
4	0.1 µM	1.862 µL/100 mL

2.13 Cytokinin stress on transgenic plants

BAP treatment was applied to seedlings having dense roots about 2 to 3 cm in length, kept in growth chamber under 16 hours light and 8 hours dark period at 25 °C. For BAP treatment seedlings were immersed in MS liquid medium (MS 4.43 g/L, sucrose 30 g/L) having pH 5.8, containing various concentrations of BAP (6-Benzyl amino purine) i.e., 80 µM, 40 µM, 20 µM and 1 µM (Table 2.8). Leaves and roots were excised after 0, 3, 6, 12, 24 and 48 hours of BAP treatment and put into X-Gluc solution for histochemical staining. Control plants were also treated under similar conditions as described for transgenic plants.

Table 2.8: Different solutions of BAP.

Sr. No.	Solutions	Quantity
1	80 µM	1802 µL/100 mL
2	40 µM	901.0 µL/100 mL
3	20 µM	450.5 µL/100 mL
4	1 µM	22.52 µL/100 mL

2.14 Abscisic acid stress on transgenic plants

Various concentrations of abscisic acid (ABA) i.e., 50 µM, 150 µM, 250 µM and 350 µM (Table 2.9) were applied to plants by using spray method. Abscisic acid solutions were prepared in distilled water and sprayed on plants in solid MS media. Leaves and roots were excised after 24 and 48 hours of treatment and put into X-Gluc solution for histochemical staining. Control plants were also treated under similar conditions as described for transgenic plants.

Table 2.9: Different solutions of ABA.

Sr. No.	Solutions	Quantity
1	350 µM	350 µL/10 mL
2	250 µM	250 µL/10 mL
3	150 µM	150 µL/10 mL
4	50 µM	50 µL/10 mL

2.15 Histochemical staining of leaves and roots

Leaves and roots isolated from hormonal treated plants were then subjected to histochemical staining in 1.5 mL eppendorf tubes. Leaves and roots were immersed in X-Gluc solution (Table 2.10) containing X-Gluc (Table 2.11) in GUS buffer (Table 2.12) having pH 7 and then incubated for 12 hours at 37 °C in dark for the expression analysis of antisense *Os*PPO promoter. After incubation 2 to 3 times leaves and roots were washed with absolute methanol until the complete removal of chlorophyll. Leaves were then preserved in methanol.

Table 2.10: Composition of GUS solution.

Sr. No.	Compounds	Quantity
1	X-Gluc	5 µL
2	GUS Buffer	1 mL

Table 2.11: Composition of X-Gluc.

Sr. No.	Compounds	Quantity
1	X-Gluc	10 mg
2	DMSO	100 µL

Key: DMSO: Dimethyl sulpho oxide

2.16 Microscopy of preserved samples of leaves and roots

The preserved leaves and roots samples from NAA, BAP and ABA treated transgenic plants were examined microscopically under different magnification powers with help of light microscope. Different parts of plant e.g. leaf blade, petiole, stem and roots were visualized. Microscopic analysis helped to visualize close GUS expression in various parts of leaf e.g. guard cells, midrib and in veins.

Table 2.12: Composition of GUS buffer.

Sr. No.	Compounds	Quantity
1	NaH_2PO_4	50 mM
2	Na_2EDTA	10 mM
3	Triton X 100	0.01 %

Key: NaH_2PO_4: Sodium dihydrogen phosphate, Na_2EDTA: Disodium ethyl dimethyl tetra acetic acid

RESULTS AND DISCUSSION

3.1 Pre-incubation and selection of leaf disks

Leaf disks were cut off from young seedlings and pre-incubated on simple MS media for 48 hours (Figure 1). Leaf disks which showed bulging were selected for infection. Pre-incubation is necessary, as it helped to improve the efficiency of transformation in Arabidopsis (Sangwan *et al*., 1992).

3.2 Infection and co-cultivation of leaf disks

Leaf disks were wounded artificially and put into beaker containing starter culture (*Agrobacterium* strain EHA101 having antisense *Os*PPO promoter fused with GUS gene) for 10 to 15 minutes. Infected leaf disks were dried on autoclaved filter paper and transferred to petri plates containing co-cultivation media (Figure 2). Petri plates were wrapped with parafilm and shifted to incubator for 48 hours. Bacterial growth was monitored after 36 hours.

3.3 Regeneration of leaf disks

Leaf disks from co-cultivation media were taken and washed with cefotaxime to remove excess of bacterial growth. Leaf disks were dried and shifted to petri plates containing selection and regeneration media. Petri plated were wrapped with parafilm and put in growth chamber under control conditions.

3.4 Callus formation

Growth of leaf disk into callus (undistinguishable bulk of cells) was started after 3 weeks (Figure 3). When sizes of calli became large they were shifted to shooting media.

3.5 Generation of shoots and roots

For generating shoots, calli were transferred to glass bottles containing shooting media and kept in growth chamber under 25 °C at 16/8 light and dark period. Shooting was observed after 3 to 4 weeks (Figure 4). When enough shoots developed they were shifted to separate glass bottles having rooting media. Rooting was observed after 15 days. Dense roots were formed after 25 days (Figure 5).

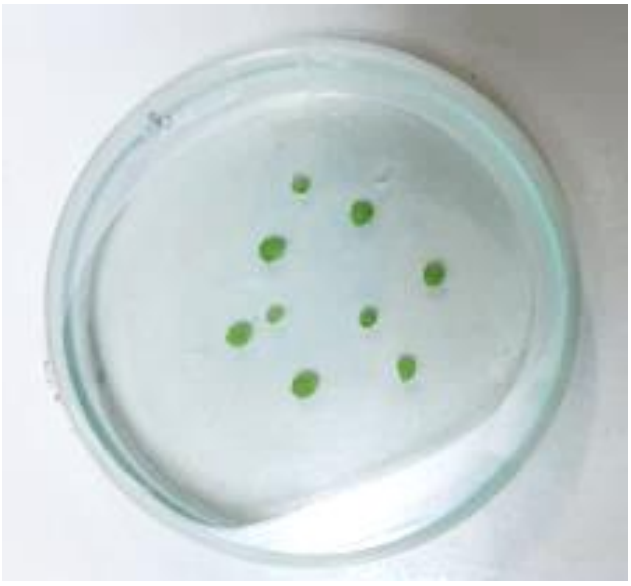

Figure 1: Incubation of tobacco leaf disks on simple MS media for 48 hours at 28°C.

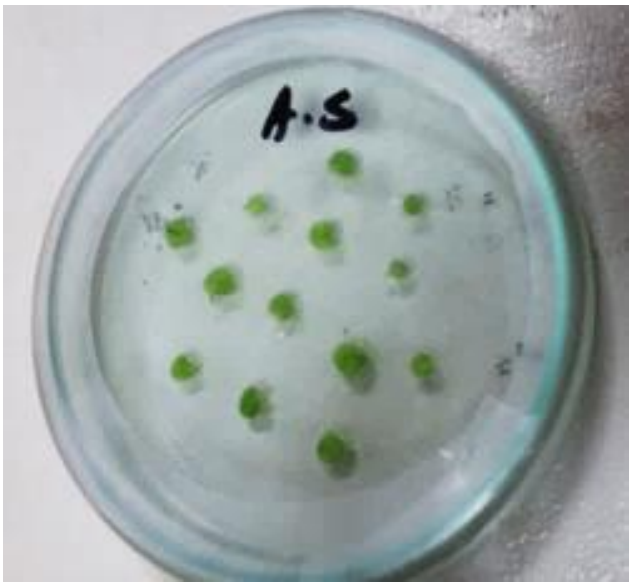

Figure 2: *Agrobacterium* infected tobacco leaf disks on co-cultivation media for incubation.

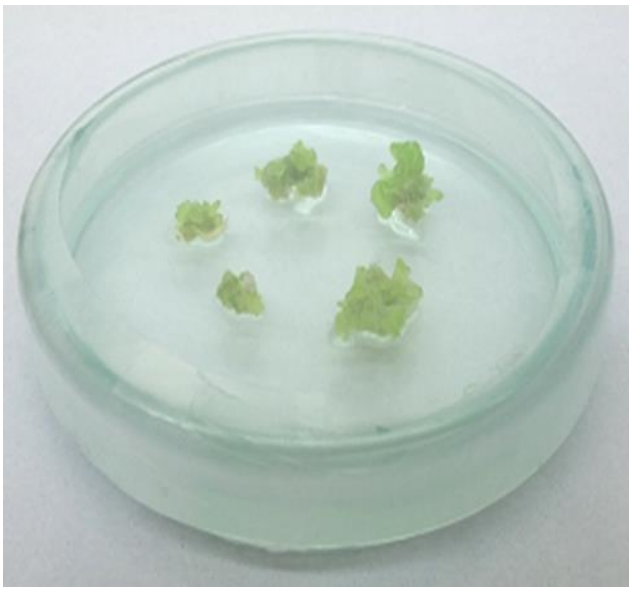

Figure 3: Formation of callus from transformed tobacco leaf disks on selection and regeneration media.

Figure 4: Generation of shoots from transgenic tobacco callus on shooting media.

Figure 5: Dense root formation of transformed tobacco plants on rooting media.

3.6 Control tobacco plants

Same methodology was utilized for producing control plants but they were not treated with *Agrobacterium.* Control plants were also shifted to fresh MS media for better growth.

3.7 Confirmation of tranformant through PCR analysis

Transformed and control tobacco plants were confirmed through PCR analysis. Genomic DNA was isolated from leaves of transgenic as well as control plants. Amplification of DNA was done by using specific set of primers and amplified product was run on agarose gel electrophoresis. Amplified DNA bands were found when gel was visualized under UV transilluminator while no amplified band was observed from the DNA sample of control tobacco plants (Figure 6).

3.8 Confirmation of transformants through GUS analysis

Leaves and roots were taken from transgenic and control plants and incubated in GUS solution for 48 hours. Leaves and root having antisense *Os*PPO promoter fused with GUS (reporter gene) showed blue colour while non transformed samples were remained same (Figure 7).

3.9 Stress of hormones

Transgenic and control tobacco plants were selected for hormonal treatments. Different concentrations of NAA and BAP were added in liquid MS media having pH 5.8. Transgenic and control tobacco plants were dipped in MS media while ABA solutions were sprayed on plants in solid MS media (Figure 8, 9 and 10). After regular intervals of time, leaves and roots were detached and put in GUS solution for histochemical analysis.

3.10 Histochemical staining

Leaves and roots were taken from NAA and BAP treated plants at 0, 3, 6, 12, 24 and 48 hours, while branches were taken from ABA treated plants after 24 and 48 hours and incubated overnight in GUS solution. After 24 hours GUS solution was discarded and methanol was added to leaves and roots for the removal of chlorophyll.

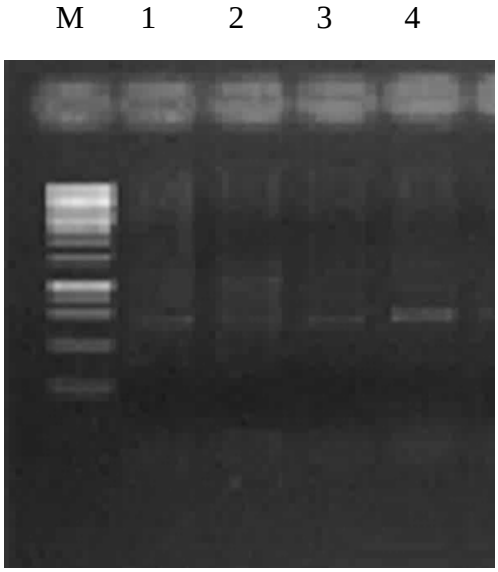

Figure 6: Confirmation of antisense *Os*PPO promoter transformation in tobacco by PCR amplification.

