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ROLE OF TOBACCO ANIONIC PEROXIDASE ON
PLANT GROWTH AND DEVELOPMENT AND THE EFFECT
ON ENDOGENOUS AUXIN PRODUCTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of
The Ohio State University

By
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*****

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ABSTRACT

Peroxidase has been shown to metabolize auxins in vitro. It would be interesting to know if peroxidase has a direct role in IAA catabolism in vivo. The objective of my dissertation research was to determine the physiological, quantitative, and molecular changes in IAA-mediated growth and development which occur in tobacco plants as results of altered peroxidase activity in vivo. Regeneration and growth of adventitious roots, the elongation rates of dark-grown young seedlings, the expansion of axillary buds after the release of apical dominance on wild type and under- and over-producing plants of N. tabacum cv. xanthi and N. sylvestris were examined. Quantitative analysis on endogenous IAA levels in root and shoot tissues of 19 d seedlings and in lateral buds of various positions before and following decapitation was also done. Simultaneous changes in the expression of pCNT103, an auxin-inducible gene, on buds of the same plant material prior and after the release of the apical dominance was investigated.

The results showed that the physiological and quantitative data of plants with altered peroxidase activity are generally consistent with the IAA theory. The underproducers had more adventitious root formation, greater stem growth, longer hypocotyl, stronger gravitropic response and more IAAs in axillary buds and shoots of 19 d seedlings when compared to wild type and the overproducers. A stronger lateral
bud outgrowth was observed in the underproducers as result of elevated endogenous IAA levels. Also, roots of the underproducer seedlings were more sensitive to exogenous IAAAs than wild types. The altered phenotypes observed in the overproducers include decreased overall growth of the plant, reduced lateral bud outgrowth, decreased adventitious root formation and slower root growth.

However, the findings that the underproducer plants had significantly longer roots, reduced apical dominance and less IAAAs in roots of 19 d seedlings are inconsistent with the conventional theory of auxin inhibition of root elongation and its involvement with apical dominance. It is also inconsistent with my hypothesis that more IAAAs are in roots of young seedlings with suppressed peroxidase activity.

No conclusion could be drawn in the attempt to compare the differences in the expression level of auxin inducible gene, pC1103, between the transgenic and wild type plants due to incomplete dataset obtained from one single Northern hybridization analysis. A repeated Northern blot analysis using shoot-tip specific cDNA probes is needed for more conclusive interpretation on the effects of peroxidase in IAA degradation in vivo.

In summary, these results suggest that tobacco anionic peroxidase may not be directly involved with IAA catabolism and that trying to explain all growth and development process with this simple hypothesis proved to be insufficient and unsuccessful. The results also suggest that IAA may not be a major factor in apical dominance as believed.
Dedicated to my parents and my beloved husband T.J. Doong
ACKNOWLEDGMENTS

The deepest appreciation is expressed to my two major professors, Dr. Mark Lagrimini and Dr. Jim Metzger, not only for their valuable counseling and assistance, but also, in particular, for their great patience during the writing of this dissertation. Without their guidance and encouragement, this thesis in its present form would never have achieved.

Acknowledgments are also extended to Dr. Mike Evans, Dr. Michael Knee, Dr. Tara VanToai, and Dr. JC Jang for their crucial advice and for serving as committee members of this thesis. I greatly thank Dr. Mike Evans and Dr. Ishikawa for their generosity and kindness of assisting the gravitropism experiments. I also greatly thank Dr. Michael Knee for his direction and technical support during data analyses using Statistical Analysis System (SAS). Tremendous gratitude is also given to Dr. JC Jang for his guidance and editorial assistance in the molecular biology part.

In addition, I would like to take this chance to thank past and present fellow graduate students John Zheng, Chamchuree, Frank Hsiao for their friendship, encouragement and assistance while at Ohio State University.
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CHAPTER 1

INTRODUCTION

PEROXIDASE MINIREVIEW

Peroxidases (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) are ubiquitous enzymes found in wide span of living organisms from fungi to animals. Most studies in the characterization of this enzyme were done in higher plants where they form a large family of isozymes (Lagrimini et al., 1987). The distribution of peroxidase isozymes in plants appears to be developmentally regulated and the expression of their genes is also tissue specific (Lagrimini et al., 1987, Klotz, 1995). Since their discovery at the turn of the 20th century, peroxidases have been widely used as markers in pathological and genetic studies due to their extreme stability and ease of enzymatic assays (Dunford, 1991).

The reasons why peroxidases are ubiquitous in eukaryotes are not known, nor is it known why so many isozymes evolved. Peroxidases are heme-containing, monomeric glycoproteins that oxidize many different compounds using hydrogen peroxide or oxygen as a source of oxidizing potential (Lagrimini et al., 1990). The active site contains a single Fe(III) protoporphyrin IX moiety, which is thought to be necessary for activity.
Peroxidase is capable of carrying out electron oxidations to form highly reactive radical species. The compound III (Fe$^{3+}$-O$_2^-$), formed after series of oxidation states of the native peroxidase enzyme (Fe$^{3+}$), also exhibits some oxidase activity. This oxidase activity is thought to enable peroxidases to oxidize substrates such as auxins without the presence of hydrogen peroxidase or by oxidation of compound II (Fe$^{3+}$-OH) by the hydrogen peroxide (Campà, 1991). In vitro studies indicated that peroxidases react with a variety of substrates, but many of the in vivo natural substrates are not known.

Peroxidase has been implicated in a number of biochemical events including polysaccharide cross-linking (Fry, 1986), indole-3-acetic acid oxidation (Hinnman and Lang, 1965; Acosta et al., '1988; Lagrimini, 1992), lignification (Bruce and West, 1989; Graham and Graham, 1991), wound-healing (Espelie, Franceschi, and Kolattukudy, 1986), pathogen defense (Hammerschmidt, Nuckles, and Kuc, 1982). Peroxidases have also been suggested as a stress indicator in plant exposed to ozone (Curtis and Howell, 1971; Curtis et al., 1976). Elevation in the activity of several peroxidase isoenzymes were found in bean leaves exposed to low doses of ozone even before symptoms of ozone injury appeared (Curtis and Howell, 1971). Studies showed that an increase in the activity of almost all peroxidase isoenzymes in the ozone-sensitive soybean cultivar, *Glycine max*(L.) *Merr.*, 'York', was observed while only a few of the peroxidase isoenzymes in ozone-sensitive cultivar, 'York', was observed (Curtis et al., 1976).

It is thought that peroxidases are involved in the cross-linking of pectin, hydroxyproline-rich glycoproteins, and lignin (Lagrimini et al., 1987; Lagrimini and Rothstein, 1987). Plant cell wall polysaccharides bind to phenolic acids or ferulic acid
through ester linkages, and it was suggested that cell wall-bound peroxidase may play an important role in catalyzing cross-linking between sugar residues and phenolic acids (Fry, 1986). It has also been suggested that peroxidases are involved in cell wall polymerization (Campa, 1991, Lagrimini et al., 1987).

Peroxidase is thought to be involved in IAA catabolism by oxidative decarboxylation of IAA (Lagrimini, 1991; McDougall, 1992). Some studies suggested that indole-3-acetic acid can be oxidized by peroxidase to form IAA cation radical and that radical, through subsequent decarboxylation, would become skatole radical. Further oxidation of skatole radical would lead to skatole peroxy radical, which when reacts with IAA would produce skatole hydroperoxide. This skatole hydroperoxide could then be degraded as indole aldehyde or as methylene-oxindole. In addition, results published by some studies showed the formation of oxindole-3-acetylaspartic acid after the oxidation of indole-3-acetylaspartic acid by horseradish peroxidase in vitro (Smith et al., 1982, Lagrimini, 1991). It was believed that cationic peroxidase isoenzymes located in the vacuole may be the most efficient in carrying out IAA oxidation in vitro (Barcelo and Munoz, 1992). Tobacco plants overproducing tobacco anionic peroxidases (TobAnPOD) also demonstrated strong capability to break down exogenous auxins, particularly IAA. Although there has been increasing evidence supporting the idea that peroxidases may be closely involved with the decomposition of auxins in vitro, the hypothesis that peroxidase mediated auxin catabolism also occurs in vivo needs further testing. Analysis of IAA degradation products from plants treated with labeled IAA have been inconclusive.
(Barcelo and Munoz, 1992). More studies on the role of peroxidase in \textit{in vivo} IAA decomposition are needed.

Peroxidase is thought to be important in certain defense mechanisms against insect and pathogen attacks in plants. Peroxidase activity was found to increase rapidly in response to viral, fungal and bacterial infections as well as to wounding (Campa, 1991; Moerschbacher, 1992). Graham and Graham (1991) observed faster accumulation of anionic peroxidases and phenolic polymers on soybean leaves with higher resistance to fungal infections than susceptible ones. Increased peroxidase activity after pathogen invasion may help induce more lignification, during the repair of cell walls, thereby creating a stronger barrier to prevent tissue from further pathogen ingress (Lagrimini and Rothstein, 1987). Moreover, peroxidase may kill pathogens by degrading substrates essential to the microbes metabolism, or may act to protect plants by detoxifying phytoalexins produced by host (Moerschbacher, 1992).

Yet, the real functions of peroxidases have not been identified partly due to their lack of substrate specificity. More than a dozen peroxidase isoenzymes have been found in higher plants (Lagrimini et al., 1990). Scientists think that plants need to have many peroxidase isoenzymes to perform various tasks within plants under different environmental conditions (Campa, 1991; Gaspar et al., 1985; Saunders et al., 1964). Various isozymes react differently to substrates, and show different expression patterns within the cell and plant (Campa, 1991). The tobacco peroxidases are the most studied and their potential functions have been extensively investigated. \textit{Nicotiana tabacum} (\textit{N.}
*tabacum*) has at least 12 isozymes that can be divided into three groups based on isoelectric point (pI): cationic, moderately anionic and anionic (Lagrimini et al., 1987).

The cationic isozymes, with pI of 8.1 to 11, are mostly located in the central vacuole in tobacco. They are most abundant in roots and rarely found in healthy leaf or pith tissue (Lagrimini and Rothstein, 1987). One of their biological functions appears to be involved in IAA catabolism since they have been shown to have some indoleacetic acid oxidase activity in the absence of hydrogen peroxide (Grambow and Langenbeck-Schwich, 1983). They were also able to catalyze production of hydrogen peroxide from NADH and water (Lagrimini et al., 1987). They do not seem to be involved in lignification since they exist primarily in vacuole and exhibit low activity towards lignin precursors.

Five moderately anionic peroxidase isozymes with pI between 4.5 and 6.5 were found in tobacco with most of them being located in the cell wall and expressed mostly in roots and callus (Campa, 1991; Lagrimini and Rothstein, 1987; Lagrimini et al., 1987). The substantial increase in expression levels after wounding suggests involvement in suberization (Lagrimini and Rothstein, 1987). They may have a role in pathogenic defense mechanisms since the infection by tobacco mosaic virus (TMV) induced significant expression of at least 2 isozymes (Lagrimini and Rothstein, 1987). Some induction of these isozymes was also seen in areas of a TMV infected plant not showing symptoms further suggests that these isozymes may be important in systemic acquired resistance in tobacco (Lagrimini and Rothstein, 1987).
Two anionic isozymes were found in *N. tabacum* with pI of 3.5 and 3.75 (Lagrimini et al., 1987). The TobAnPOD are the predominant peroxidases in tobacco. Almost 90% of peroxidase activity in all tissues except root has been attributed to the anionic peroxidases. They are the major peroxidase isoenzymes in mature stem tissue (Klotz et al., 1998, Lagrimini et al., 1990; Lagrimini and Rothstein, 1987). They are extracellular, and are thought to be important for lignification and cell wall cross-linking. Although TobAnPOD had demonstrated high affinity with lignin precursors *in vitro*, there has been little evidence to associate their involvement in lignification *in vivo*. Due to lack of secondary cell wall, TobAnPOD were not easily detected in callus tissue, and they were not induced by wounding (Lagrimini et al., 1987). It is not clear what other roles this isoenzyme may have.