Figure 7: Histochemical analysis of transformed tobacco leaves showing GUS expression.

Figure 8: Application of NAA treatments (0.1 µM, 1 µM, 10 µM, and 100 µM) to transformed tobacco plants in liquid MS media.

Figure 9: Application of BAP treatments (80 µM, 60 µM, 20 µM, and 1 µM) to transformed tobacco plants in liquid MS media.

Figure 10: Application of ABA treatments (50 µM, 150 µM, 250 µM, and 350 µM) to transformed tobacco plants in solid MS media.

When chlorophyll was completely eliminated, leaves and roots samples were preserved in methanol for further examination. Leaves and roots of transgenic tobacco showed blue colour upon induction of antisense *Os*PPO promoter while non-transformed tobacco samples were remained same. Promoter activity was examined to be changed with the increasing concentrations of hormones and with the passage of time.

3.11 Application of NAA

NAA, a synthetic auxin is commonly utilized in lower amount to provoke auxin-type responses in fruit setting, cell division, cell growth and in rooting (Sun and Hong, 2010). Different concentrations of NAA i.e., 0.1 µM, 1 µM, 10 µM and 100 µM as described by Wu *et al*. (2009) were applied to transgenic as well as control tobacco plants in 100 mL of liquid MS media with pH 5.8.

3.11.1 Effect of 0.1 µM NAA on promoter activity

Promoter was weakly induced after 0, 3 and 6 hours of 0.1 µM NAA application. Less expression was observed on midrib, petiole and in veins after 0 and 3 and 6 hours. However strong GUS expression was observed after 12 hours on overall leaf surface which was decreased after 24 and 48 hours and expression was found to be strong on petiole and base of leaf but decreased towards apex (Figure 11), while maximum expression on roots was observed only after 12 hours (Figure 12). Differential response of antisense *Os*PPO promoter with respect to time of treatment was observed (Table 3.1 and Figure 13). Control plants did not show any expression.

3.11.2 Effect of 1 µM NAA on promoter activity

Less GUS expression was found on petiole and edges of leaf at 0 hour. Promoter was strongly induced after 3, 6 12 and 24 hours and maximum GUS expression was observed in veins, which was decreased after 48 hours and very less expression was found on margins of leaf (Figure 10). Expression on root was increased up to 12 hours and then decreased (Figure 11). No GUS expression was found on control plants. Discontinuous pattern of GUS expression induced by antisense *Os*PPO promoter with the passage of time was observed (Table 3.2 and Figure 14).

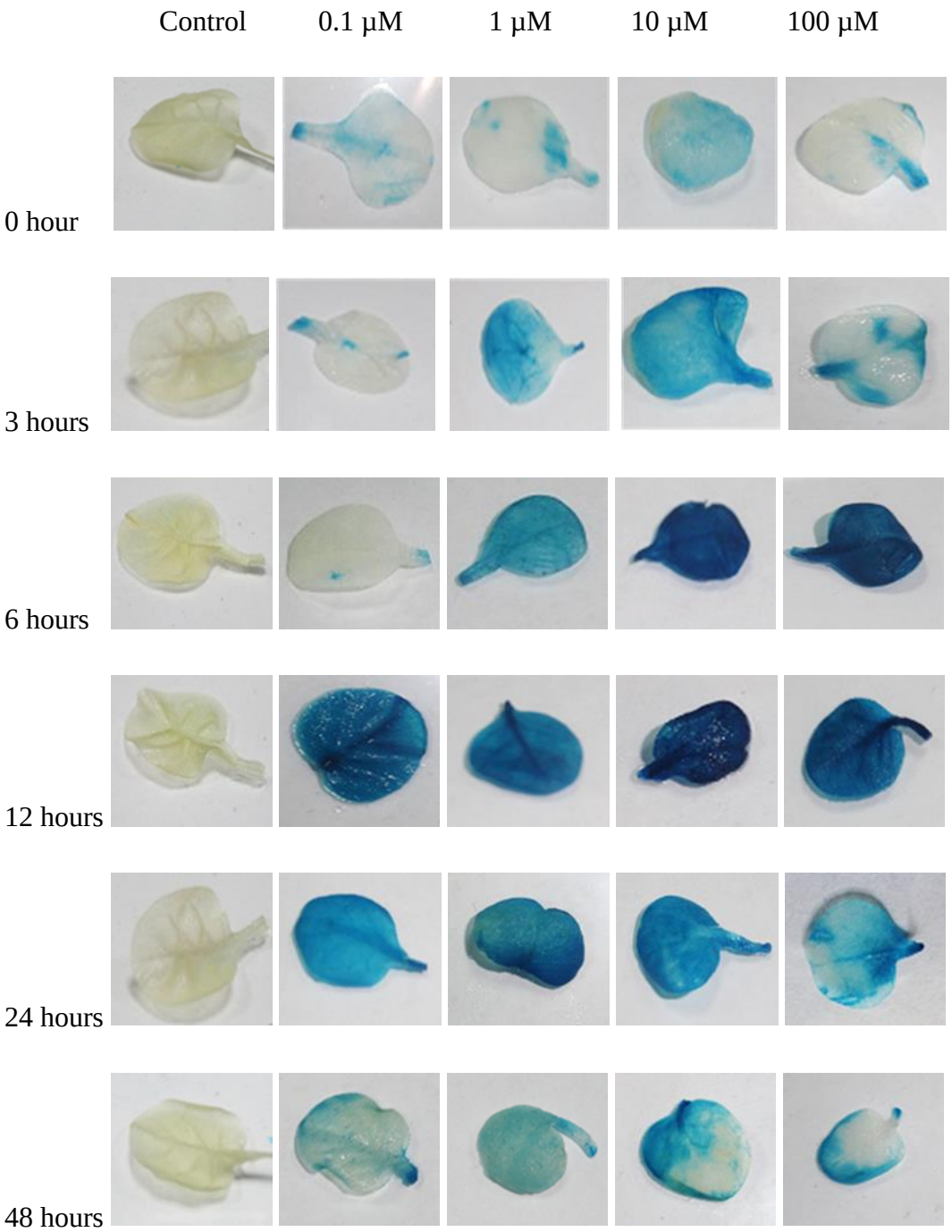

Figure 11: GUS expression analysis of transgenic and control tobacco leaves treated with various concentrations of NAA, at different time intervals.

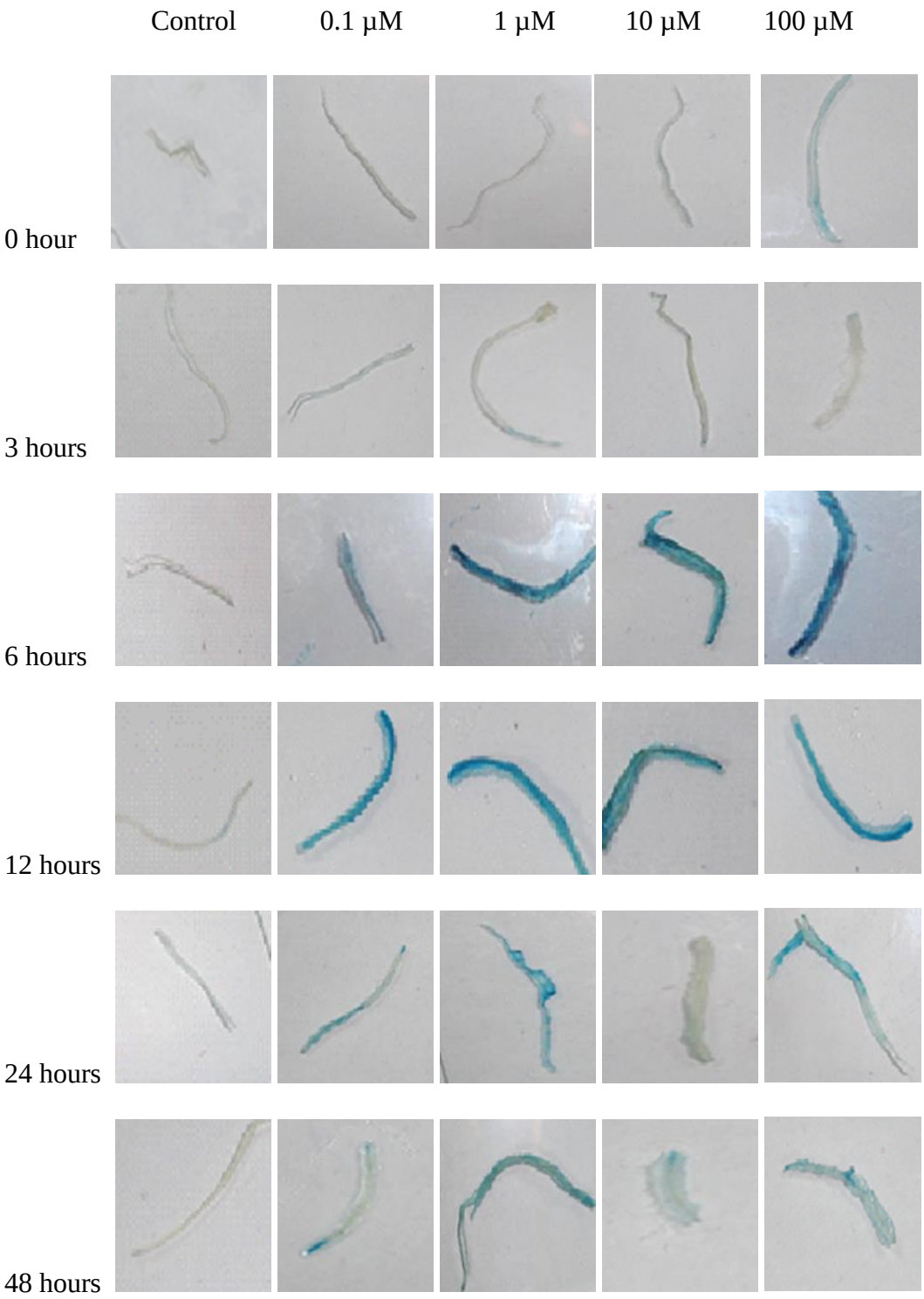

Figure 12: GUS expression analysis of transgenic and control tobacco roots treated with various concentrations of NAA, at different time intervals.