To explore the *in planta* functions of tobacco peroxidases, it is necessary to first understand the regulation and expression of the isozymes and their possible function in plant growth and development. Delineating the precise roles of the different isoenzymes has been slow due to the lack of reliable quantitative methods of separating isozymes as well as their multiple reactivity for endogenous substrates (Lagrimini and Rothstein, 1987). Alternative approaches include molecular biology in conjunction with biochemistry and plant physiology to better understand the roles of individual peroxidase isozymes. Manipulating the levels of individual isoenzymes through genetic engineering may help clarify their *in vivo* functions. By using tobacco plants transformed with a chimeric gene in which TobAnPOD promoter was fused to the reporter gene – *E. coli* β-glucuronidase (GUS) coding region followed by the nopaline synthase terminator, Klotz
et al. (1998) was able to demonstrate the expression pattern of this gene at various
developmental stages.

A single gene encoding two TobAnPODs with pl of 3.5 and 3.75 was isolated and
cloned by Lagrimini et al. (1987). The difference in isoelectric points between the two
isoenzymes is the result of post-translational modification (Lagrimini et al., 1990). Based
on the cDNA sequence, the protein is first synthesized as a pre-protein with a 22 amino
acid signal peptide sequence at the amino terminal, primarily hydrophobic, which directs
the secretion of the protein to the extracellular space. The predicted molecular mass of
this pre-protein is 34.6 kD. The apparent molecular masses of the two anionic peroxidase
isoenzymes, as determined by SDS-PAGE, are 36 kD and 37 kD for TobAnPOD 3.5 and
TobAnPOD 3.75, respectively (Lagrimini et al., 1987). However, it is believed that the
two isoenzymes have the same functions. The difference between the predicted and the
apparent molecular masses is due, in part, to glycosylation. It is known that the anionic
peroxidases are secreted into the cell wall as glycoproteins. The anionic peroxidases
contain four potential sites for N-glycosylation. Another known post-translational
modification is found at the amino end of the protein where the glutamine residue is self-
condensed into a pyroglutamate (Lagrimini et al., 1987).

* N. sylvestris* has two copies of the gene encoding TobAnPOD with pl 3.75 and *N.
tomentosiformis* has two copies of the same gene with pl 3.5. However, *N. tabacum*, an
allotetraploid, is believed to be an interspecific hybrid of *N. sylvestris* and *N.
tomentosiformis* originating over a million years ago (Lagrimini et al., 1987). Southern
hybridization analysis further confirmed that the *N. sylvestris* and *N. tomentosiformis* are
the two diploid progenitors of the tetraploid *N. tabacum*; and they were both expressed in equal amount (Lagrimini et al., 1990). Clearly, both peroxidase isoenzymes are not necessary in *N. tabacum* since both parent species function normally with only one anionic peroxidase isozyme (Lagrimini et al., 1987).

To confirm its role in lignification and to elucidate any other functions that the anionic peroxidase may have *in planta*, tobacco plants (*N. tabacum* and *N. sylvestris*) were genetically engineered to overproduce or underproduce these two isoenzymes (Lagrimini et al., 1990; Lagrimini, 1992). The cDNA clone for the anionic peroxidase containing the entire coding region including the signal peptide that allows for secretion into the cell wall and the polyA addition site was ligated to the cauliflower mosaic virus (CaMV) 35S promoter and terminator (Lagrimini et al., 1990; Lagrimini, 1992). The CaMV 35S promoter is a constitutive promoter that causes a high level of expression in all or nearly all tissues, and all stages of development (Benfey and Chua, 1990). CaMV 35S-promoted expression has been reported to be greatest in epidermis and phloem (Benfey and Chua. 1990). Tobacco plants over- and under-expressing TobAnPOD were constructed using sense and antisense technology in which the 5' half of the cDNA clones was inserted between the CaMV35S promoter and terminator in a normal or inverted orientation (Lagrimini et al., 1992). This gene construct was inserted into an *Agrobacterium tumefaciens* (*A. tumefaciens*) binary vector containing a gene for neomycin phosphotransferase (NPTII) expression. Both the peroxidase chimeric gene, which consists of 35S promoter and the entire coding region of the anionic peroxidase gene and the terminator, and the NPTII gene were transformed into tobacco with _A._
tumefaciens. NPTII allows for selection of transformed plants by their resistance to kanamycin. Two subsequent generations were obtained by self-fertilization to achieve homozygous R2 tobacco plants that had up to a ten-fold increase in total peroxidase activity throughout the entire life cycle of the plant, while plants transformed with the antisense construct displayed three to twenty-fold less total peroxidase activity than the wild type plants (Lagrimini et al., 1990). Isoelectric focusing (IEF) gels showed that changes in total peroxidase activity were the result of changes in expression levels of TobAnPOD gene (Lagrimini et al., 1990).

Peroxidase overproducing and underproducing plants exhibited unique and unexpected phenotypes that provide clues to the in vivo function of the TobAnPOD. Prior to flowering, peroxidase overproducing plants were phenotypically similar to wild type plants with a few exceptions (Lagrimini et al., 1990). The leaves of peroxidase overproducers were slightly smaller and 30% thinner than those of wild type plants (Lagrimini et al., 1990; Lagrimini, 1991). The difference in leaf size was attributable to a difference in cell size. Leaf mesophyll cells were significantly smaller and more densely packed in peroxidase overproducing plants than in wild-type plants (Lagrimini, 1991). The number of cells per leaf was unchanged. Internodes of peroxidase overproducing plants were also slightly shorter than control plants (Lagrimini et al., 1990).

At the onset of flowering, the phenotype of the overproducer was unmistakably different from wild type tobacco plants. The TobAnPOD overproducing plants wilted severely, with all leaves wilting equally, regardless of their age (Lagrimini et al., 1990). The plants regained turgor overnight, only to wilt again after sunrise. Wilting was less
severe on overcast days. Increasing the frequency of watering did not alleviate the wilting. No wilting was seen in wild type plants or transformants with less than a two-fold increase in peroxidase activity. Wilting was not a symptom of increased water loss from the leaf surface. Microscopic examination of the epidermis and stomatal conductance measurements revealed that the stomates of the wilted plants were closed. The vascular tissue of the transformed plants also appeared normal in number and structure (Lagrimini et al., 1990). There is evidence to indicate that the wilting of TobAnPOD overproducing plants originates in the roots. Pressure bomb and xylem conductivity measurements show that the vascular tissue of the shoot is able to conduct water as efficiently as wild type plants (Lagrimini, 1991b). Moreover, shoots from TobAnPOD overproducing plants grafted onto wild type root stocks did not wilt (Lagrimini, 1991b). It appears that overproduction of TobAnPOD in the roots reduced or altered the development of the root system by some unknown mechanism. At the time of flowering, the root mass of TobAnPOD overproducing plants was one third the root mass of wild type tobacco plants (Lagrimini, 1994). The growth rate of roots of the overproducing plants slowed considerably 40 days after germination. Shoot growth rate, however, was unaffected. The wilting phenotype may simply have been the result of a reduced root system that was incapable of meeting the water demands of a healthy and transpiring shoot.

It was not surprising that the TobAnPOD overproducing plants had a higher lignin content than wild type controls. This was demonstrated, qualitatively, by staining leaves of overproducers and wild type plants for lignin with phloroglucinol (Lagrimini, 1991a).
The extent of lignification of cell walls and vascular tissues was directly proportional to the peroxidase level of the plant. Quantitatively, tobacco plants overproducing TobAnPOD by ten-fold, had a 2 to 10 times higher lignin content depending on the tissue examined (Chabbert et al., 1992). The overproducers also exhibited severe browning of wounded tissue (Lagrimini, 1991a, b). Browning involves the deposition of polyphenolic acids which can cross-link not only to other polyphenolic acids but also to proteins and other cell wall constituents (Lagrimini, 1991a). Wound-induced browning had previously been attributed to polyphenol peroxidase. Although the TobAnPOD has no polyphenol oxidase activity, it is capable of polymerizing phenolic acids.

TobAnPOD underproducing tobacco plants, like the overproducers, had unique characteristics. The plants were phenotypically similar to wild type plants. The leaves of TobAnPOD underproducing plants, however, were 17% thicker than normal (Lagrimini, 1991a). The cells of these leaves were larger and more loosely packed than cells of wild type leaves. The number of cells in a leaf was unchanged. The lignin content was reduced by less than 10% in plants with a 20-fold reduction in total peroxidase activity. It is unknown if the structure or size of the lignin polymers was altered by the reduction of the TobAnPOD isoenzymes. The phenotypes of TobAnPOD over-expressing and underexpressing plants are consistent with the hypothesized role for the TobAnPOD in lignin formation. There is a direct correlation in the amount of TobAnPOD and lignification. It is somewhat surprising, however, that lignin was reduced by less than 10% in plants with a 95% reduction in peroxidase levels. Perhaps expression of the antisense construct by the CaMV 35S promoter does not occur at the proper level or
correct developmental stage to suppress peroxidase adequately in the cells responsible for lignification. It is also possible that other peroxidase isoenzymes or other enzymes, such as laccase, are capable of lignin formation, especially in the absence of the anionic peroxidase isoenzymes. Lignification is of such importance to the development and life of a plant that plants may have evolved multiple genes and multiple enzymes with differential regulation to carry out this reaction. Further study is necessary to elucidate the role of the anionic peroxidase in lignin formation.

The difference in leaf and cell size in plants with altered peroxidase levels also suggests a developmental role for the anionic peroxidase. Cell size may be controlled, in part, by cell wall extensibility. A role for the anionic peroxidase in limiting cell wall extensibility by cross-linking of cell wall components can easily be envisioned. The possibility also exists that the anionic peroxidase may limit growth by catabolism of IAA. The TobAnPOD can catabolize IAA in vitro as well as exogenous IAA in vivo (Lagrimini, 1992). However, examination of IAA concentrations in wild type, TobAnPOD overproducing and underproducing plants revealed no quantitative differences (Lagrimini, 1991).

The use of the CaMV 35S promoter to over- or under-express the TobAnPOD complicates the interpretation of the unique phenotypes of the transformed plants. It is not known whether the unusual characteristics observed in peroxidase overproducing transformants were the consequence of an increased level of anionic peroxidase, or due to the expression of this protein in cells, tissues or developmental states where it is not normally found. Likewise, it is unknown whether the antisense construct was able to
suppress expression in all the tissues and developmental stages where anionic peroxidase expression occurs. The situation is further complicated by ignorance of the tissue specificity of anionic peroxidase gene expression, as well as the environmental and developmental signals regulating its expression.

Little is known about the expression patterns of peroxidases and their regulation. It is very likely that peroxidase levels and location of expression are tightly controlled since it has high activity towards wide variety of substrates and many of its reaction intermediate products or final products are toxic to the cell (Lagrimini et al., 1990). Regulation of peroxidase expression can occur at many levels. Regulation may occur at the level of gene expression, post translational regulation, cellular location, availability of cofactors, or availability of substrates.

There is sufficient evidence, however, to believe that regulation at this level is important. The existence of unique patterns of peroxidase isoenzymes in different tissues, at different developmental stages, and in response to environmental or chemical stimuli suggest the regulation of peroxidases at the level of gene expression. Gene expression, however, is a multi-step process, and at what steps regulation occurs is unknown. Mohan et al. (1993) have shown that transcriptional regulation is important, at least for a tomato anionic peroxidase. They demonstrated that gene promoter elements control tissue and developmental expression of this peroxidase. Environmental and chemical stimuli, such as wounding, and exposure to abscisic acid or a fungal elicitor also affected transcription. There is evidence that the tobacco anionic peroxidase gene is also regulated, at least in part, at the level of gene expression. Lagrimini et al. (1987) have shown by Northern
hybridization analysis that there are differences in mRNA levels for this gene in different tissues. The difference in mRNA abundance may be due to differences in the rate of transcription of DNA into RNA or in the stability of the mRNA intermediate. It was also observed that the induction of certain tobacco peroxidase isoenzymes required de novo synthesis of protein. This also is indicative of regulation at the genetic level.

Controlling gene expression is not the only mechanism for control of peroxidase activity. Peroxidase activity can be controlled by the availability of cofactors. Peroxidase requires a heme moiety for activity. Biosynthesis of active peroxidase is, therefore, dependent on the coordinate biosynthesis of the heme group (Lagrimini, 1991). Heme synthesis occurs in the mitochondria is dependent on the availability of soluble iron (Lagrimini, 1991 a, b).