Table 3.1: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 0.1 µM NAA application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	10
2	3	20
3	6	10
4	12	90
5	24	80
6	48	30

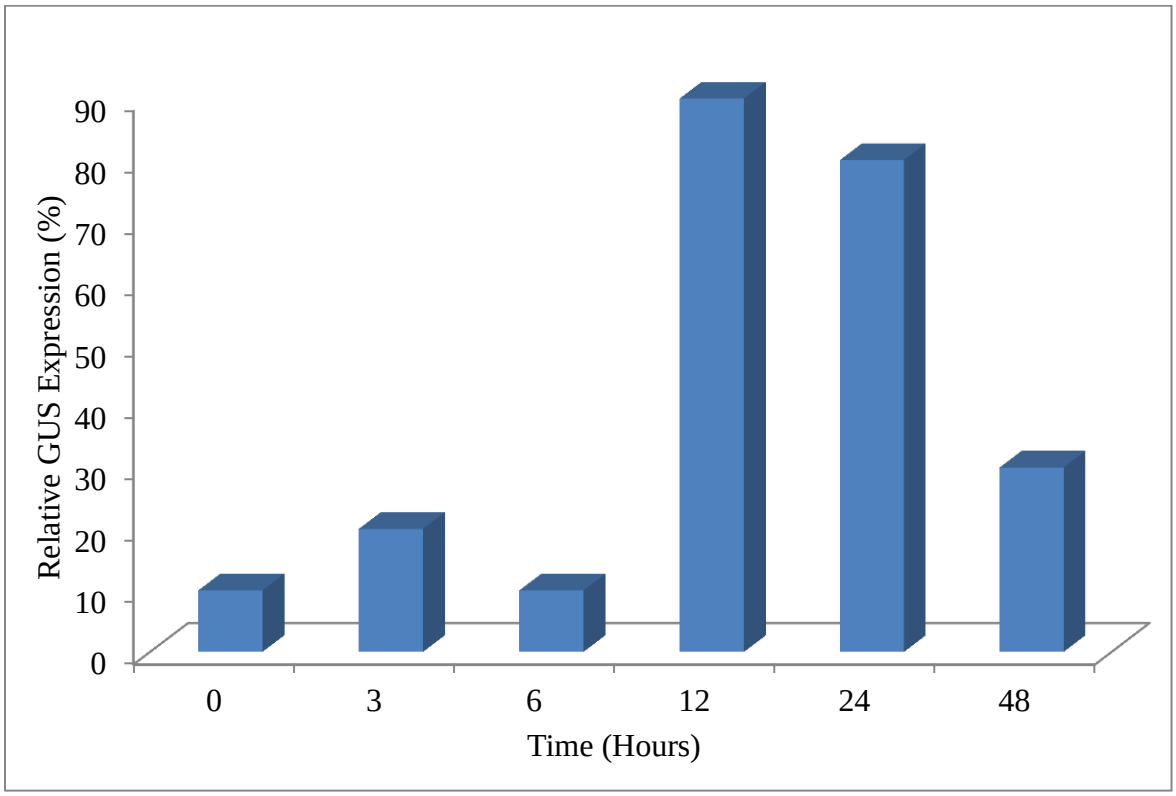

Figure 13: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 0.1 µM NAA treatment.

Table 3.2: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 1 µM NAA application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	20
2	3	60
3	6	70
4	12	90
5	24	80
6	48	30

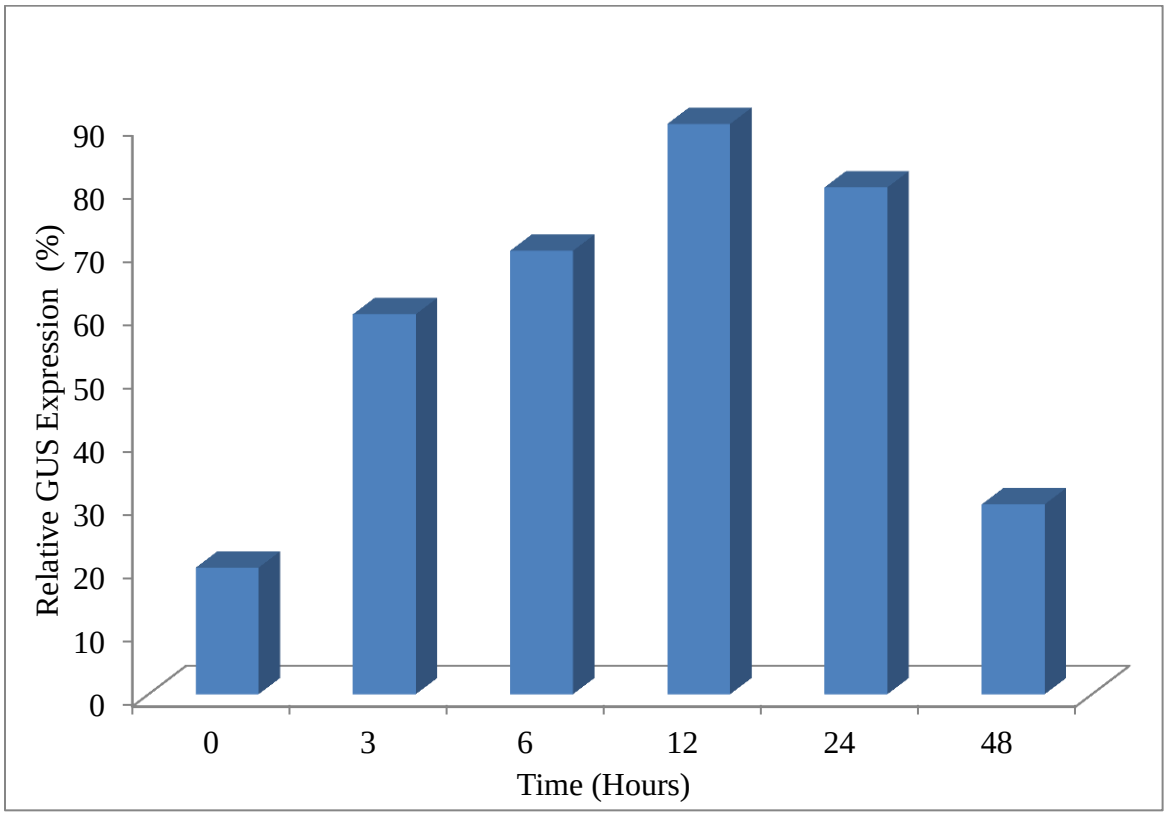

Figure 14: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 1 µM NAA.

3.11.3 Effect of 10 µM NAA on promoter activity

Increase in GUS expression was observed with increasing the concentration of hormone. Diffused expression was found on the whole surface of leaf after 0 and 3 hours. Promoter was strongly induced after 6, 12 and 24 hours of treatment and strong expression was observed on overall leaf. Induction of promoter was observed to be decreased after 48 hours and expression was discontinuous, only found on apex and reduced towards base of leaf (Figure 9). This result showed the increase of GUS expression up to 12 hours of treatment and then started decreasing (Table 3.3 and Figure 15). Strong expression was achieved on roots after 6 and 12 hours while promoter did not show any considerable activity after 24 and 48 hours. No expression was found on roots after 0 and 3 hours and also on control samples of leaves and roots.

3.11.4 Effect of 100 µM NAA on promoter activity

Promoter was strongly induced after 6 and 12 hours and maximum GUS expression was found on petiole, midrib and in veins. Expression decreased with the passage of time and was only observed on leaf margins after 24 and 48 hours (Figure 9). Antisense *Os*PPO promoter showed discontinuous GUS expression in response to 100 µM NAA treatment with the passage of time (Table 3.4 and Figure 16). In roots promoter activity was highest after 6 and 12 hours and overall root showed GUS expression. Control plants did not show any expression.

3.12 Stress of BAP

Different concentrations of BAP (6-benzyl amino purine) i.e., 1 µM, 20 µM, 40 µM and 80 µM were added to 100 mL of liquid MS media and pH was set to 5.8. Transgenic and control plants were immersed in media and functioning of promoter was observed after 0, 3, 6, 12, 24 and 48 hours.

Table 3.3: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 10 µM NAA application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	40
2	3	70
3	6	80
4	12	90
5	24	70
6	48	60

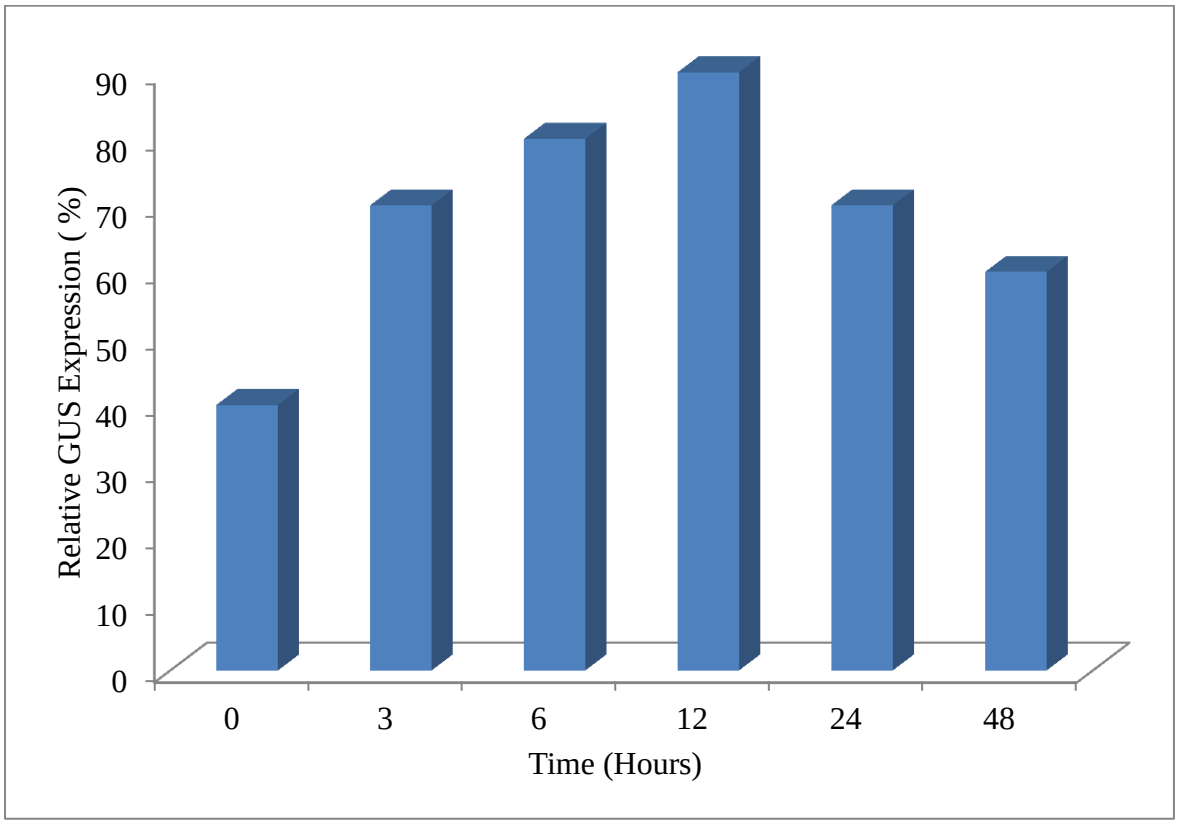

Figure 15: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 10 µM NAA treatment.

Table 3.4: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 100 µM NAA application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	20
2	3	40
3	6	80
4	12	90
5	24	70
6	48	50

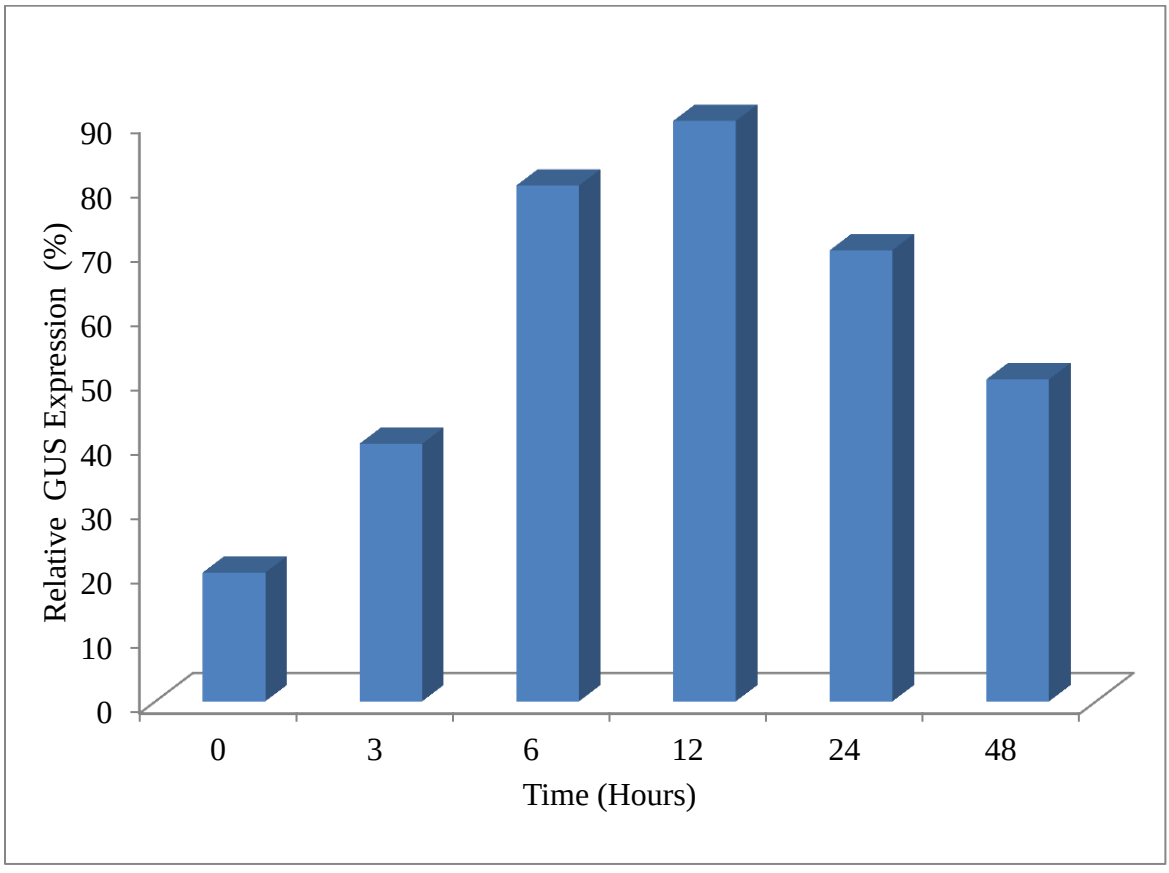

Figure 16: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 100 µM NAA treatment.

3.12.1 Effect of 1 µM BAP on promoter activity

Promoter was weakly induced after 0 and 3 hours and very less expression was found in veins and wounded part of petiole. Considerable increase in promoter activity was observed after 6, 12, 24 and 48 hours and GUS expression was observed on whole surface of leaf but expression was stronger in petiole, midrib and in veins of leaf while fade expression was shown by margins of leaf (Figure 17). Roots also showed GUS expression (Figure 18). It was estimated that GUS expression was upregulated by *Os*PPO promoter with respect to time (Table 3.5 and Figure 19). No expression was observed in control plants.

3.12.2 Effect of 20 µM BAP on promoter activity

Induction of promoter was observed to be increased on 20 µM BAP application as compared to 1 µM. Expression was observed on mid rib, leaf base, petiole and in veins after 3, 6 and 12 hours. Expression was further increased after 24 and 48 of treatment and strong expression was observed on whole leaf (Figure 17). Gus expression was also found on roots while control plants were remained same. Increase in the activity of promoter was observed with the passage of time (Table 3.6 and Figure 20).

3.12.3 Effect of 40 µM BAP on promoter activity

Relative decrease in GUS expression was observed on 40 µM BAP treatment. Promoter did not show any considerable activity after 0, 3, 6 and 12 hours. Strong expression was found on overall leaf surface after 24 and 48 hours (Figure 17). Differential activity of *Os*PPO promoter was observed with the passage of time (Table 3.7 and Figure 21). Expression was also observed on roots and control plants did not show any expression.

3.12.4 Effect of 80 µM BAP on promoter activity

Strong GUS expression was observed after 6 hours in veins however petiole, base and apex of leaf showed diffused expression. Expression was increased after 12 hours and dark expression was observed in veins and apex of leaf. Expression further increased after 24 and 48 hours and observed on whole surface of leaf (Figure 17). Result has revealed an upregulation of promoter activity after different time interval (Table 3.8 and Figure 22). Expression was also found on roots while control plants did not show any expression.

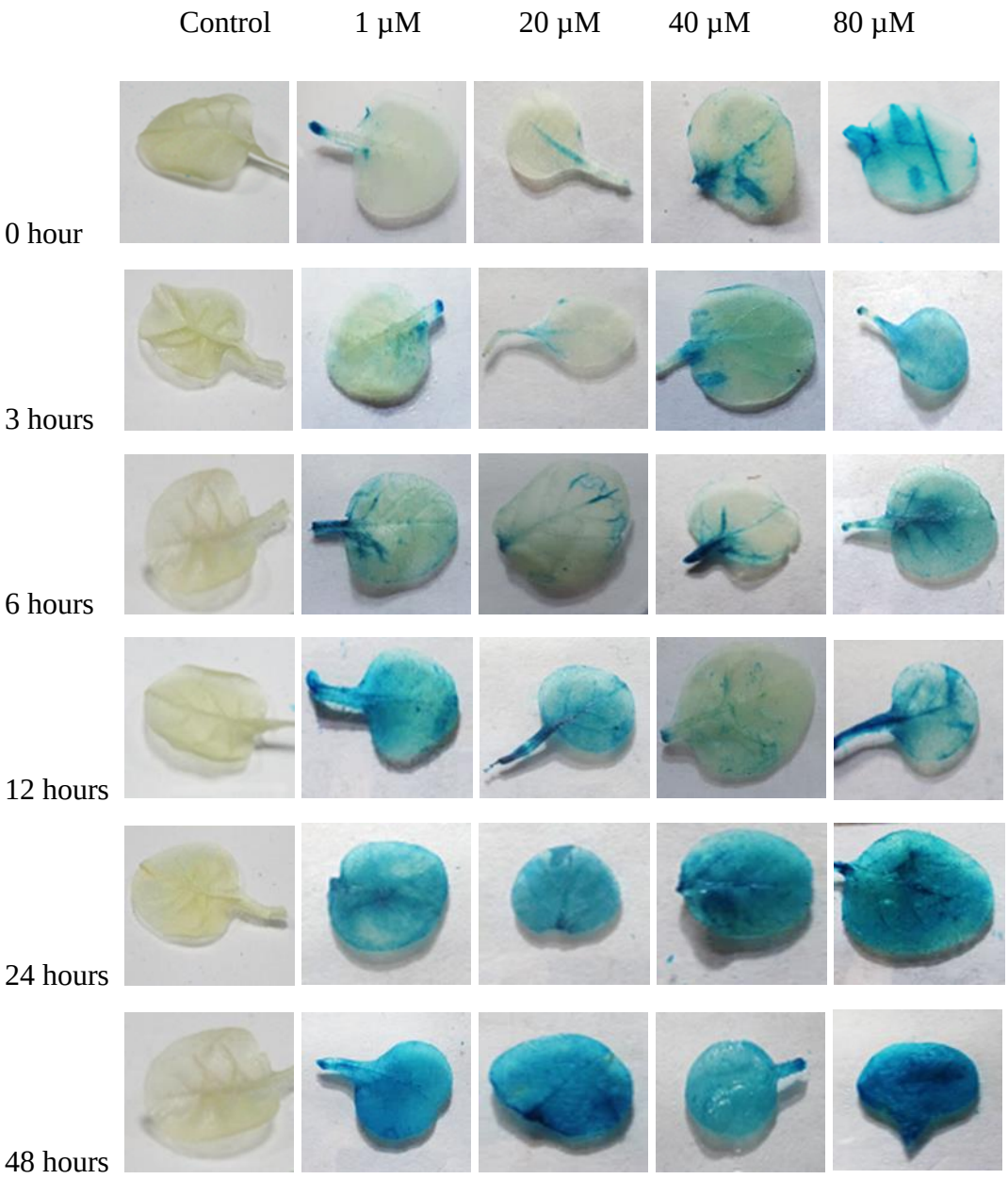

Figure 17: GUS expression analysis of transgenic and control tobacco leaves treated with various concentrations of BAP, at different time intervals.

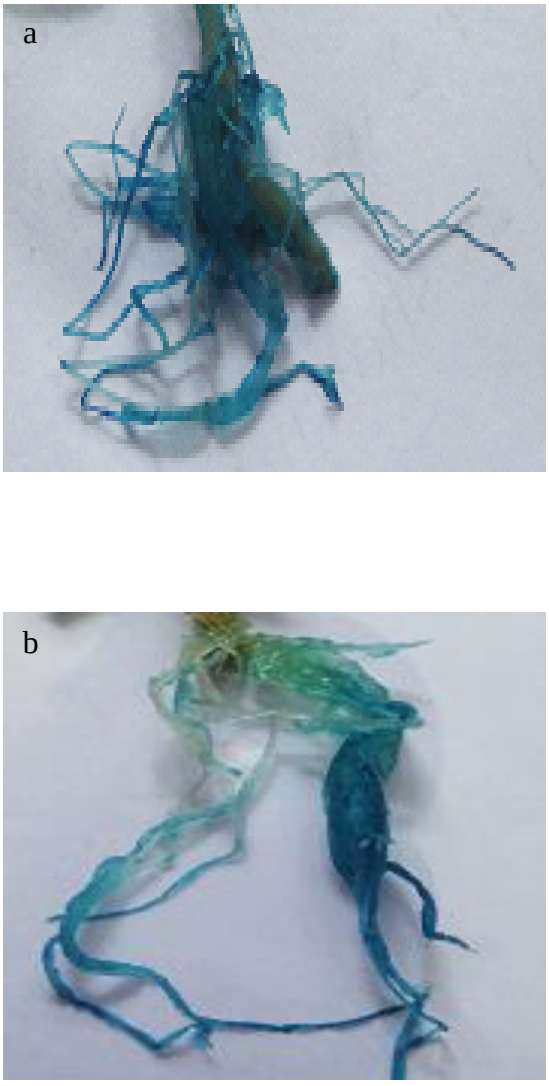

Figure 18: Transformed tobacco roots showing GUS expression after treated with BAP (a: strong GUS expression, b: discontinuous GUS expression, roots tips are showing strong expression while base of the root has diffused expression).