Calcium levels are also known to affect peroxidase activity. Calcium, although not a cofactor, is known to be important for stability and increased activity of peroxidase. Secretion of peroxidase into the cell wall is dependent on calcium levels (Campa, 1991; Van Huystee, 1987). Auxin has also been suggested as a regulator of peroxidase activity directly or indirectly (Lagrimini et al., 1987; Jones, 1986). A direct effect was observed in pea epicotyls where exogenous IAA inhibited the appearance of newly synthesized peroxidases in the cell wall (Jones, 1986). It is not known if IAA inhibited the synthesis or the secretion of peroxidases in this system. Peroxidase activity may be regulated indirectly by auxins by altering the pH of the cell wall. The acid-growth theory asserts that auxin acidifies the extracellular matrix by stimulating hydrogen ion excretion
The kinetics and stability of peroxidase isoenzymes present in the cell wall are likely to be affected by changes of pH.

The availability of substrates may also regulate peroxidase activity. The importance of this regulatory mechanism, however, has not been demonstrated. Substrate availability is important for peroxidase-catalyzed lignin formation. An efflux of phenolic compounds, which presumably includes lignin precursors, coincides with peroxidase secretion into the cell wall (Gaspar et al., 1985).

**AUXIN AND APICAL DOMINANCE**

It has long been speculated that apically-produced auxin plays a major role in maintaining apical dominance and suppressing further development of axillary buds (Morris, 1977; Thimann and Skoog, 1934; Thimann, 1977). The direct auxin inhibition hypothesis proposed by Thimann (Thimann and Skoog, 1934) claimed apically produced auxin would move into lateral buds at very high concentrations and suppress their growth. Results of many studies on the effects of auxin in apical dominance seemed to support this hypothesis. Prasad et al., (1989) reported the growth of axillary buds was greatly inhibited after auxin was applied to the stump of a decapitated shoot. Outgrowth of lateral buds observed by Tamas et. al, (1989) below the point where auxin transport inhibitors were applied to the stem also added the strength to the theory. Supporting this hypothesis is the observation that strong apical dominance was observed in transgenic petunia plants containing higher endogenous auxin levels (Medford and Klee, 1989). Others found auxin-induced apical dominance was significantly reversed in auxin
resistant mutants (Estelle and Somerville, 1987; Blonstein et. al., 1988). However, other studies failed to show a correlation between auxin transport into buds and apical dominance. Morris (1977) and Lim and Tamas (1989) could not explain why there was not sufficient transport of labeled IAA into buds to inhibit their growth while the apical dominance of tested plants still remained strong. Although Thimann (1977) managed to trace labeled auxin in the buds, he was unable to show that the label remained as IAA during the experimental period.

The biggest challenge for the direct auxin-inhibition hypothesis perhaps is the lack of appropriate measurements of endogenous auxin in nodes and buds before, during and after the release of apical dominance. Modern analytical tools such as gas chromatography-mass spectrometry (GC-MS) have made it possible to now take measurements and trace the metabolic pathway before and after the treatment. Nevertheless, most of the studies failed to match physiological observation with the changes of endogenous auxin level, and thus no conclusive support to the hypothesis was obtained.

AUXIN AND GRAVITROPISM

It is believed that directional longitudinal and lateral transport of auxin induced by gravity would lead to asymmetrical elongation pattern and cause the downward curvature of roots or upward curvature in shoots (Went, 1926; Wilkins, 1984). Traditional physiological studies in exploring the effects of auxin transport on root growth and gravitropism using auxin transport inhibitors support this hypothesis (Katekar and
Geissler, 1977; Muday and Haworth, 1994). Li et al., (1991) took a different approach to study the correlation between auxin transport and gravitropism. They used genetically-engineered tobacco seedlings to examine the expression of GUS gene from the auxin-inducible SAUR promoter during tropism. They observed differential induction of the SAUR promoter GUS chimeric gene was correlated with the direction of gravistimulation. Their results were consistent with Cholodny-Went theory and add support to the importance of auxin polar transport to gravitropism.

Several other investigations, however, have been launched to challenge the validity of this hypothesis because the magnitude of changes in auxin asymmetry were usually too small to promote any observable differential growth shortly after gravistimulation (Evans, 1991; Trewavas, 1981; Firm and Digby, 1980). Ishikawa and Evans (1993) used a video digitizer system to measure and compare changes in maize primary root longitudinal cell elongation patterns with and without the pretreatment of auxin. They have observed rapid gravitropic responses even in roots in which elongation was completely inhibited by high concentrations of exogenous auxin. This suggests that there are mechanisms other than auxin transport involved with gravitropic responses (Evans, 1991; Trewavas, 1992).

**AUXIN TRANSPORT**

Auxins are believed to be delivered to various tissues and cells by a polar transport mechanism (Goldsmith, 1977; Lomax, 1994). Most of the studies of auxin transport utilized etiolated coleoptiles and hypocotyls (Goldsmith, 1977; Kaldeman,
Although auxin transport also occurs in roots, the direction of the polarity is still unclear (Evans, 1991). The chemi-osmotic hypothesis has been widely accepted in explaining the mechanism of auxin polar transport (Jacobs and Gilbert, 1983; Jacobs and Rubery, 1988; Rubery, 1987). It proposes that the proton gradient across the plasma membrane of plant cells drives auxin uptake by the next basipetally positioned cell. Polar transport of auxin appears to involve both auxin uptake carrier (IAA\(^-\) / 2H\(^+\)) and auxin efflux carrier. It is the basal location of IAA efflux carrier which plays major role for proper transport to take place (Jacobs and Gilbert, 1983).

Several phytotropins or auxin transport inhibitors have been identified and characterized in the past decades (Katekar and Geissler, 1980; Jacobs and Rubery, 1988; Rubery and Jacobs, 1990). Among those, phenylacetic acid (PAA) and flavonoids such as quercetin are naturally occurring compounds that have been demonstrated to interfere with auxin transport in intact coleoptiles and segments of hypocotyls (Johnson and Morris, 1989). Synthetic compounds such as N-1-naphthylphthalamic acid (NPA) and triiodobenzoic acid (TIBA) are much stronger inhibitors than natural ones.

The site of action of both natural and synthetic inhibitors was studied by measuring the amount of labeled auxin uptake into stem segments and isolated hypocotyl membrane vesicles (Rubery and Jacobs, 1990; Brunn et. al., 1992; Michalke et. al., 1992). Evidence indicates these inhibitors act by binding with high infinity to a site on the auxin efflux carrier distinct from auxin binding site in the plasma membrane. A single NPA binding site has been identified on the membrane in many plant species including corn, pea and sunflower (Trillmich and Michalke, 1979; Suttle, 1988, 1991).
Regardless of the lack of direct evidence on how phytotropins affect auxin polar transport in roots, it was clear that these compounds were able to reduce IAA efflux from root segments and inhibit gravitropic responses (Evans, 1991).

**AUXIN REGULATED GENES**

Numerous studies have been done to explore physiological effects of auxins on various aspects of plant growth and development including cell expansion, cell division, adventitious root formation, tropic responses and the control of apical dominance (Briggs, 1963; Evans, 1985; Brummell and Hall, 1987; Rayle and Cleland, 1992; Tamas, 1995). These responses must be associated with direct or indirect changes in expression of various auxin regulated genes. Very little is known, however, about the regulation of these genes by auxins at molecular level. Although several gene products associated with plant signal transduction pathways have been identified, the clear function of these genes and how they are regulated by auxins remains largely unknown. Successful cloning of these genes and further identification and characterization is necessary to better understand the role of auxin plays in regulating plant growth and development.

Several auxin-regulated genes have been cloned from various systems and cell types (Table 1.1). To date, most genes cloned and studied were those that are activated rapidly and up-regulated by auxins. Genes that are down-regulated by auxins have not been investigated much and will not be emphasized in this review. The following section described in detail what these genes are, their putative function, and from which tissues they were isolated.
Auxin Activated Genes in Dividing Cells Elongating

Ainley et al. (1988) reported the isolation of aux22 and aux28 genes from soybean hypocotyls. Around the same time, other auxin up-regulated genes such as GH2-4 (Czarnecka et al., 1988; Hagen et al., 1988), GH3 (Hagen et al., 1991) and SAUR - Small Auxin Up RNAs (McClure et al., 1989) were also isolated from studies using the same plant system. A few years later, PS-IAA 4/5 (Oeller et al., 1993) and arg1-4 (Yamamoto et al., 1992) were cloned from pea epicotyls and mung bean hypocotyls, respectively. Changes in level of mRNAs transcribed from these genes can be detected within 20 minutes after treatment with auxin or under inductive condition such as gravistimulation. It seems that aux22, aux28, PS-1AA4/5, arg3 and arg4 comprise a gene family and that the PS-1AA4/5 proteins are involved with auxin-mediated cell elongation processes (Abel et al., 1994; Takahashi et al., 1995).

Elevated expression in some of these genes was detected as early as 2-5 min after the addition of auxins indicating auxin may have positive effects in the transcription level. aux22, aux28, PS-1AA 4/5, PS-1AA6, arg3 and arg4 are from the same super gene family and are highly homologous at amino acid level. Expression patterns of GH3 and SAUR genes are quite different despite the same source from where GH3 and SAUR genes were isolated. The expression of GH3 was found predominantly in vascular tissues of all organs, in developing mesophyll cells and cotyledons after the induction by auxin. On the other hand, increased transcription of SAUR genes were often observed in the epidermis, cortex, and pith tissues of epicotyls and hypocotyls soon after given the
treatment with 2,4-D. Two other isolated cDNA clones, *arg1* and *arg2*, encode proteins that may be important for rapid hypocotyl growth; their protein sequences are similar to a fatty acid esterase in Arabidopsis, *fad3*. 
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Source/Plant System</th>
<th>Potential Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>aux22</td>
<td>Soybean Hypocotyls</td>
<td>N/A</td>
<td>Ainley et al., (1988)</td>
</tr>
<tr>
<td>aux28</td>
<td>Soybean Hypocotyls</td>
<td>N/A</td>
<td>Ainley et al., (1988)</td>
</tr>
<tr>
<td>GH3</td>
<td>Soybean Hypocotyls</td>
<td>Cell Elongation</td>
<td>Hagen et al., (1991)</td>
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<tr>
<td>SAUR</td>
<td>Soybean Hypocotyls</td>
<td>Nuclear Protein</td>
<td>McClure et al., (1989)</td>
</tr>
<tr>
<td>arg1</td>
<td>Mung Bean Hypocotyls</td>
<td>Fatty Acid Desaturase</td>
<td>Yamamoto et al., (1992)</td>
</tr>
<tr>
<td>arg2</td>
<td>Mung Bean Hypocotyls</td>
<td>N/A</td>
<td>Yamamoto et al., (1992)</td>
</tr>
<tr>
<td>arg3</td>
<td>Mung Bean Hypocotyls</td>
<td>N/A</td>
<td>Yamamoto et al., (1992)</td>
</tr>
<tr>
<td>arg4</td>
<td>Mung Bean Hypocotyls</td>
<td>N/A</td>
<td>Yamamoto et al., (1992)</td>
</tr>
<tr>
<td>parA</td>
<td>Tobacco Mesophyll Protoplasts</td>
<td>Nuclear Protein(p24)</td>
<td>Takahashi et al., (1989)</td>
</tr>
<tr>
<td>parB</td>
<td>Tobacco Mesophyll Protoplasts</td>
<td>GST</td>
<td>Takahashi and Nagata (1992)</td>
</tr>
<tr>
<td>parC</td>
<td>Tobacco Mesophyll Protoplasts</td>
<td>p24</td>
<td>Takahashi and Nagata (1992)</td>
</tr>
<tr>
<td>PCNT103</td>
<td>Tobacco Cell Suspension Culture</td>
<td>GST (p24)</td>
<td>van der Zaal et al., (1991)</td>
</tr>
</tbody>
</table>

Table 1.1 Up-regulated auxin-regulated genes mentioned in this research
Auxin Activated Genes in Dividing Cells of Mesophyll Protoplasts

Solid evidence has been accumulated over the past several decades suggesting that auxin is necessary for the initiation of cell division. Much of this research was done with tobacco mesophyll protoplasts because they proliferate rapidly under proper conditions and require exogenous auxins and cytokinins for the induction of cell division.