Table 3.5: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 1 µM BAP application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	10
2	3	10
3	6	20
4	12	40
5	24	70
6	48	80

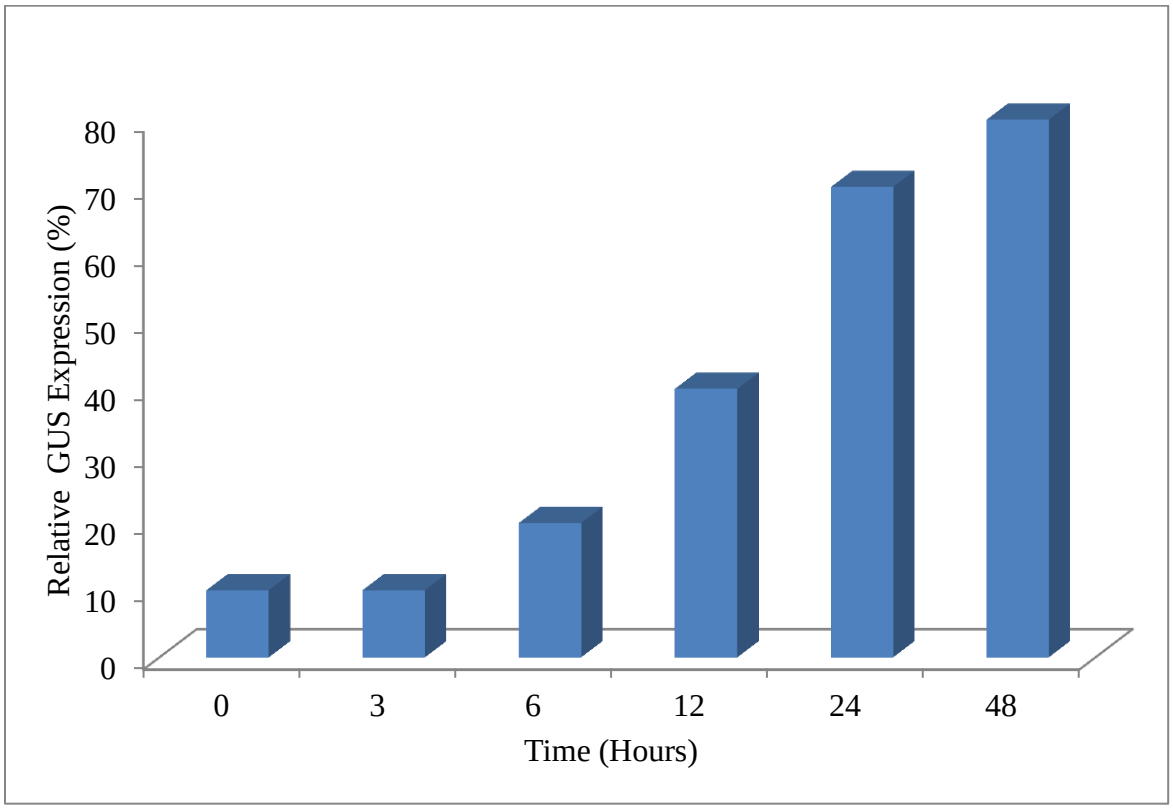

Figure 19: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 1 µM BAP treatment.

Table 3.6: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 20 µM BAP application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	10
2	3	10
3	6	40
4	12	60
5	24	70
6	48	80

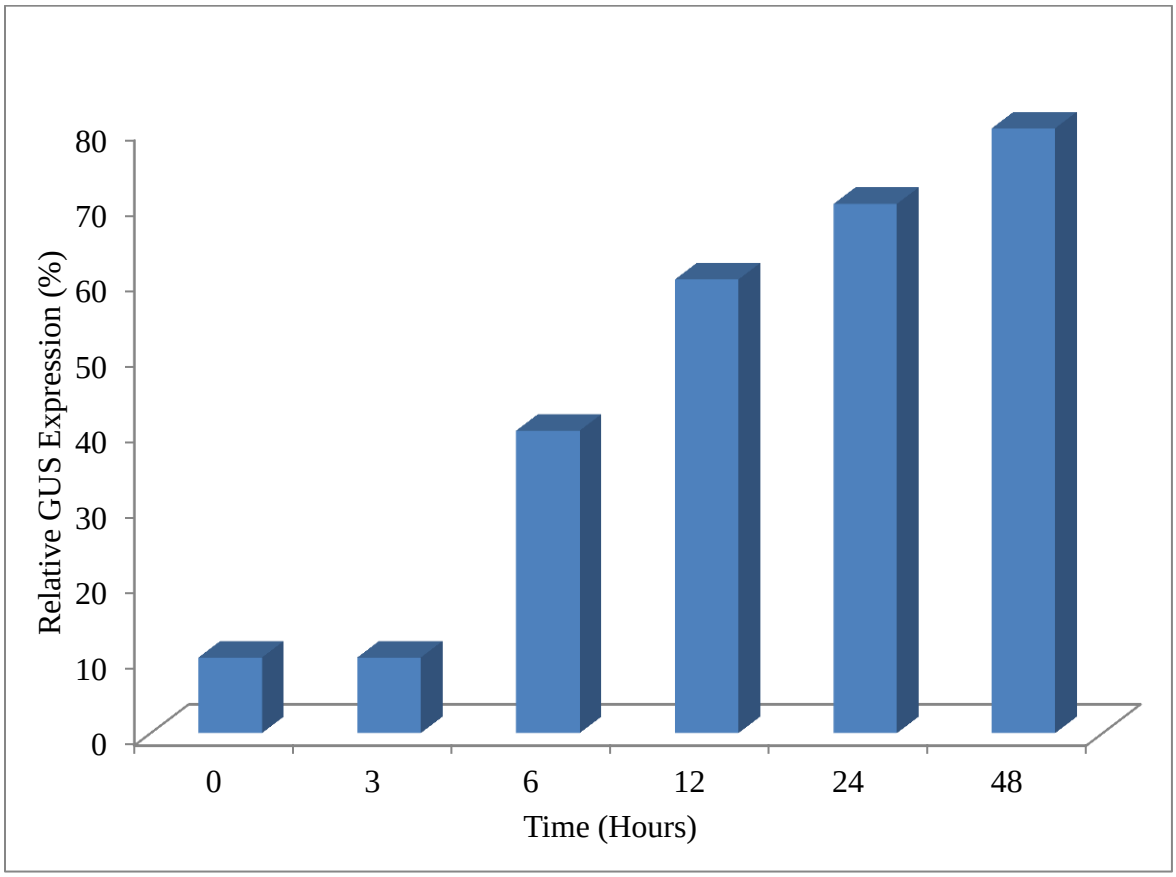

Figure 20: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 20 µM BAP treatment.

Table 3.7: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 40 µM BAP application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	30
2	3	20
3	6	30
4	12	10
5	24	80
6	48	70

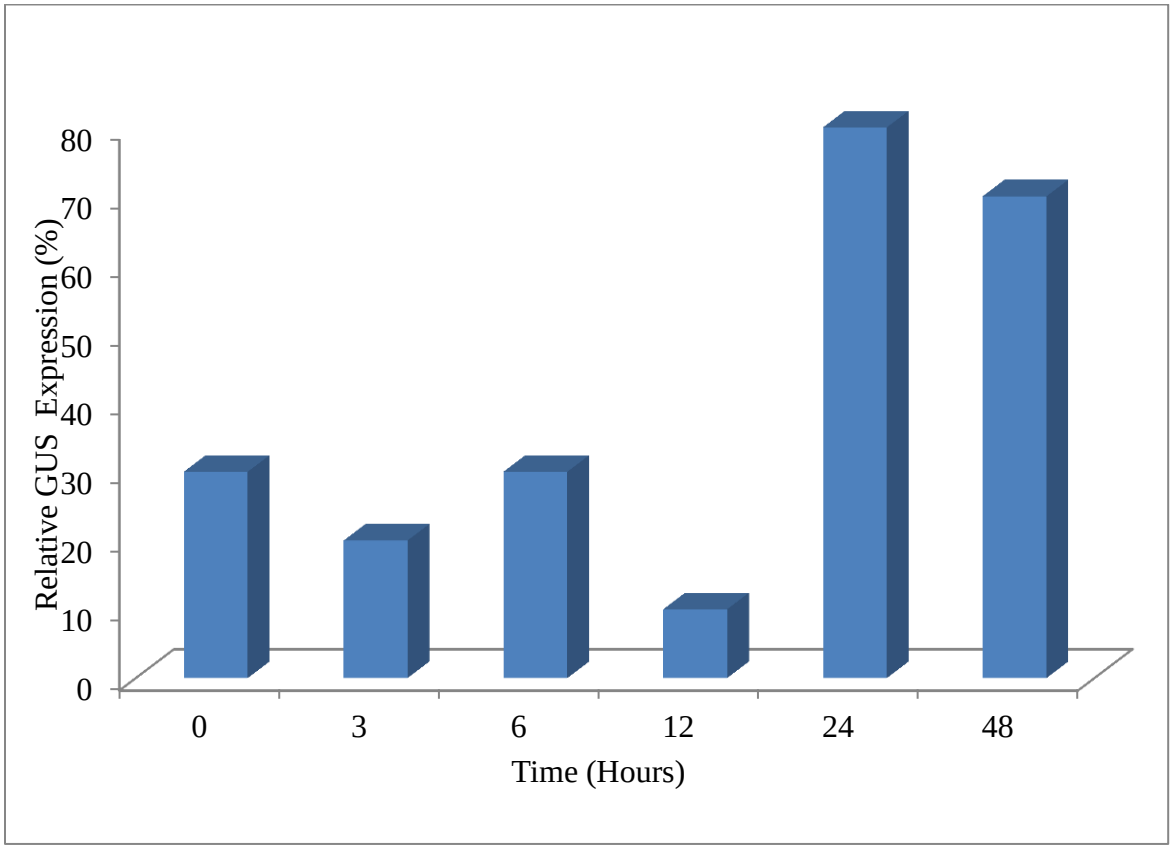

Figure 21: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 40 µM BAP treatment.

Table 3.8: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to 80µM BAP application.

S.No.	Time (Hours)	Relative GUS expression (%)
1	0	30
2	3	50
3	6	60
4	12	70
5	24	80
6	48	90

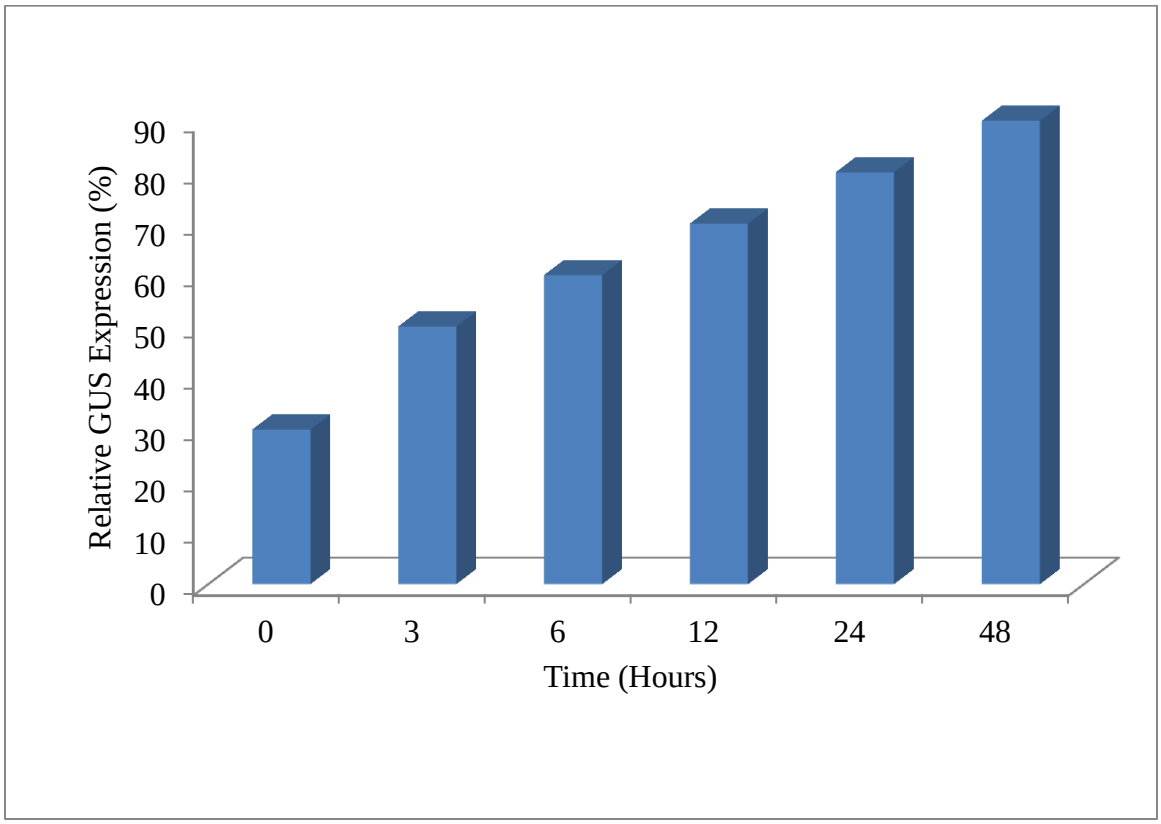

Figure 22: Regulation of GUS expression by antisense *Os*PPO promoter at different time intervals after 80 µM BAP treatment.

3.13 Application of ABA

Different treatments i.e. 50 µM, 150 µM, 250 µM and 350 µM of ABA were sprayed on transgenic and control plants on solid MS media. Antisense *Os*PPO promoter activity was observed after 24 and 48 hours of application.

3.13.1 Effect of 50 µM ABA on promoter activity

Comparatively, promoter was weakly induced in response to 50 µM ABA treatment. Diffused expression was observed on base and apex of leaf while spotted on stem after 24 hours. However it was increased after 48 hours and whole surface of leaf showed strong expression while considerable GUS expression was also observed on stem and root (Figure 23 and 24). No expression was observed on control plants. Relative increase in the activity of antisense *Os*PPO promoter from 24 to 48 hours of treatment was estimated (Table 3.9 and Figure 25).

3.13.2 Effect of 150 µM ABA on promoter activity

An increase in the induction of Promoter was observed from 24 to 48 hours and strong GUS expression was found on stem while diffused in veins and midrib of leaves (Figure 23). It was examined that GUS expression was upregulated by *Os*PPO promoter with increasing time interval. Roots also showed strong GUS expression. No expression was observed on control plants.

3.13.3 Effect of 250 µM ABA on promoter activity

Promoter was induced after 24 hours of treatment and discontinuous GUS expression was observed all over leaf surface i.e. strongest in veins and margins while faded on the rest of leaf surface. However expression was reduced after 48 hours and found only in veins and midrib (Figure 23). Decrease in GUS expression driven by antisense *Os*PPO promoter from 24 to 48 hours of treatment was examined. Roots also showed strong GUS expression.

3.13.4 Effect of 350 µM ABA on promoter activity

Promoter was strongly induced by 350 µM ABA treatment. GUS expression was observed on stem, leaves and roots after 24 and 48 hours. Expression was continuous throughout the surface of leaves (Figure 23) while control plants did not show any expression Results have revealed the increase in antisense *Os*PPO promoter activity with the passage of time.

Figure 23: GUS expression analysis of transgenic tobacco plant treated with various concentrations (50 µM, 150 µM, 250 µM & 350 µM) of ABA, after 24 and 48 hours (a: 24 hours, b: 48 hours).

Figure 24: Differentially regulated GUS expression in transformed tobacco roots by antisense *Os*PPO promoter in response to ABA treatment (a: strong GUS expression, b: diffused GUS expression).

Table 3.9: Relative percentage of GUS expression regulated by antisense *Os*PPO promoter in response to various ABA treatments.

S.No.	Time (Hours)	Relative GUS expression (%) at 50 µM	Relative GUS expression (%) at 150 µM	Relative GUS expression (%) at 250 µM	Relative GUS expression (%) at 350 µM
1	24	20	30	60	70
2	48	60	50	40	80

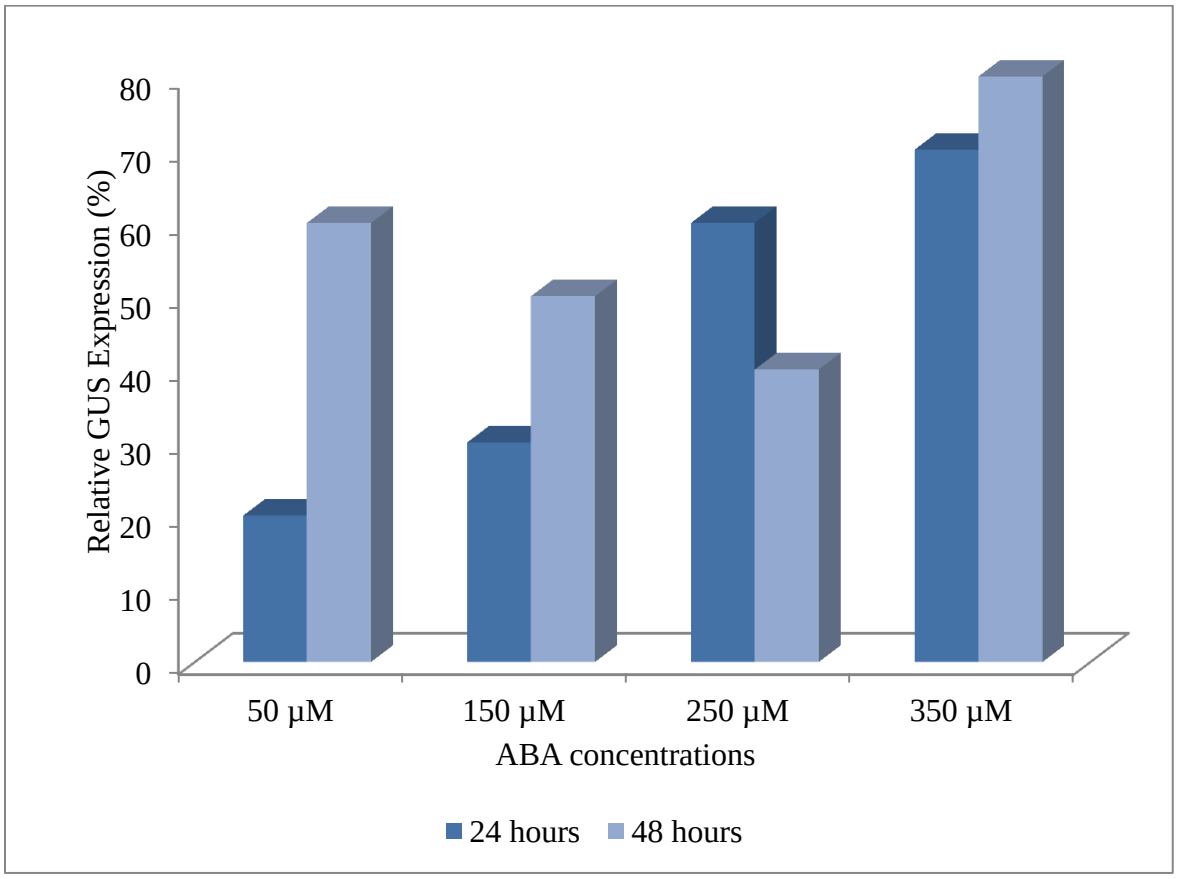

Figure 25: Regulation of GUS expression by antisense *Os*PPO promoter in response to various concentrations of ABA after 24 and 48 hours of treatments.

3.14 Hormonal stress and activity of PPO

Polyphenol oxidases (PPOs) are nuclear encoded, ubiquitous and copper binding enzymes present in all angiosperms (Steffens *et al*., 1994). PPOs are involved in the oxidation of phenolic compounds into quinones which causes browning of fruits and vegetables. PPOs are induced by both abiotic and biotic stresses (Thipyapong *et al*., 2007). Polyphenol oxidase activity is habitually hidden until disturbing agents such as senescence, wounding and attack by pathogens or insects releases it from thylakoid to react with its (mono or *o*-diphenolic) substrates that were earlier kept in vacuole (Steffens *et al*., 1994).

Plant growth regulators (PGRs) or exogenous plant hormones are used to persuade shoot organogenesis. They interact with the tissue specific endogenous hormones, therefore level of the endogenous plant hormones in explants and resulted calli may play maximum role in shoot organogenesis. PGRs have a vital role in differentiation and growth of cells. Both endogenous and exogenous levels of plant growth regulators are extremely associated to shoot organogenesis (Yin *et al*., 2008; Zhang *et al*., 2008; Feng *et al*., 2010; Huang *et al*., 2012). Abscisic acid, cytokinin and auxin are considered vital factors for the shoot differentiation in callus culture (Brown *et al*., 1989; Pernisova *et al*., 2009; Su *et al*., 2009; Vanneste and Friml, 2009; Cheng *et al*., 2010; Zhao *et al*., 2010).

The present study is about the effects of different hormones i.e. NAA, BAP and ABA on antisense *Os*PPO promoter activity. *Nicotiana tabacum* cv. Samsun plants (containing antisense *Os*PPO promoter fused with GUS reporter gene) were treated with exogenous NAA, BAP and ABA and activity of promoter was examined. Trangenic tobacco plants showed differential GUS expression towards hormonal stresses. Results revealed that antisense *Os*PPO promoter was induced by applying different concentrations of these hormones.

Antisense *Os*PPO promoter was strongly induced by exogenous NAA application, signifying that antisense *Os*PPO gene promoter is reactive towards exogenous NAA. Strong GUS expression was observed in almost all regions of leaves. Antisense *Os*PPO promoter was induced at all NAA concentrations (0.1µM, 1µM, 10µM and 100µM) used in present work. Maximum expression was observed at 10 µM of NAA. These results

revealed that the activity of PPO was greater at 10 µM NAA as compared to 100 µM. The present result was consistent with the results of Yan *et al*. (2014) which reported the regulation in PPO activity in stem cutting of *Hemarthria compressa* after different treatments of NAA were applied. The activity of PPO was increased when cuttings were treated with 100 mg/l and 200 mg/l of NAA usages. PPO showed maximum activity on 200 mg/l NAA treatment. When NAA was used in 400 mg/l, a decreased in PPO activity was observed.

The present results also indicated that activity of PPO was maximum in 10 µM NAA after 12 hours of treatment, and it was greater than that of 10 µM NAA after 3 and 6 hours of treatment. These results showed similarity to the results of Yan *et al*., 2014, which indicated that the activity of PPO was maximum in 200 mg/l NAA for 20 minutes soaking period treated cuttings, which was higher than that of 200 mg/l NAA for 10 minutes soaking period and 100 mg/l NAA for 30 minutes soaking period.

Ling *et al*. (2013) reported same results that exogenous IBA treatment increases the polyphenol oxidase activity in softwood cutting of Tetraploid Locust. Present results showed that activity of antisense *Os*PPO promoter was sensitive to quantity of NAA and timings of application, related to the results of James, (1983) and Hunt *et al*. (2011) which investigated that auxin concentration and timings of treatment are crucial factors in effective rooting and related physiological changes.

Antisense *Os*PPO promoter activity was also examined by applying different treatments of ABA. The present study showed that ABA application induced antisense *Os*PPO promoter and strong GUS expression was observed in plants, just like the results of Jia *et al*. (2015) which stated that ABA (exogenous) treatments promote the functioning of *FaPPO1* and *FaPPO2* genes in strawberry and enhance their expression levels. Chai *et al*. (2013) reported the upregulation of soya bean *GmaPPO1* promoter in response to ABA and results showed 2.4 fold increase in mRNA (transcript) levels of *GmaPPO1*, when ABA treatment was applied.

The present research also examined the functioning of *Os*PPO promoter during BAP stress. GUS expression was observed in transgenic plants while control plants did not show any expression. This study revealed that cytokinin (BAP) can induce *Os*PPO activity and is involved in the up regulation of activity of PPO. This result is related with

the study of Blee *et al*. (2001), which reported that an increase in endogenous cytokinin level causes the upregulation of polyphenol oxidase (laccase) and peroxidases in tobacco, transformed with *Tcyt* of *A. tumefacians*. Some guidance from literature concerning the link between plant phenolics and cytokinin metabolism comes from the research that biosynthesis expression of cytokinin in *A. tumefacians* is induced by phenolic compounds in plant (Powel *et al*., 1988).