At least three auxin-regulated genes (parA, parB and parC) have been isolated from mesophyll protoplasts of *N. tabacum* L. (Takahashi et al., 1989; Takahashi and Nagata, 1992a; Takahashi and Nagata, 1992b). The accumulation of transcripts of all three genes was seen within 20 min after induction by 2,4-D in concentrations as low as $4.5 \times 10^{-8}$ M. The accumulation reached its peak about 4 hr after the treatment. This finding suggested that active expression of three *par* genes might be associated during G₀ to S phase of the cell cycle since not much newly synthesized DNA was detected until 20 to 48 hr after the treatment with 2,4-D in cultured mesophyll protoplasts. It is also worth mentioning that these three genes were only expressed in response to exogenous synthetic and natural auxins and not to other plant hormones.

In contrast to most other auxin-regulated genes, the expression of *parB* was observed in the shoot apex, as well as in the root meristems. Moreover, drastic changes in auxin-responsiveness of *parB* observed throughout plant growth and development suggested gene activation by auxin is rather tissue specific and regulated temporally.

Sequence data for *parB* exhibited homology with glutathione S-transferases (GST; EC 2.5.1.18) and expression of *parB* cDNA in *E. coli* gave a product with GST activity (Takahashi and Nagata, 1992a). The main function of GSTs is detoxification of
xenobiotics through the formation of conjugates with glutathione and subsequent export of the complex to the vacuole or plasma membrane (Martinoia et al., 1993). Although IAA is very unlikely to be a substrate at this site, it was proposed that it may bind to the non-catalytic site, and GSTs may be associated with auxin transport from cell to cell through ATP-driven pumps at the plasma membrane (Napier and Venis, 1995). High similarity at the amino acid level to an auxin-binding protein from the plasma membrane of Arabidopsis indicated possible involvement of parB in the proliferation of tobacco mesophyll protoplasts (Zettl et al., 1994).

Studies showed parA, parC and C7, another parA-related gene, exhibited high DNA sequence homology. Unlike parA and parC, however, C7 did not respond to exogenous auxins and it was expressed mainly in mature leaves. The expression of parA and parC was detected primarily in root tips. It was suggested that parA-related genes are members of the GST family since the product of pCNT103 exhibited 42% homology to parA and had strong GST activity. The actual role of parA protein remains to be determined.

**Auxin Activated Genes in Dividing Cells of Suspension Culture**

The initiation of cell division in suspension culture requires a supplement of exogenous auxins. This unique feature plus a well-controlled growth environment made cell suspension cultures very good systems to study gene expression regulated by plant growth regulators. Several auxin-induced genes have been identified in cell lines of tobacco suspension culture. Nagata and coworkers isolated an auxin-inducible gene,
arcA, from suspension cells derived from *N. tabacum* L. cv. Bright Yellow 2 (Ishida et al., 1993). The initiation of *arcA* expression by the application of auxin preceded the induction of cell division. Sequence analysis of *arcA* product revealed a 40 amino acid series with internal repeats suggesting that it could be structurally related to the β subunit of the heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) (Ishida et al., 1993). Members of this gene family all seem to have segments of internal repeats of 40 amino acids called the WD-40 repeat where W is the code for tryptophan and D is the code for aspartic acid. There has been evidence indicating some members of the G-protein (or WD-40 repeat) family are involved in a signal transduction pathway, regulation of gene expression and cell cycling (Neer et al., 1994). Therefore, as a member of the G protein β subunit family, arcA is thought to be involved with cell division processes.

**CURRENT HYPOTHESIS**

Auxins are involved in many physiological processes in plants such as cellular elongation, phototropism, geotropism, apical dominance, root initiation, and fruit development. The amount of IAA present in tissues at a particular time can be regulated through 1) control of the biosynthesis of the hormone, 2) the reversible conjugation of IAA (Salibury and Ross, 1992) and 3) degradation of auxin. If peroxidase can catabolize IAA, one would expect to see peroxidase underproducing plants have more short adventitious roots, stronger apical dominance, gravitropic responses and growth of young seedlings than wild type plants. On the other hand, the overproducers are expected to
have phenotypes of less and shorter adventitious root formation, slower hypocotyl elongation growth, weaker apical dominance and gravitropic responses.

RESEARCH OBJECTIVE

The objective of my dissertation research was to determine the physiological, quantitative, and molecular changes in IAA-mediated growth and development which occur in tobacco plants as results of altered peroxidase activity \textit{in vivo}. Growth rates of adventitious roots, the elongation rates of dark-grown young seedlings, the expansion of axillary buds after the release of apical dominance on wild type and under- and over-producing plants of \textit{N. tabacum} cv. xanthi and \textit{N. sylvestris} were examined. Quantitative analysis on endogenous IAA levels on lateral buds of various positions before and following decapitation was also done. Simultaneous changes in the expression of \textit{pCNT103}, an auxin-inducible gene, on buds of the same plant material prior and after the release of the apical dominance was investigated. Implications for whether TobAnPOD affects endogenous IAA levels and what roles IAA may play in the apical dominance were discussed.
CHAPTER 2

ROLE OF TOBACCO ANIONIC PEROXIDASE IN PLANT GROWTH AND DEVELOPMENT AND EFFECTS OF ALTERED PEROXIDASE ACTIVITY ON THE EXPRESSION OF AUXIN-INDUCIBLE GENES

INTRODUCTION

Peroxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7, POD), is predominantly found in xylogenic and epidermal tissue of higher plants and is believed to play an important role in cell wall synthesis (Fry, 1986), lignin formation (Lagrimini, 1991; Mader et al., 1982), wound healing (Roberts et al., 1988), and pathogen defense (Lagrimini et al., 1987; Dowd and Lagrimini, 1997; Felton et al., 1989). In addition, the involvement of peroxidase in the control of endogenous auxin levels has been proposed (Gazaryan and Lagrimini, 1996; Hinnman, 1965). This hypothesis is based on results gathered from in vitro investigations (Acosta et al., 1988, Gasper et al., 1982; Lagrimini, 1992; Li et al., 1991; Mohamed et al., 1989; O’Neil and Scott, 1987; Pressey, 1990).

However, there is no convincing evidence that peroxidases degrade auxin in vivo.

The many potential substrates and multitude of isoenzyme forms have made it difficult to determine the actual in vivo role for the various peroxidase isozymes using
standard biochemical techniques (Lagrimini et al., 1987). The application of recombinant DNA technology is a complementary approach that potentially could uncover some of the complicated issues like this. Among the twelve peroxidase isoenzymes found in tobacco, the gene encoding an anionic isoenzyme with a $\pi$ of 3.5 (MW 36,000) was cloned by Lagrimini and Rothstein (1987). Studies have suggested that this isoenzyme is found mainly in xylem and epidemal tissue and is necessary for lignin formation (Mader et al., 1975). Generation of transgenic tobacco plants with altered expression of this gene may enable a better understanding of other potential functions of peroxidase (Lagrimini and Rothstein, 1987; Lagrimini et al., 1990; Lagrimini 1991). Tobacco plants transformed with TobAnPOD under the control of cauliflower mosaic virus 35S promoter have been generated (Lagrimini et al., 1990). Depending on the orientation of the gene relative to promoter sequences, peroxidase activity in the transgenic plants relative to wild type ranged from a tenfold increase to twenty-fold suppression.

By using transgenic plants that either under- or over-produce TobAnPOD, one may investigate the function(s) of this isoenzyme, especially in terms of effects on overall plant growth and development. Root growth and lateral development is highly auxin-regulated (Cline, 1994; Muday and Haworth, 1994). It has been reported that root growth pattern varies in plants treated with different concentrations of IAA (Katekar and Geissler, 1980; Gaspar et al., 1982). Evans et al. (1994) reported that auxin at very low concentration promotes root elongation, but inhibits growth at higher concentrations. In contrast, auxin has also been found to enhance root branching at concentrations that inhibit root elongation (Hobbie and Estelle, 1995; Muday and Haworth, 1994). These
phenomena may result from alteration of auxin sensitivity or availability in specific cells or tissues (Ishikawa and Evans, 1993; Li et al., 1991). If peroxidases are indeed involved in auxin metabolism in planta, changes in their activities will lead to alteration of endogenous IAA levels. This in turn should lead to different root growth pattern compared to wild type plant. Plants containing higher levels of endogenous auxin should, in theory, generate a greater number of shorter roots than plants with lower auxin levels. This suggests many predicted phenotypes associated with TobAnPOD under- and over-producers with altered endogenous IAA levels.

In addition to rooting, auxin is also considered an important mediator for gravitropic responses (Ishikawa and Evans, 1993; Li et al., 1991). According to the Cholodny-Went theory, tropisms are mediated by stimulus-induced redistribution of auxin (Ishikawa and Evans, 1993). In the case of gravitropism, auxin accumulates on the lower side, inhibits lower-side cell growth and leads to downward curvature of the roots (Ishikawa and Evans, 1993; Li et al., 1991). Altered endogenous auxin levels resulting from the over- or under-expression of the TobAnPOD gene may result in changes to gravitropic responses of roots (Lagrimini, 1992). The association of peroxidases and in vivo IAA metabolism could be further strengthened if differences in other auxin-associated growth responses such as lateral bud development and hypocotyl elongation between transgenic and wild types were observed.

Furthermore, it is generally believed that the mechanisms by which auxin affects such diverse biological processes are made possible through changes in the expression of a large group of auxin-regulated genes throughout the plant's growth and development.
cycle (Hagen, 1995; Li et al., 1999; Sitbon et al., 1996). Several auxin-regulated genes have been isolated from various plant systems including soybean hypocotyls, pea epicotyls, tobacco mesophyll protoplasts and Nicotiana suspension culture cells among others (Li et al., 1999). Expression of some of these genes occurs either before or shortly after an auxin-mediated growth response, primarily, cell elongation and cell division.

In soybean, the expression of auxin-inducible genes such as aux22 and saur was found to be in close relation to the process of hypocotyl elongation (Ainley et al., 1998; McClure and Guilfoyle, 1987). cDNAs corresponding to cell division processes and showing high homology to the sequences of saur and aux22 such as parA, pCNT103 and pCNT107 genes have been isolated from tobacco protoplasts and cell suspension cultures (Hagen, 1995). Although a great deal of data have has been generated from these studies, most of the results were based on the consequences after the addition of exogenous substances to excised organs.

There have been few studies on how these genes are regulated by endogenous auxin, and to my knowledge even fewer reports of simultaneous measurements of endogenous auxin levels and corresponding auxin-regulated gene expression using transgenic plants with altered peroxidase activity.

Sitbon et al., (1996) reported the simultaneous analysis of auxin-inducible gene expression and endogenous IAA levels in wild type and IAA overproducing plants. They analyzed mRNA levels of four auxin-inducible genes coupled with the endogenous IAA levels detected in different organs of mature plants. Their results indicated that older buds of the IAA overproducers had twice as much IAA as wild type. No significant difference
in IAA levels was found in young buds of either genotype. The mRNA level of the auxin-inducible genes investigated was also higher.

It is therefore reasonable to extend this kind of molecular approach to further investigate the relationship between auxin levels and expression of auxin-inducible genes in the context of apical dominance, among plants that either over- or under-produce TobAnPOD. Investigating the kinetics of expression levels of one of the auxin inducible genes, *pCNT103*, in wild type and the transgenic plants may provide some indication whether peroxidases have a role in IAA catabolism.

Total RNA was extracted from buds harvested on the same plants as used in IAA quantitative analysis and the mRNA levels of the *pCNT103* gene were analyzed. *pCNT103* cDNA, which is one of the most studied auxin-inducible genes, was isolated from tobacco cell suspension cultures by van der Zaal’s group (van der Zaal et al., 1991). It often exhibits highest expression in the root tip (van der Zaal, et. al., 1991). The fact that the transcript of *pCNT103* gene only became detectable shortly after auxin was added to the medium in an attempt to induce cell division makes it an ideal target gene for my research. In addition, unlike other auxin-inducible genes such as *saur* whose expression can be induced by substances other than auxins, the expression of the *pCNT103* gene is most strongly induced by auxin. It sometimes can be induced by high concentrations of salicylic acid and heavy metals (van der Zaal et. al., 1992). But most importantly, it was the only auxin-inducible gene made available for us at the time to conduct the experiments.
The objectives of this chapter were to 1) investigate the effects of altered TobAnPOD activity on auxin-mediated growth and development such as adventitious root formation, hypocotyl elongation, whole plant growth rate, gravitropic response, axillary bud growth rate before and following decapitation and 2) study the effects of altered peroxidase activity on expression of an auxin-inducible gene, pCNT103. before and after the release of apical dominance.
MATERIALS AND METHODS

Plant Material

Tobacco plants (*N. tabacum* and *N. sylvestris*) were genetically engineered by Lagrimini and his colleagues (Lagrimini and Rothstein, 1987) to overproduce or underproduce TobAnPOD. Tobacco plants over-expressing TobAnPOD were constructed in which the full length cDNA clone with the signal peptide and the poly A tail was ligated between the CaMV35S promoter and terminator in a normal orientation (Lagrimini et al., 1992). Tobacco plants under-producing anionic peroxidase were constructed using antisense technology in which the 5' half of the TobAnPOD cDNA clone was inserted between the CaMV35S promoter and terminator in an inverted orientation (Lagrimini et al., 1992). The resulting plasmids were transferred into the *A. tumefaciens* and selection of transformed plants was based on kanamycin resistance at 100 mg L⁻¹. Putative lines under- and over-expressing TobAnPOD were screened by the altered total peroxidase activity and IEF gel analysis. Subsequent generations were obtained by self-fertilization to achieve homozygous T2 tobacco plants that had up to a ten-fold increase in total peroxidase activity for the overproducers, while plants transformed with the antisense construct displayed three to twenty-fold less total peroxidase activity than the wild type plants (Lagrimini et al., 1990). Isoelectric focusing (IEF) gels showed that changes in total peroxidase activity were the result of changes in expression levels of TobAnPOD gene (Lagrimini et al., 1990).
All seeds used in my research were harvested from the offsprings of the homozygous lines of *N. tabacum* cv. Xanthi and *N. sylvestris* described above. The abbreviations used for *N. tabacum* Xanthi TobAnPOD underproducers and overproducers are xan427 and xan507, respectively. In *N. sylvestris*, the underproducing plants are called syl427, while the overproducing plants are abbreviated as syl507. All wild type plants of both species were indicated as xanWT and sylWT.

Assay of total peroxidase assay was carried out as follows (Lagrimini et. al., 1997). About 400-500 mg of fresh weight leaf tissue was homogenized with a Polytron blender (Brinkman Industries) in a 15 ml tube placed on ice in three volumes of cold grinding buffer (10 mM sodium phosphate pH 6.0, 5 mM sodium metabisulfite). A cleared extract was obtained by centrifugation at 10,000 x g for 20 min. Total peroxidase activity was determined by the increase (or changes) in absorbance at 470 nm in 0.28% guaiacol, 0.05 M NaPO₄ pH 6.0, 0.3% H₂O₂ and 150 ul of enzyme extract from the leaf tissue. Total leaf peroxidase activity in each seedling of transformed *N. tabacum* and *N. sylvestris* plants was measured in each experiment. Genotypes in which total peroxidase was increased by less than 10-fold in overexpression lines and that of which was reduced less than 20-fold in antisense plants were excluded from the study. All plant materials used in the following studies were screened on their total peroxidase activities prior to use based on the previously stated criteria unless mentioned otherwise.

Plants for RNA extraction were grown from seeds. Seedlings of xan427, xanWT and xan507 were initially grown in tissue culture for four weeks. They were subsequently transferred and grown in the greenhouse. Plants were decapitated to release apical
dominance using a razor blade when flower buds became visible. At 0, 1, and 3 days after decapitation, the first five axillary buds from the apex of each plant were excised and immediately frozen into liquid N₂. Buds were stored at -80°C until extraction.

**Adventitious Root Formation**

Seeds of xan427, xanWT, xan507, syl427, sylWT and syl507 were sterilized with 20% NaOCl for 15 min followed by 3 rinses with sterile distilled water. Seeds were then germinated on a medium, abbreviated as OMS, containing only Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), B5 vitamins, 30% sucrose and 2.4 g L⁻¹ Gelrite. No plant growth regulators were added, and the pH was adjusted to 5.7 with 1M KOH before autoclaving. All media were autoclaved at 120°C for 20 min. Seedlings were grown at 25°C in a 16 h of light from fluorescent tubes. Ten days after germination, seedlings were transferred to GA7 jars (Magenta box) containing the same medium for continuous growth. Three weeks after transfer, shoot tips were excised and put on new OMS medium in GA7 jars. Observations on the number of root tips and length of the selected target root were made in each explant 4, 5, 6, 7, 8, 9, 10, 11 and 12 days after the final transfer. The selection of the target root in each shoot tip was made 4 days after the final transfer. In most cases, root which appeared to be the longest or the most prominent (thickest) when compared with other roots regenerated from the same explant at day 4 was chosen as the target root. In cases which all roots had about the same size and appearance, a target root was chosen at random. Length of the same target root from each
explant was measured 4, 5, 6, 7, 8, 9, 10, 11 and 12 days after the final transfer. Experiments were repeated twice.

**Hypocotyl growth**

Seeds of xan427, xanWT and xan507 were surface sterilized as previously described and germinated under light in GA7 jars containing OMS medium for 18 hr and then placed in the dark at 28°C in incubator. There were 6 jars per genotype at the beginning of the experiment and between 20 and 40 seeds per jar. To improve and synchronize germination, especially in *N. sylvestris*, cultured seeds were placed under fluorescent light for 24 hr prior to transferring to complete darkness at 28°C for continuous growth. Each day, beginning at day 7, samples of seedlings were carefully removed from the jars and placed on transparent adhesive 3M Scotch tape for hypocotyl length measurements. The tape containing the seedlings was then covered by a 2-cm wide transparency overhead noted with date and corresponding genotype. These seedlings were then stored at -80°C until analysis. Lengths were measured from images projected from a photographic enlarger (3x magnification) (Knee and Hangarter, 1996). The length of each hypocotyl was measured and the growth rate calculated. The experiments were repeated three times and data was analyzed using one way ANOVA at $p=0.05$.

**Stem growth**

Seedlings of xan427, xanWT and xan507 plants were initially grown in tissue culture for four weeks. They were subsequently transferred into 22 cm pots containing a
commercial soilless media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH) and fertilized 2 times per week with 200 ppm of Peters 20-10-20 complete fertilizer. Plant height was measured when flower buds became visible and data was analyzed using one way ANOVA at $p=0.05$.

**Tobacco anionic peroxidase and apical dominance**

Plant material was prepared as described before. Plants were decapitated at the appearance of flower buds using a razor blade to release apical dominance. Lengths of the first five axillary buds were measured at 0, 1, and 3 days after decapitation. Samples and data were collected about the same time on each sampling day.

**Anionic peroxidase and gravitropism**

**Growth of vertical roots before and after treatment with IAA**

Seedlings of xan427 and xanWT were sterilized as described in the adventitious root formation section. Seeds were sown in a row (5 to 6 seeds per row) on sterile OMS medium in petri plates (60mm in diameter, 15mm in height) sealed with parafilm (American Can, Greenwich, CT). The seeds were placed into the medium perpendicular to the cylindrical axis of the dish. The petri plates were placed in a culture room under continuous white light from fluorescent lamps (F30T8-CW, Sylvania) at 23°C. Three to five day old seedlings with root lengths of 30-50 mm were used for the experiments.

Before applying IAA, a Petri plate containing 3 to 5 day-old seedlings was mounted vertically in a plastic chamber with roots immersed in an aerated buffer solution.
(1 mM KCl, 1 mM CaCl$_2$, MES pH 5.8). The roots were allowed to grow under these conditions for about 80-100 min. To analyze root growth, seedlings were viewed by a CCD (charge-coupled device) camera (Marchall Electronics, Culver City, CA) connected to a computer via a frame-grabber circuit board (ImageNation, Beaverton, OR). The roots were illuminated from behind with an IR light-emitting diode (Radio Shack, Fort Worth, TX). Elongation rate was recorded using a custom software program called ADAPT (Ishikawa and Evans, 1990).

A small volume of IAA stock was then added to obtain the desired final concentration of $10^{-9}$, $5 \times 10^{-9}$, $10^{-8}$, $5 \times 10^{-8}$, $10^{-7}$, $3 \times 10^{-7}$, $5 \times 10^{-7}$, $10^{-6}$, or $10^{-5}$ M. Roots continued to grow for an additional 500-520 min and growth rate after the addition of IAA was measured using the same digital video system. Four to eight 3-5 d old seedlings were used for each IAA concentration. Some experimental results contain data from only a single root since growth data from other seedlings were lost due to technical issues such as failure to capture root images due to mispositioning of the Petri plate during the course of the experiment.

**Root growth and curvature changes before and after gravistimulation**

Seedlings of xan427, xanWT and xan507 were sterilized as described in the section of adventitious root formation part of p.33. Seeds were sown in a row (5 to 6 seeds per row) on sterile agar (1%, w/v) in petri plates (60mm in diameter, 15mm in height) sealed with parafilm (American Can, Greenwich, CT). The medium contained 1/2 strength MS salts and 1% (w/v) sucrose. The row of seeds were placed on the agar
surface perpendicular to the cylindrical axis of the dish. The petri plates were placed in a culture room under continuous white light from fluorescent lamps (F30T8-CW, Sylvania) at 23°C.

Root growth was first analyzed by the same system described earlier with the seedling growing vertically for 180 min. The petri dish containing the seedlings was then turned 90° in the clockwise direction for gravi-stimulation and the roots were allowed to grow for an additional 600-900 min. Changes in angle of curvature over the first 180 min upon gravistimulation were measured. All data regarding measurements of elongation rate and curvature before and after gravistimulation were recorded and calculated.

**RNA extraction**

Two ml of phenol/CHCl₃ (Ambion) and 1 ml of grinding buffer consisting of 50 mM Tris-HCl pH8.0, 4% sodium p-amino salicylic acid (Sigma) and 1% sodium Naphthalene 1,5-disulphonic acid (Sigma) were added to 15 ml Falcon tube in which approximately 50-200 mg of frozen buds were collected. Samples were kept at approximately 0 to 4°C during the extraction procedures. Samples were homogenized using a Polytron tissue grinder for 1 min and then centrifuged for 16 min at 9,000 rpm (10,000g). The aqueous phase was transferred into a clean tube and 2 ml of CHCl₃ were added. The samples were vortexed at full speed for 2 min followed by centrifugation at 9000 rpm for 5 min. The aqueous phase was transferred into a clean tube and RNA precipitated overnight at 4°C by adding 8M LiCl, 8M urea and 500 mM EDTA. Pellets were obtained by centrifuging 30 min at 9,000 rpm and resuspended with 300 µl of
resuspension buffer (40mM Tris-HCl pH7.5, 20mM sodium acetate (NaOAc), 5mM EDTA and 1% SDS). The samples were centrifuged once more for 5 min at 9000 rpm and the supernatant was transferred to clean eppendorf tubes. RNA was reprecipitated by adding 3M of NaOAc and 100% ethanol (EtOH) to the aqueous phase and stored overnight at -20°C. Samples were then centrifuged for 30 minutes at 9000 rpm to recover the pellet. The pellets containing RNA were resuspended with 20 ul of 0.1x TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). All RNA samples were then stored at -80°C until analysis.

**RNA blot analysis**

Total RNA was separated on a 1.2% agarose gel containing 1.8 g agarose and 0.14 g iodoacetamide as a RNAase inhibitor in 150 ml gel running buffer consisting of 40 mM MOPS (pH 7.0) and 2mM EDTA (pH 8.0). Equal amount of total RNA (around 5 μg) was loaded and the gel was run in the same running buffer at 120 V for 75 min or until the running dye was 9 cm away from the base.