3.15 Microscopic analysis of stress treated tobacco plants

Stress treated transformed leaf and root samples were examined under compound microscope at low and high magnification powers and photographs were taken. Microscopic analysis facilitated the visualization of close GUS expression in various parts of leaves. GUS expression was strong in regions like petiole, guard cells, midrib and in veins of leaves while in roots GUS expression was obvious in root hairs (Figure 26 and 27).

3.16 Conclusion

The present study showed that antisense *Os*PPO promoter is inducible towards different hormonal stresses. Strong GUS expression was regulated by antisense *Os*PPO promoter in response to 10 µM NAA, 80 µM BAP and 350 µM ABA application as compared to the other treatments of these hormones.

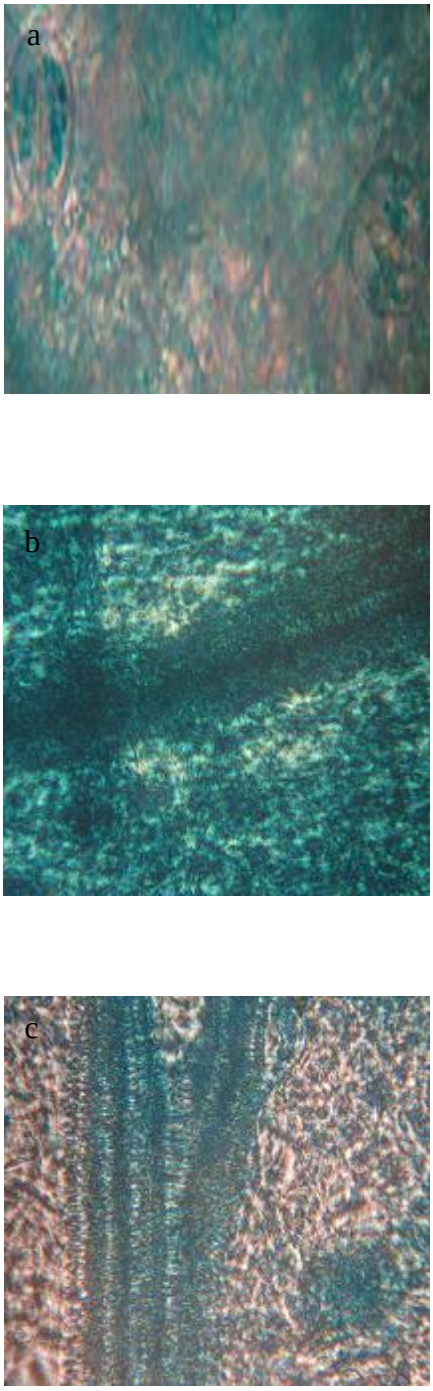

Figure 26: Microscopy of treated transformed tobacco leaves showing strong GUS expression (a: guard cells, b: midrib and c: veins).

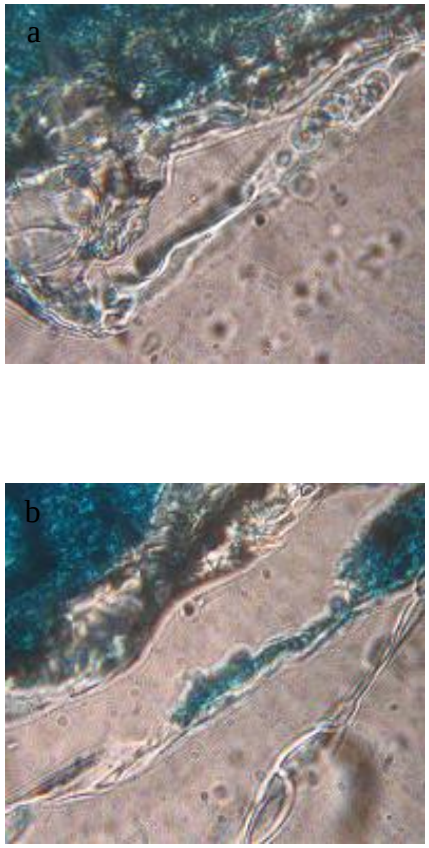

Figure 27: Microscopy of treated transformed tobacco roots showing strong GUS expression (a: root, b: root hairs).

LITERATURE CITED

Altunkaya, A., and Gokmen, V. (2012). Partial purification and characterization of polyphenoloxidase from durum wheat (Triticum durum L.). *Journal of Cereal Science*, *55*(3), 300-304.

Barbehenn, R. V., Jones, C. P., Yip, L., Tran, L., and Constabel, C. P. (2007). Limited impact of elevated levels of polyphenol oxidase on tree-feeding caterpillars: assessing individual plant defenses with transgenic poplar. *Oecologia*, *154*(1), 129-140.

Bergey, D. R., Howe, G. A., and Ryan, C. A. (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proceedings of the National Academy of Sciences*, *93*(22), 12053-12058.

Blee, K. A., Wheatley, E. R., Bonham, V. A., Mitchell, G. P., Robertson, D., Slabas, A. R., and Bolwell, G. P. (2001). Proteomic analysis reveals a novel set of cell wall proteins in a transformed tobacco cell culture that synthesises secondary walls as determined by biochemical and morphological parameters. *Planta*, *212*(3), 404-415.

Brown, C., Brooks, F. J., Pearson, D., and Mathias, R. J. (1989). Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic acid. *Journal of plant physiology*, *133*(6), 727-733.

Cabanes, J., Escribano, J., Gandia-Herrero, F., Garcia-Carmona, F., and Jimenez-Atienzar, M. (2007). Partial purification of latent polyphenol oxidase from peach (Prunus persica L. Cv. Catherina). Molecular properties and kinetic characterization of soluble and membrane-bound forms. *Journal of agricultural and food chemistry*, *55*(25), 10446-10451.

Castanera, P., Steffens, J. C., and Tingey, W. M. (1996). Biological performance of Colorado potato beetle larvae on potato genotypes with differing levels of polyphenol oxidase. *Journal of chemical ecology*, *22*(1), 91-101.

Chai, C., Lin, Y., Shen, D., Wu, Y., Li, H., and Dou, D. (2013). Identification and functional characterization of the Soybean GmaPPO12 promoter conferring Phytophthora sojae induced expression. *PloS one*, *8*(6), e67670.

Chen, H., Wilkerson, C. G., Kuchar, J. A., Phinney, B. S., and Howe, G. A. (2005). Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore

midgut. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(52), 19237-19242.

Cheng, Z. J., Zhu, S. S., Gao, X. Q., and Zhang, X. S. (2010). Cytokinin and auxin regulates WUS induction and inflorescence regeneration in vitro in Arabidopsis. *Plant cell reports*, *29*(8), 927-933.

Cho, M. H., Moinuddin, S. G., Helms, G. L., Hishiyama, S., Eichinger, D., Davin, L. B., and Lewis, N. G. (2003). (+)-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the creosote bush (Larrea tridentata). *Proceedings of the National Academy of Sciences*, *100*(19), 10641-10646.

Constabel, C. P., and Ryan, C. A. (1998). A survey of wound-and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. *Phytochemistry*, *47*(4), 507-511.

Constabel, C. P., Bergey, D. R., and Ryan, C. A. (1995). Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proceedings of the National Academy of Sciences*, *92*(2), 407-411.

Constabel, C. P., Yip, L., Patton, J. J., and Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiology*, *124*(1), 285-296.

Dogan, M., Arslan, O., and Dogan, S. (2002). Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. *International journal of food science & technology*, *37*(4), 415-423.

Dogru, Y. Z., and Erat, M. (2012). Investigation of some kinetic properties of polyphenol oxidase from parsley (Petroselinum crispum, Apiaceae). *Food Research International*, *49*(1), 411-415.

Duangmal, K., and Apenten, R. K. O. (1999). A comparative study of polyphenoloxidases from taro (Colocasia esculenta) and potato (Solanum tuberosum var. Romano). *Food Chemistry*, *64*(3), 351-359.

English-Loeb, G., Stout, M. J., and Duffey, S. S. (1997). Drought stress in tomatoes: changes in plant chemistry and potential nonlinear consequences for insect herbivores. *Oikos*, 79(3) 456-468.

Erat, M., Sahin, Y. N., Aksoy, G., and Demirkol, A. (2010). Partial characterization of polyphenoloxidase from a hybridized wheat (Triticum aestivum L.). *European Food Research and Technology*, *231*(6), 899-905.

Felton, G. W., Donato, K., Del Vecchio, R. J., and Duffey, S. S. (1989). Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *Journal of Chemical Ecology*, *15*(12), 2667-2694.

Felton, G. W., Workman, J., and Duffey, S. S. (1992). Avoidance of antinutritive plant defense: role of midgut pH in Colorado potato beetle. *Journal of chemical ecology*, *18*(4), 571-583.

Feng, J. C., Yu, X. M., Shang, X. L., Li, J. D., and Wu, Y. X. (2010). Factors influencing efficiency of shoot regeneration in Ziziphus jujuba Mill.'Huizao. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *101*(1), 111-117.

Galeazzi, M. A. M., and Sgarbieri, V. C. (1981). Substrate specificity and inhibition of polyphenoloxidase (PPO) from a dwarf variety of banana (Muss Cavendishii, L.). *Journal of Food Science*, *46*(5), 1404-1406.

Gandia-Herrero, F., Garcia-Carmona, F., and Escribano, J. (2004). Purification and characterization of a latent polyphenol oxidase from beet root (Beta vulgaris L.). *Journal of agricultural and food chemistry*, *52*(3), 609-615.

Gao, Z. J., Liu, J. B., and Xiao, X. G. (2011). Purification and characterisation of polyphenol oxidase from leaves of Cleome gynandra L. *Food chemistry*, *129*(3), 1012-1018.

Haruta, M., Pedersen, J. A., and Constabel, C. P. (2001). Polyphenol oxidase and herbivore defense in trembling aspen (Populus tremuloides): cDNA cloning, expression, and potential substrates. *Physiologia Plantarum*, *112*(4), 552-558.

Huang, W. L., Lee, C. H., and Chen, Y. R. (2012). Levels of endogenous abscisic acid and indole-3-acetic acid influence shoot organogenesis in callus cultures of rice subjected to osmotic stress. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *108*(2), 257-263.

Hunt, M. A., Trueman, S. J., and Rasmussen, A. (2011). Indole-3-butyric acid accelerates adventitious root formation and impedes shoot growth of Pinus elliottii var. elliottii× P. caribaea var. hondurensis cuttings. *New Forests*, *41*(3), 349-360.

Hunt, M. A., Trueman, S. J., and Rasmussen, A. (2011). Indole-3-butyric acid accelerates adventitious root formation and impedes shoot growth of Pinus elliottii var. elliottii× P. caribaea var. hondurensis cuttings. *New Forests*, *41*(3), 349-360.

Ionita, E. (2013). Plant polyphenol oxidases: isolation and characterization. *Innovative Romanian Food Biotechnology*, *13*.

James, D. J. (1983). Adventitious root formation 'in vitro'in apple rootstocks (Malus pumila) I. Factors affecting the length of the auxin-sensitive phase in M. 9. *Physiologia Plantarum*, *57*(1), 149-153.

Jia, H., Zhao, P., Wang, B., Tariq, P., Zhao, F., Zhao, M., and Fang, J. (2015). Overexpression of Polyphenol Oxidase Gene in Strawberry Fruit Delays the Fungus Infection Process. *Plant Molecular Biology Reporter*, pp: 1-15.