The gel was rinsed in several changes of double distilled water for 5 min followed by soaking in fresh 50 mM NaOH and 10 mM NaCl for 45 min. The RNA gel was then transferred to 0.1 M Tris-HCl (pH 7.5) solution and continued to soak for additional 45 min. The RNA was then transferred to the nylon membrane (Hybond-N; Amersham) overnight and cross-linked with UV light. The pNT103 probe used in this experiment was the 0.9 Kb EcoRI insert of pBluescript (Van Zarr, 1991), obtained from Dr. Van Zarr’s group at Leiden University in the Netherlands. Radiolabeled probes were generated by
either PCR or random primed $^{32}$P-labeling of pCNT103 insert in pBluescript. RNA was pre-hybridized with 5 ml of Church hybridization buffer (Church and Gilbert, 1984) at 65°C for 1 h. The pCNT103 probe was then added for overnight hybridization using the same buffer as used in the pre-hybridization procedure. Membrane was then washed once at the following day with 2x SSC at room temperature for 15 min. Second and third washes were done with 0.2x SSC, 0.1% SDS at 60°C for 15 min each. The X-ray films were then exposed to the membrane for 3 d.
RESULTS

Effects of altered peroxidase levels on root growth

Significant differences in the number of total root tips among the three genotypes of *N. tabacum* plants (Fig. 2.1) were observed. More roots were generated from xan427 shoot tips than those obtained from the other two genotypes. While xanWT shoot tips had fewer roots than xan427, they appeared to have generated significantly more roots than those of the xan507 cuttings. Similarly, the highest number of roots generated from *N. sylvestris* cultures was in syl427 when compared with the other two genotypes (Fig. 2.3). Unlike *N. tabacum*, no significant differences in either number of roots or growth rates were observed between sylWT and syl507 explants (Fig. 2.3).

The lines in Figs 2.2 and 2.4 are regression lines and the predicted root growth rates are indicated by the slopes in the equations. For *N. tabacum* plants, root elongation rates were the highest in xan427 (Fig. 2.2) with the average growth rate of 0.55 cm per day. In contrast, adventitious roots of xan507 grew the slowest with an average rate of less than 0.2 cm per day. Likewise, syl427 roots elongated at a faster rate with the average growth of 0.7 cm per day (Fig. 2.4) which was in agreement with the results shown in xan427 plants.

However, no significant differences in root lengths were observed between sylWT and syl507 shoot tip explants (Fig. 2.4), which is not consistent with the data obtained in
those of xanWT and xan507 cuttings. This indicates that the effects of altered
TobAnPOD level on auxin-mediated adventitious root growth may be more profound in
xan507 than in sy1507 plants.
Figure 2.1. Root initiation on xan427, xanWT and xan507 shoot tips. All values represent the mean and standard errors for 58 explants per genotype from 3 experiments.
Figure 2.2. Growth of selected adventitious roots of xan427, xanWT, and xan507. All values represent the mean and standard errors for 58 explants per genotype from 3 experiments.
Figure 2.3. Root initiation on syl427, sylWT and syl507. All values represent the mean and standard errors for 58 explants per genotype from 3 experiments.
Effects of altered peroxidase level on hypocotyl growth

No significant differences in the average hypocotyl length among xan427, xanWT and xan507 seedlings were observed (Fig. 2.5). In *N. sylvestris*, no differences in hypocotyl lengths among three genotypes were observed on the first day of measurement.
(Fig 2.6). However, the gap in hypocotyl growth between syl507 and sylWT further widened with time (Fig 2.6). After 11 d in the dark, syl507 seedlings showed the greatest increase in length, followed by syl427. Nevertheless, sylWT hypocotyls elongated the least when compared to that of syl427 and syl507 (Fig. 2.6). Wild type hypocotyls, on average, elongated at only half the rate (0.8 mm/day) as compared to syl427 (1.5 mm/day) and to syl507 (1.8 mm/day).

**Effects of altered peroxidase level on whole plant growth**

Results of whole plant growth study are shown in Fig. 2.7. Statistical analysis using one way ANOVA at $p=0.05$ showed significant differences in stem growth among the three genotypes. The xan427 plants were, on average, taller than overexpressing plants. The average height for xanWT plants was 101 cm, which was about 10% taller than that of the xan507 plants, but was approximately 15% shorter than the xan427 plants (Fig. 2.7). The distinctive wilting phenotype of the transgenic plants reported by Lagrimini was not observed (Lagrimini, 1991).
Figure 2.5. Hypocotyl growth of dark-grown seedlings of xan427, xanWT and xan507. Values represent the means and standard errors of at least 25 seedlings per genotype from 3 experiments.
Figure 2.6. Hypocotyl growth of dark-grown seedlings of syl427, sylWT and syl507. Values represented the means and standard errors of at least 25 seedlings per genotype from 3 experiments.
Figure 2.7. Comparison of stem growth in xan427, xanWT and xan507 plants. Values represented the means and standard errors of 60 plants per genotype from 3 experiments.

Study of tobacco anionic peroxidase and apical dominance

Axillary buds elongated in all three genotypes during the course of the experiment. Lateral buds 1, 2, and 3 of xan427 plants were significantly longer than the
other two genotype plants prior to decapitation (Fig 2.8). No significant differences in the lengths of the axillary buds were found between xanWT and xan507 plants before decapitation (Fig 2.8). Nonetheless, no differences in the lengths among lateral buds of the same genotype were observed regardless their position relative to the apex (Fig 2.8).

Axillary bud growth rates as a function of time after decapitation are presented in Fig. 2.9. Growth rates of all buds regardless of location were 3 times greater in xan427 plants than those in xan507 plants. All xanWT buds grew at 50% slower rate than corresponding buds of xan427 plants. While there were large differences in lateral bud growth among the three genotypes over the 72h period, almost identical growth rates of the first, second and the third buds of each genotype were observed during the same time period (Fig. 2.9). This is consistent with previous results (Fig 2.8) where no differences in lengths among bud 1, 2 and 3 of each genotype were observed.
Figure 2.8. Comparison of axillary bud length in xan427, xanWT and xan507 plants before decapitation. Data represents the mean axillary length of at least 60 plants ± SE for each replicate.
Figure 2.9. Axillary bud growth rates in wild-type and transgenic tobacco plants. Values represented the means of at least 12 plants per experiment per genotype from 3 experiments.
Tobacco anionic peroxidase and gravitropism

Effects of exogenous IAA on root growth

The effects of IAA on root growth of xan427 and xanWT young seedlings are shown in Figs. 2.10 and 2.11, respectively. Root growth rate was almost identical between the two genotypes prior to the application of IAA with the average growth rates of 0.05 mm/hr for xan427 roots and 0.06 mm/hr for the roots of xanWT seedlings. Root growth of xan427 seedling was inhibited by IAA at all concentrations with the exception of $10^{-9}$ M (Fig 2.10). A continuous decline in growth rates of the xan427 seedlings was observed as the concentrations of exogenous IAA increased. Growth was completely inhibited at IAA concentrations of $10^{-6}$ M and higher (Fig 2.10).
Figure 2.10. IAA dose response curves for root growth in xan427 seedlings grown vertically while submerged in the presence of IAA. Root growth rate is expressed as a percentage change of the IAA-free control of the xan427 root. The line was generated using MS Excel. Data were pooled from roots of various sample size with the standard deviation of set of 1-8 sample measurements. Root growth rates for controls were 0.05±0.01 mm h⁻¹.
Figure 2.11. IAA dose response curves for root growth in xanWT seedlings grown vertically while submerged in the presence of IAA. Root growth rate is expressed as a percentage change of the IAA-free control of xanWT root. The line was generated using MS Excel. Data were pooled from various sample size with the standard deviation of set of 1-8 sample measurements. Root growth rates for controls were 0.06±0.03 mm h⁻¹.
Despite similar growth rates between the xanWT and xan427 seedlings prior to the application of IAA, xanWT seedlings were less sensitive to exogenous IAA than the TobAnPOD underproducers (Fig. 2.11). In contrast to the xan427 seedlings, root growth in xanWT seedlings was stimulated by IAA of $5 \times 10^{-8}$ M and lower (Fig. 2.11). A 50% growth inhibition was observed at $10^{-6}$ M in xanWT roots followed by an 80% growth suppression by IAA of $10^{-5}$ M (Fig 2.11) suggesting that xanWT roots were very insensitive to IAA when compared with roots of xan427 seedlings. No xan507 seedlings were used in this experiment.

A comparison of root elongation before and after gravistimulation in three genotypes

Root elongation prior to gravistimulation was greater in xan427 seedlings than in xanWT seedlings (Fig 2.12). Xan427 seedlings elongated approximately 4 times faster than in xanWT plants. No significant differences in root growth between xanWT and xan507 seedlings was observed when grown in their original orientation before rotation (Fig 2.12). These findings are not entirely consistent with the results obtained in previous studies where root elongation root was almost identical between xanWT seedlings and the xan427 seedlings (Figs 2.10 and 2.11). This apparent contradiction may be due to differences in how the experiments were conducted and the conditions under which the seedlings were grown. In IAA studies, seeds were germinated and grown into the medium in petri plates containing GelRite, a different support material than agar and the roots were completely submerged into growth solution in an aerated chamber during the entire course of the experiment. In the gravitropic studies, seeds were germinated and
grown on the surface of the MS medium containing agar as support material. Seedlings then stayed enclosed inside the plate during the course of the experiment before and after gravistimulation in an attempt to avoid the shock of exposing the root to new medium and to obtain more consistent root growth. It has been suggested that roots grown on the surface of a solid medium provide the most consistent growth and gravitropism results (Muday and Haworth, 1994). A study conducted by Mullen et al., (1998) suggested that the kinetics of the gravitropic responses and root growth behavior were affected by how experimental roots were grown on the surface of agar in plates tilted by various angles from the vertical axis of the plate.

Following gravitropic stimulation, roots of xan427 seedlings grew about 6 and 13 times faster than the xanWT and xan507 seedlings, respectively (Fig. 2.12). Root growth of xan507 seedlings was significantly reduced compared with wild type seedlings and showed almost identical growth rates as measured before roots were subjected to gravistimulation (Fig. 2.12).

A comparison of gravitropic responses in three genotypes

Three hours following gravitropic stimulation at 90 degrees to the vertical, xan427 seedlings yielded the greater curvature changes than xanWT and xan507 seedlings (Fig. 2.13). XanWT seedlings curved the least while xan507 seedlings demonstrated a moderate downward curvature change (Fig 2.13). Although additional experiments were conducted, obtaining results which were consistent and reproducible proved to be difficult (data not shown). The gravitropic results obtained from those experiments in
which only xanWT and xan427 genotypes were included showed that 1) more time was needed for xan427 seedlings to exhibit gravitropic responses; 2) less time, however, was needed to reach the final angle; 3) maximal slopes obtained from both genotypes were identical.

Gravitropic response studies were also conducted on syl427, sylWT and syl507 seedlings. In contrast to the results obtained on gravitropic studies using *N. tabacum* seedlings, the greatest curvature rate occurred with syl507 seedlings while no gravitropic response was observed in roots of syl427 seedlings (data not shown).
Figure 2.12. Elongation of roots in xan427, xanWT and xan507 seedlings before and after gravi-stimulation. Each value represents the mean root growth of at least 15 seedlings.
Figure 2.13. Development of gravitropic curvatures in xan427, xanWT and xan507 seedlings stimulated at 90 degree. Each value represents about 10-15 seedlings.

Expression of *pCNT103* gene

Because only 15 axillary buds per genotype were collected for RNA analysis at the time samples used for the quantitative analysis of IAA were harvested for the second experiment in Chapter 3, most of the sample groups (of 15 buds) had less than 20mg of dry weight available for RNA extractions. This made it very difficult to extract RNA in quantities sufficient for quality Northern blot analysis. In some instances, no RNA was detected in the samples, while in other instances, the concentration of total RNA was so low that it required too large a volume for them to be loaded in the gel. Therefore, I was not able to detect expression of *pCNT103* in all samples. The expression of *pCNT103* gene prior to, and at various times after decapitation is shown in Fig 2.14. The attempt to interpret the results from the RNA analysis, however, was unsuccessful due to lack of
consistency in the expression of the ubiquitin gene as an internal control despite the fact that the total RNA of each sample was equal based on the concentrations calculated from the readings of the spectrophotometer. Therefore, the study needs to be repeated and no further description of the results are reported.
Fig 2.14. The expression of \textit{pCNT103} gene prior to, and various times after decapitation.
DISCUSSION

Excised shoot tips of TobAnPOD underproducers tended to generate a greater number of adventitious roots. One explanation of this observation is that there is a lower rate of IAA catabolism, and hence, higher endogenous IAA levels in the TobAnPOD underproducing plants stimulate root formation. Moreover, the significant decrease in lateral root formation observed in peroxidase overproducing seedlings (xan507) when compared to the wild type seedlings is consistent with this. Studies conducted by Lagrimini et al. (1997) to investigate the effects of peroxidase activity on root growth and development revealed a significant decrease in root mass of tobacco plants overexpressing peroxidase which is consistent with the results shown in Figs 2.1 and 2.3. One possible explanation is that different levels of TobAnPOD result in altered levels of endogenous IAA, which in turn, led to the differences in the number of adventitious roots observed on the explants.

However, xan427 shoot explants theoretically should have more endogenous IAA as result of the suppressed expression of the TobAnPOD gene had significantly longer roots than xanWT and xan507 plants. This is inconsistent with the conventional theory of auxin inhibition of root elongation. The reasons why xan427 shoots generated significantly longer adventitious roots than the xanWT plants are not clear. Nonetheless, possible explanations for the increased elongation rate observed in the xan427 and syl427 explants include: 1) elevated auxin stimulates cell division which in turn have increased cell numbers and thus increases root growth; and 2) elevated IAA level has been known
to activate elongation in the distal elongation zone (DEZ) and this could lead to overall increase in rate.

One can also speculate on the possibility that xan427 plants may be less sensitive to auxin than the other two genotypes. This insensitivity to auxin might have enabled xan427 plants to better adapt themselves to inhibitory levels of auxins and stimulated root elongation. However, this explanation conflicts with the results shown in another root growth study in which case xan427 roots appear to be highly sensitive to auxin in short term tests (as seen in Fig. 2.10). Since I did not conduct a study to compare the long-term adaptation of roots of these three types, it is difficult to predict how would xan427 roots behave in high auxin environment long-term than xanWT roots. Nevertheless, roots are known to adapt to elevated auxin levels in long term growth (Gougler and Evans, 1981). In other words, the ability for xan427 roots to adapt to inhibitory levels of auxins over a long period of time and to use them to stimulate cell division and expansion may be one of the reasons why they had promoted root growth instead of the inhibited growth as hypothesized.

Another possibility could be that the IAA levels in xan427 roots were not elevated as originally hypothesized. In fact, there could be much less IAAs in xan427 roots and therefore they had greater root growth as lower IAA levels are known to stimulate elongation. This speculation would be further examined in the quantitative studies in Chapter 3. It is also possible that the increased elongation rate seen in underproducer roots were result of other complex physiological processes that had little to do with
altered auxin levels by the TobAnPOD. Therefore, the simple peroxidase and IAA catabolism model may not be enough to explain in these situations.

Although changes in hypocotyl elongation were often observed in seedlings that were genetically engineered to under- and over-produce the TobAnPOD (Grambow and Langenbock-Schwich, 1983; Lagrimini et al., 1997), no differences in the average hypocotyl length among xan427, xanWT and xan507 were observed (Fig. 2.5). This is inconsistent with the results published by some studies in which they found that auxin is able to increase hypocotyl elongation in plants with reduced free IAA levels or less sensitive to exogenous auxin (Collett et al., 2000). However, their findings are consistent with the syl507 hypocotyl growth data (Fig. 2.6). Perhaps IAA is not the only factor in controlling hypocotyl growth. Auxins appears to stimulate hypocotyl growth in some studies and to inhibit in others (Collett et al., 2000; Jensen et al., 1998; Smalle et al., 1997). There have been studies suggested that auxin may not act alone in the control of stem elongation and that it was controlled by the interactions between auxins, ethylene and gibberellins (Collett et al., 2000; Yang et al., 1996). It is also possible that peroxidase does not regulate IAA catabolism in young seedlings as hypothesized.

However, the findings in whole plant growth study (as shown in Fig. 2.7) are consistent with the conventional IAA theory (Evans, 1985) and with my hypothesis that peroxidase can metabolite IAAs in vivo. Xan427 plants grew at a faster rate, possibly, as a result of elevated endogenous auxin levels from the underexpression of the tobacco anionic peroxidase activity in the plants. Similarly, growth rates in xan507 were lower as a result of a higher peroxidase activity in the overproducing plants.
From the physiological point of view, axillary bud development is usually suppressed in the presence of the growing apical bud. According to the most widely accepted theory (Morris, 1977; Thimann and Skoog, 1934; Thimann, 1977), removal of the apical bud from the shoot tip often leads to rapid growth of axillary buds as a result of less auxin transported from the tip. My hypothesis predicts a stronger apical dominance and thus less lateral bud outgrowth in the TobAnPOD underproducing plants with elevated IAA levels. Although differences in axillary bud growth among genotypes were observed, it is not appropriate to use this data alone to correlate peroxidase and axillary bud growth after the release of the apical dominance because some lateral buds in xan427 plants were already growing even before removal of the apex. The outgrowth of the top two axillary buds observed in xan427 plants even before decapitation is inconsistent with my original hypothesis and with the conventional theory which holds that the high concentrations of auxin produced in the apex inhibits the growth of the neighboring axillary buds (Bangerth, 1989; Gruber and Bangerth, 1990; Thimann and Skoog, 1934). It may be better to compare growth rates of axillary buds in xanWT and transgenic lines (xan427 and xan507) over time to better understand the effects of decapitation on lateral shoot growth of plants with altered peroxidase activity. The results shown in Fig. 2.9 indicate that average axillary bud growth rates in xanWT plants were significantly lower than that of xan427 plants. Therefore, apical dominance was less obvious in xan427 than in xanWT.

A great difference in sensitivity to exogenous IAA on root growth was observed between xan427 and xanWT seedlings (Figs. 2.10 and 2.11). Xan427 roots were much
more sensitive to exogenous IAA than xanWT roots. The amount of IAA that was needed
to cause inhibition of growth in xanWT roots was almost 1000 times more than that
required for the xan427 roots. Also, xanWT seedlings can take up to 10 times more IAA
than xan427 seedlings before their root growth was completely stopped. Root growth of
xanWT plants was promoted when low concentrations of IAA were added, which is
consistent with results in other published studies. However, that same level of IAA
showed no effects in promoting root elongation in underproducers, indicating that the
availability of endogenous IAA level in xan427 seedlings prior to treatment may have
already been optimal for normal root growth compared to the wild types seedlings.
Therefore, the application of IAA at higher concentration may have adverse effect on root
growth in peroxidase underproducing seedlings. Indirect evidence from a variety of
studies suggests that auxin plays a critical role in cell division and elongation in growing
roots (Hobbie and Estelle, 1995; Pickard, 1985). In other species, very low concentrations
of auxin stimulate root elongation, while higher concentrations invariably inhibit
elongation (Burström, 1969; Evans et al., 1994).

Differences in elongation of roots in xan427, xanWT and xan507 seedlings before
and after gravi-stimulation were observed (Fig 2.12). Following gravitropic stimulation,
roots of xan427 seedlings grew about 6 and 13 times faster than the xanWT and xan507
seedlings, respectively (Fig. 2.12). Root growth of xan507 seedlings was significantly
reduced compared with wild type seedlings and showed almost identical growth rates as
measured before roots were subjected to gravistimulation (Fig. 2.12). This finding is
consistent with the hypothesis that overproduction of TobAnPOD in xan507 plants

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reduced endogenous IAA levels and thus created a suboptimal condition for elongation even after gravistimulation, whereas the decreased endogenous auxin level in the upper side of the seedling in peroxidase underproducing plants after the rotation may help release the inhibition and stimulate growth.

Root gravitropism is thought to involve the effects of auxin on cell elongation. According to the Cholodny-Went hypothesis, differential growth of the upper and lower sides of a gravistimulated roots is caused by an asymmetric auxin concentration across the root (Pickard, 1985). Results obtained from studies on gravitropic responses involving *N. tabacum* plants with altered peroxidase activity demonstrated differences in the angle of curvature under gravistimulation. This correlated well with the findings gathered from similar studies which looked at the effects of auxin on gravitropism involving mutant plants with altered responses to auxin (Evans et al., 1994). However, the gravitropic response mechanisms are rather complex. In an investigation of the effects of auxin on growth and gravitropic responses of maize roots, Ishikawa and Evans (1993) reported that cells of the DEZ in maize root behaved differently than those in the main elongation zone in response to auxin and to gravistimulation and that they may play an important role in gravitropism. They found that 1) upon exposure to exogenous auxin, rapid elongation was resumed (or initiated) in DEZ cells while growth of cells in the rest of the elongation zone remained suppressed by the presence of auxin in the solution; 2) removal of auxin during the period of strong inhibition resulted in rapid root growth which was initiated by the rapid elongation in the DEZ cells along with the recovery in the main elongation zone; and 3) gravistimulation of auxin-inhibited roots stimulated
rapid elongation in DEZ cells on the upper side which in turn resulted in rapid curvature even in roots that showed zero growth before gravistimulation. Their findings were consistent with results reported by other workers which also demonstrated the early involvement of a group of cells in the apical region of elongation zone of the root in the response to gravity and auxin (Burström, 1957; Zieschang and Sievers, 1991).

Those findings may help us explain the faster growth and curvature changes of xan427 roots before and after gravistimulation when compared with that of xanWT and xan507 roots. If tobacco roots behave similarly to maize roots (Ishikawa and Evans, 1993), one may assume that cells in the DEZ of xan427 roots were less sensitive to auxin and therefore adapted to elevated auxin levels than those of xanWT and xan507 roots for greater elongation and growth before stimulation (Fig 2.12). Similarly, stronger gravitropic response observed in xan427 roots could have resulted from enhancement of elongation in the DEZ on the upper side of the roots, coupled with a slower growth along the lower side than that of xanWT and xan507 roots. Although this conflicts with the results shown in Fig. 2.10 because short term responses to IAA indicate that xan427 roots were quite sensitive to sudden step up in IAA, it seems likely that the more rapid long term growth of xan427 roots may result from more long term developmental changes in the root such as increased cell growth throughout the elongation zone. This would be consistent both with high sensitivity to auxin and with more rapid growth and with greater gravitropism, that is, focus on a developmental change in the root rather than just immediate effects of altered auxin level.
Although some of the results shown are consistent with the possible link between peroxidases and auxin metabolism, some however are inconsistent or in contrary with my original hypothesis. Therefore, it is difficult to conclude that peroxidase plays a direct role in \textit{in vivo} oxidation of IAA.
CHAPTER 3

ENDOGENOUS IAA LEVELS IN TRANSGENIC TOBACCO PLANTS WITH ALTERED PEROXIDASE ACTIVITY

INTRODUCTION

It is widely accepted that the plant hormone auxin (indole-3-acetic acid) has effects on almost every aspect of the plant growth and development. Some of the common physiological processes suggested to be regulated by auxin include cell elongation, cell division, stem growth (Davies, 1995), adventitious root initiation, apical dominance (Tamas, 1995) and tropic responses to gravity (Kaufman and Song, 1987).

The growing shoot apex is considered to regulate a wide range of developmental processes in plants, and among them, apical dominance or the suppression of axillary bud growth, is the most significant. Cline (1994) defines apical dominance as the control over the outgrowth of the axillary buds by the apex. The primary source of bud suppression is the growing shoot apex. The more vigorous the growth of the apex, the greater the suppression of the axillary bud growth. There are also indications that the degree of bud growth suppression is under genetic control, and the inhibition may be stronger in one genotype than another (Cline, 1997).
Thimann and Skoog (1934) discovered that decapitation of *Vicia faba* plants led the outgrowth of the axillary buds, but the growth was stopped if the cut surface was treated with auxin. These results have since been confirmed in numerous plant species. However, there have been few analyses of endogenous IAA levels in the lateral buds in relation to apical dominance.

The hypothesis that peroxidase is involved in auxin metabolism is supported by results obtained by several studies conducted by several research groups. Hinman and Lang in 1965 were the first to identify the intermediates of IAA oxidation by horseradish peroxidase. The mechanism of this peroxidase and IAA reaction was later proposed by Richard and Job in 1974. An *in vivo* role of peroxidase in IAA catabolism has been hypothesized, and evidence has accumulated to support this idea. Lagrimini and Gazaryan (1996) found that TobAnPOD was capable of oxidizing IAA without the presence of other cofactors such as hydrogen peroxide or phenols. The oxidation cycle is thought to be functioning separately from peroxidation. It was consistent with the oxidation reaction reported by Smith et al., (1982).