John, K. S., Bhat, S. G., and Rao, U. P. (2011). Isolation and partial characterization of phenol oxidases from Mangifera indica L. sap (latex). *Journal of Molecular Catalysis B: Enzymatic*, *68*(1), 30-36.

Kessler, A., and Baldwin, I. T. (2002). Manduca quinquemaculata's optimization of intra-plant oviposition to predation, food quality, and thermal constraints. *Ecology*, *83*(8), 2346-2354.

Kojima, M., and Takeuchi, W. (1989). Detection and characterization of p-coumaric acid hydroxylase in mung bean, Vigna mungo, seedlings. *Journal of biochemistry*, *105*(2), 265-270.

Lee, P. M., Lee, K. H., Ismail, M., and Karim, A. (1991). Biochemical studies of cocoa bean polyphenol oxidase. *Journal of the Science of Food and Agriculture*, *55*(2), 251-260.

Li, L., and Steffens, J. C. (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta*, *215*(2), 239-247.

Ling, W. X., Zhu, G., and Zhong, Z. (2013). Effect of IBA on rooting from softwood cuttings of'tetraploid locust'and associated biochemical changes. *Pakistan Journal of Botany, Karachi*, *45*(5), 1801-1806.

Liu, N. N., Liu, W., Wang, D. J., Zhou, Y. B., Lin, X. J., Wang, X., and Li, S. B. (2013). Purification and partial characterization of polyphenol oxidase from the flower buds of Lonicera japonica Thunb. *Food chemistry*, *138*(1), 478-483.

Madani, I., Lee, P. M., and Hung, L. K. (2011). Partial purification and characterisation of polyphenol oxidase from Hibiscus rosa–sinensis L. In *2nd International Conference on Biotechnology and Food Science. Singapore*, 7.

Mayer, A. M. (1986). Polyphenol oxidases in plants-recent progress. *Phytochemistry*, *26*(1), 11-20.

Mayer, A. M. (2006). Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry*, *67*(21), 2318-2331.

Melo, G. A., Shimizu, M. M., and Mazzafera, P. (2006). Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust. *Phytochemistry*, *67*(3), 277-285.

Mishra, B. B., Gautam, S., and Sharma, A. (2012). Purification and characterisation of polyphenol oxidase (PPO) from eggplant (Solanum melongena). *Food chemistry*, *134*(4), 1855-1861.

Nagai, T., and Suzuki, N. (2001). Partial purification of polyphenol oxidase from Chinese cabbage Brassica rapa L. *Journal of agricultural and food chemistry*, *49*(8), 3922-3926.

Nakayama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., and Nishino, T. (2000). Aureusidin synthase: a polyphenol oxidase homolog responsible for flower coloration. *Science*, *290*(5494), 1163-1166.

Oktay, M., Kufreviolu, I., Kocacaliskan, I., and Saklrolu, H. (1995). Polyphenoloxidase from Amasya apple. *Journal of Food Science*, *60*(3), 494-496.

Ono, E., Hatayama, M., Isono, Y., Sato, T., Watanabe, R., Yonekura-Sakakibara, K., and Nakayama, T. (2006). Localization of a flavonoid biosynthetic polyphenol oxidase in vacuoles. *The Plant Journal*, *45*(2), 133-143.

Palma-Orozco, G., Ortiz-Moreno, A., Dorantes-Alvarez, L., Sampedro, J. G., and Najera, H. (2011). Purification and partial biochemical characterization of polyphenol oxidase from mamey (Pouteria sapota). *Phytochemistry*, *72*(1), 82-88.

Paul, B., and Gowda, L. R. (2000). Purification and characterization of a polyphenol oxidase from the seeds of field bean (Dolichos lablab). *Journal of agricultural and food chemistry*, *48*(9), 3839-3846.

Paul, B., and Gowda, L. R. (2000). Purification and characterization of a polyphenol oxidase from the seeds of field bean (Dolichos lablab). *Journal of agricultural and food chemistry*, *48*(9), 3839-3846.

Pernisova, M., Klima, P., Horak, J., Valkova, M., Malbeck, J., Soucek, P., and Za, E. (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. *Proceedings of the National Academy of Sciences*, *106*(9), 3609-3614.

Powell, G. K., Hommes, N. G., Kuo, J., Castle, L. A., and Morris, R. O. (1988). Inducible expression of cytokinin biosynthesis in Agrobacterium tumefaciens by plant phenolics. *Molecular Plant Microbe Interaction*, 1(6), 235-242.

Queiroz, C., da Silva, A. J. R., Lopes, M. L. M., Fialho, E., and Valente-Mesquita, V. L. (2011). Polyphenol oxidase activity, phenolic acid composition and browning in cashew apple (Anacardium occidentale, L.) after processing. *Food Chemistry*, *125*(1), 128-132.

Ren, F., and Lu, Y. T. (2006). Overexpression of tobacco hydroxyproline-rich glycopeptide systemin precursor A gene in transgenic tobacco enhances resistance against Helicoverpa armigera larvae. *Plant science*, *171*(2), 286-292.

Rout, G. R. (2006). Effect of auxins on adventitious root development from single node cuttings of Camellia sinensis (L.) Kuntze and associated biochemical changes. *Plant Growth Regulation*, *48*(2), 111-117.

Sakiroglu, H., Kufrevioglu, O. I., Kocacaliskan, I., Oktay, M., and Onganer, Y. (1996). Purification and characterization of dog-rose (Rosa dumalis Rechst.) polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, *44*(10), 2982-2986.

Sangwan, R. S., Bourgeois, Y., Brown, S., Vasseur, G., and Sangwan-Norreel, B. (1992). Characterization of competent cells and early events of Agrobacterium-mediated genetic transformation in Arabidopsis thaliana.*Planta*, *188*(3), 439-456.

Selles-Marchart, S., Casado-Vela, J., and Bru-Martínez, R. (2006). Isolation of a latent polyphenol oxidase from loquat fruit (Eriobotrya japonica Lindl): Kinetic characterization and comparison with the active form. *Archives of Biochemistry and Biophysics*, *446*(2), 175-185.

Sherman, T. D., Vaughn, K. C., and Duke, S. O. (1991). A limited survey of the phylogenetic distribution of polyphenol oxidase. *Phytochemistry*, *30*(8), 2499-2506.

Shivishankar, S. (1988). Polyphenol oxidase isozymes in coconut genotypes under water stress. *Plant physiology & biochemistry*, 15(1), 87-91.

Siddiq, M., Sinha, N. K., and Cash, J. N. (1992). Characterization of polyphenoloxidase from Stanley plums. *Journal of Food Science*, *57*(5), 1177-1179.

Steffens, J. C., Harel, E., and Hunt, M. D. (1994). Polyphenol oxidase. *Genetic engineering of plant secondary metabolism*, 28, pp: 275-312.

Stout, M. J., Workman, K. V., Bostock, R. M., and Duffey, S. S. (1997). Specificity of induced resistance in the tomato, Lycopersicon esculentum. *Oecologia*, *113*(1), 74-8.

Su, Y. H., Zhao, X. Y., Liu, Y. B., Zhang, C. L., O'Neill, S. D., and Zhang, X. S. (2009). Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. *The Plant Journal*, *59*(3), 448-460.

Sullivan, M. L., Hatfield, R. D., Thoma, S. L., and Samac, D. A. (2004). Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in Escherichia coli and transgenic alfalfa. *Plant Physiology*, *136*(2), 3234-3244.

Sun, Y. L., and Hong, S. K. (2010). Effects of plant growth regulators and L-glutamic acid on shoot organogenesis in the halophyte Leymus chinensis (Trin.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *100*(3), 317-328.

Thaler, J. S., Karban, R., Ullman, D. E., Boege, K., and Bostock, R. M. (2002). Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. *Oecologia*, *131*(2), 227-235.

Thaler, J. S., Stout, M. J., Karban, R., and Duffey, S. S. (1996). Exogenous jasmonates simulate insect wounding in tomato plants (Lycopersicon esculentum) in the laboratory and field. *Journal of Chemical Ecology*, *22*(10), 1767-1781.

Thipyapong, P., and Steffens, J. C. (1997). Tomato polyphenol oxidase (differential response of the polyphenol oxidase F promoter to injuries and wound signals). *Plant Physiology*, *115*(2), 409-418.

Thipyapong, P., Hunt, M. D., and Steffens, J. C. (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta*, *220*(1), 105-117.

Thipyapong, P., Stout, M. J., and Attajarusit, J. (2007). Functional analysis of polyphenol oxidases by antisense/sense technology. *Molecules*, *12*(8), 1569-1595.

Tsurutani, M., Yanagida, Y., Hagiwara, S., Murata, M., and Homma, S. (2002). Comparison of Soluble and Plastidal Polyphenol Oxidase in Mature Apples. *Food Science and Technology Research*, *8*(1), 42-44.

Unal, M. U., Yabacı, S. N., and Sener, A. (2011). Extraction, Partial Purification and Characterisation of Polyphenol Oxidase from Tea Leaf (Camellia sinensis). *The Journal of Food*, *36*(3), 137-144.

Vanneste, S., and Friml, J. (2009). Auxin: a trigger for change in plant development. *Cell*, *136*(6), 1005-1016.

Vaughn, K. C., and Duke, S. O. (1984). Function of polyphenol oxidase in higher plants. *Physiologia Plantarum*, *60*(1), 106-112.

Wang, J., and Constabel, C. P. (2004). Polyphenol oxidase overexpression in transgenic Populus enhances resistance to herbivory by forest tent caterpillar (Malacosoma disstria). *Planta*, *220*(1), 87-96.

Wesche-Ebeling, P. E. D. R. O., and Montgomery, M. W. (1990). Strawberry polyphenoloxidase: extraction and partial characterization. *Journal of Food Science*, *55*(5), 1320-1324.

Wu, A. M., Hu, J. S., and Liu, J. Y. (2009). Functional analysis of a cotton cellulose synthase A4 gene promoter in transgenic tobacco plants. *Plant cell reports*, *28*(10), 1539-1548.

Yan, Y. H., Li, J. L., Zhang, X. Q., Yang, W. Y., Wan, Y., Ma, Y. M. and Huang, L. K. (2014). Effect of naphthalene acetic acid on adventitious root development and associated physiological changes in stem cutting of Hemarthria compressa. *PloS one*, *9*(3), e90700.

Yin, L., Lan, Y., and Zhu, L. (2008). Analysis of the protein expression profiling during rice callus differentiation under different plant hormone conditions. *Plant molecular biology*, *68*(6), 597-617.

Zaini, N. A. M., Osman, A., Hamid, A. A., Ebrahimpour, A., and Saari, N. (2013). Purification and characterization of membrane-bound polyphenoloxidase (mPPO) from Snake fruit [Salacca zalacca (Gaertn.) Voss]. *Food chemistry*, *136*(2), 407-414.

Zawistowski, J., Biliaderis, C. G., and Eskin, N. M. (1991). Polyphenol oxidase. *Oxidative enzymes in foods*, pp: 217-273.

Zhang, Y., Zhou, J., Wu, T., and Cao, J. (2008). Shoot regeneration and the relationship between organogenic capacity and endogenous hormonal contents in pumpkin. *Plant Cell, Tissue and Organ Culture*, *93*(3), 323-331.

Zhao, Z., Andersen, S. U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S. J., and Lohmann, J. U. (2010). Hormonal control of the shoot stem-cell niche. *Nature*, *465*(7301), 1089-1092.

Zhou, H. W., and Feng, X. (1991). Polyphenol oxidase from Yali pear (Pyrus bretschneideri). *Journal of the Science of Food and Agriculture*, *57*(3), 307-313.