A molecular approach was undertaken by Klotz and Lagrimini (1996) to investigate the expression and regulation of the tobacco anionic peroxidase. The TobAnPOD promoter – GUS gene chimera was transformed into tobacco plants and its pattern of regulation by examining transient expression of the chimeric gene following application of auxins and other chemicals. Positive and negative regulatory responsive regions to auxins were then identified. Auxins were shown to effectively suppress TobAnPOD expression in both transiently transformed tobacco protoplast and in stably
transformed root tissue cultures. However, an inverse relationship between exogenous auxin levels and TobAnPOD expression was observed, suggesting a more complicated interrelationship between the regulation of this gene and auxins. Little or no promoter-driven expression of this gene was detected in young tissues such as shoot apices or root tips, where auxin concentrations are expected to be high. In contrast, the highest expression was observed in mature tissues where the auxin level is thought to be much lower.

Most of the conclusions drawn from these studies into the relationship between peroxidase and IAA oxidation were based on *in vitro* observations. There have been few studies published to date offering strong evidence that the oxidation of IAA by peroxidases occurs *in planta*. Lagrimini and his colleagues (1990) were the first to take a more direct approach by using transgenic tobacco plants to investigate the effects of changes in internal peroxidase activity on auxin-mediated growth and development in intact plants. They found that transgenic tobacco plants, xan507, which overproduce the anionic peroxidase displayed chronic wilting as a result of underdeveloped root mass with fewer root branches (Lagrimini, 1991; Lagrimini et. al., 1997). In addition, their results indicated a relationship between leaf thickness and peroxidase expression. It appeared that xan507 had thinner leaves and smaller and more compact cells, while the transgenic underproducing plants, xan427, displayed thicker and larger, loosely arranged leaf cells (Lagrimini, 1991). They concluded that the developmental changes seen in transgenic plants may be a consequence of the metabolism of IAA by the altered expression of this gene.
Studies conducted to further investigate the effects of this gene on other auxin-mediated physiological responses such as adventitious root initiation, hypocotyl elongation, gravitropism, auxin-mediated gene expression in the release of apical dominance and axillary bud growth following decapitation were described in the previous chapter. The results reported in the previous chapter provided indirect physiological evidence suggesting different phenotypes were the result of altered IAA levels. Nonetheless, quantitative analyses in endogenous IAA levels on buds of all genotypes before and following decapitation may be helpful to further test the hypothesis.

The objective of the present investigation was to further examine the relationship and to clarify the role of auxin in apical dominance by conducting quantitative analysis of endogenous free IAA levels in axillary buds of xanWT and transgenic plants with altered peroxidase activity before and following decapitation. Specifically, I wished to determine whether 1) the outgrowth rates of axillary buds at different positions correlate with changes in their IAA levels and 2) the degree of apical dominance release is different in xanWT than in transgenic plants with altered TobAnPOD activity.
MATERIALS AND METHODS

Plant Material

Plants for IAA extraction were grown from seeds. Seedlings of xan427, xanWT and xan507 were initially grown in tissue culture for four weeks. They were subsequently transferred to 10" pots and grown in greenhouse. Plants were decapitated when flower buds were first seen using razor blade to release apical dominance. The first five axillary buds from the apex of each plant were excised at 0, 1, and 3 days after decapitation and immediately frozen in liquid nitrogen. Buds were lyophilized and stored at -80°C until extraction.

Endogenous IAA Extraction

Approximately 0.02 to 0.3 g of 15 dried axillary buds from plants grown as previously described were ground in a Bio-Homogenizer (Biospec Products, Inc.; Bartlesville, OK) with 5 ml of 80 % acetone. $[^{13}]$IAA (Cambridge Isotope Laboratories; Andover, MA), in a range of 100 – 200 ng as an internal standard was added. The extract was then centrifuged at 10,000 g for 7 min and the supernatant transferred to a clean 50 ml boiling flask. The remaining pellet was extracted twice more with 5 ml of 80% acetone. The supernatants were combined and the acetone was removed under reduced pressure using rotary evaporator (Büchi, Switzerland). Distilled water was added to the aqueous residue to bring the final volume to 15 ml in the flask and the pH was adjusted to 2.5 with 1 N HCl. The aqueous solution was extracted three times with 15 ml of CH$_2$Cl$_2$. The organic phases were combined, 5 ml of water added, and the CH$_2$Cl$_2$ removed under reduced pressure. The aqueous extract was then passed through a
10 ml C-18 column, pre-conditioned with 5 ml of 100% MeOH and 5 ml of water to desalt. The C-18 column was rinsed with 5 ml of distilled water, and the IAA eluted with 5 ml of 50% methanol (MeOH). The acidic MeOH eluate was evaporated to less than 100 µl using a rotatory evaporator. The HPLC procedure was the same as described in Chen et al., (1988) except that initial conditions were 30% MeOH for 1 min followed by a linear increase in the proportion of MeOH to 100% in 25 min. Elution with MeOH continued for 4 min and returned to 30% MeOH in 2 min span. The fractions containing IAA were reduced to near dryness (<200 ul) under reduced pressure at room temperature then to complete dryness using the Speed Vac Concentrator (Savant Instrument, Inc.). The samples were then dissolved in 10 ul MSTFA (N-Methyl-n-trimethylsilyltrifluoroacetamide; Pierce; Rockford, IL) to provide the trimethylsilyl ester-trimethyl silyl amide derivative of IAA.

The methylated samples were dissolved in acetonitrile and an aliquot analyzed by gas-chromatography-mass spectrometry (GC-MS). The GC is a Hewlett-Packard 5890 series II equipped with a split/splitless injector operating at 250°C in splitless mode, and is linked to a HP5972 Mass Selective Detector. The GC is fitted with a BP5 capillary column (30m, 0.25 mm id; 0.25 um film, 5% phenyl-95% dimethyl siloxane; Hewlett-Packard). The temperature program for gas chromatography was 150°C for 1 min, followed by 10 C min⁻¹ to 300° C, which was maintained for 1 min. Free endogenous IAA and the corresponding isotope-labeled [¹³C₆] IAA internal standards were detected and measured by selected ion monitoring (SIM). Ions at m/z 208 and 202 were monitored for the base peak of the [¹³C₆] IAA internal standard and of the plant IAA, respectively.
The ratio of 202:208 was used to calculate the amount of endogenous IAAAs. The amount of free IAA is calculated from the data using the equation shown below:

\[ Y = \left( \frac{C_i}{C_f} - 1 \right) \cdot X \]

where \( Y \) is the amount of the free IAA in the tissue in ng; \( C_i \) is the initial percentage of the internal standard, or 97.3% in this case; \( C_f \) is the final percentage of the \(^{13}\text{C}_6\)IAA, which is obtained by calculating the ratio of areas of m/z 202 over m/z 202 +208; \( X \) is the amount of \(^{13}\text{C}_6\)IAA added in ng.
RESULTS

Endogenous IAA level expressed on an individual bud basis

Although there were total of three experiments performed in this study, only the data set obtained from the second experiment is presented in here. More consistent and higher quality data in determining both internal standard and endogenous IAA levels were obtained in this experiment than the others.

Much lower levels of IAA were observed in lower buds (4th and 5th buds from the apex) of xanWT and xan427 plants before decapitation while only a modest reduction in the IAA levels in comparable buds was found in xan507 plants (Table 3.1). Axillary buds of xan427 plants contained the highest level of IAA prior to excision of the apex whereas the least amount of endogenous IAA was determined in xanWT buds. The IAA concentrations determined from xan427 lateral buds of all positions were at least 30 times greater than those measured in xanWT plants before decapitation (Table 3.1).
Table 3.1. IAA Quantitation in Wild-Type and Transgenic Tobacco Plants before decapitation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Concentration of Endogenous IAA (ng/bud)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xan427</td>
<td>Bud 1^a 396 268 157 46 37</td>
</tr>
<tr>
<td>xanWT</td>
<td>11 8 2 2 *</td>
</tr>
<tr>
<td>xan507</td>
<td>20 15 13 12 12</td>
</tr>
</tbody>
</table>

^a: The location of the axillary bud from the apex.
*: Less than 1 ng of IAA

Although the highest endogenous IAA levels were observed in underproducing plants, an approximately 40% reduction in the concentrations of IAA were determined in xan427 bud 1, 2 and 3 by 24h following decapitation (Table 3.2). Meanwhile, a sharp, two to six fold increase in IAA levels were observed in the first three buds of xanWT plants by 24h after decapitation (Table 3.2). A dramatic drop in IAA levels was found in all xan507 lateral buds. A progressive reduction in the level of IAA relative to the position of the buds from the apex was observed among all genotypes (Table 3.2).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Concentration of Endogenous IAA (ng/bud)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xan427</td>
<td>Bud 1 228, Bud 2 160, Bud 3 113, Bud 4 17, Bud 5 40</td>
</tr>
<tr>
<td>xanWT</td>
<td>Bud 1 67, Bud 2 49, Bud 3 3, Bud 4 5, Bud 5 3</td>
</tr>
<tr>
<td>xan507</td>
<td>Bud 1 5, Bud 2 2, Bud 3 *</td>
</tr>
</tbody>
</table>

a: The location of the axillary bud from the apex.
*: Less than 1 ng of IAA

Table 3.2. IAA Quantitation in Wild-Type and Transgenic Tobacco Plants 24 Hrs after Decapitation

A further 80 to 90% decrease in IAA levels was observed in buds 1, 2 and 3 of xan427 plants between 24 and 72h following decapitation, whereas a 50% drop was found in the fourth and the fifth buds (Table 3.3) during the same period. Similarly, a 40% decrease between 24h and 72h after decapitation in concentrations of IAA in the first and second axillary buds of xanWT plants was observed (Table 3.3). No clear pattern in IAA levels in bud 1 through 5 of xan507 plants during the same time frame was observed (Table 3.3). A similar phenomenon was observed 72h following decapitation in which IAA concentration decreased as the buds were located further away from the apex. This was true for all three genotypes.
Table 3.3. IAA Quantitation in Wild-Type and Transgenic Tobacco Plants 72 Hrs after Decapitation

Endogenous IAA levels on a unit dry weight basis

Before decapitation, xan427 buds contained higher levels of IAA per mg dry weight than comparable buds of xanWT and xan507 plants; this is consistent with previous data expressed on a per bud basis (Fig. 3.1). In addition, the levels of IAA found in the first axillary bud of each genotype before decapitation were the highest with a progressive decline in IAA levels in buds further away from the apex (Fig. 3.1).

Significantly lower levels of IAA were found in the first two buds of xan427 and xan507 plants by 24h following decapitation. An even larger reduction was observed between 24 and 72h after decapitation on xan427 and xan507 lines (Fig. 3.1). It is
interesting to point that changes in the IAA distribution patterns in xanWT plants on weight basis is almost identical to those found on a per bud basis throughout the entire course of study (Table 3.1, 3.2, 3.3 and Fig. 3.1) with the exception of second bud which exhibited a slight increase, rather than a reduction, in IAA level on weight basis by 72h following decapitation (Fig. 3.1).

**IAA concentrations in young seedlings**

The concentration of IAA was the highest in the shoot of xan427 19-day seedlings whereas no significant difference in IAA levels was found in shoot of wild type and xan507 seedlings (Table 3.4). On the other hand, xan507 roots contained the greatest amount of IAA while no significant difference in IAA levels was observed in root of xanWT and xan427 seedlings (Table 3.5).
Fig 3.1. Effects of Decapitation on Lateral Bud IAA Levels Expressed on a Per Mg Dry Weight Bases in Transgenic Tobacco Plants with Altered Peroxidase Activity.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total IAA (ng)</th>
<th>Shoot Dry Wt. (mg)</th>
<th>IAA (ng mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First experiment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xan 427</td>
<td>210</td>
<td>135</td>
<td>1.6</td>
</tr>
<tr>
<td>xan WT</td>
<td>31</td>
<td>177</td>
<td>0.2</td>
</tr>
<tr>
<td>xan 507</td>
<td>32</td>
<td>144</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Second experiment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xan 427</td>
<td>1894</td>
<td>449</td>
<td>4.2</td>
</tr>
<tr>
<td>xan WT</td>
<td>454</td>
<td>420</td>
<td>1.1</td>
</tr>
<tr>
<td>xan 507</td>
<td>225</td>
<td>230</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Third experiment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xan 427</td>
<td>1996</td>
<td>357</td>
<td>5.6</td>
</tr>
<tr>
<td>xan WT</td>
<td>367</td>
<td>311</td>
<td>1.2</td>
</tr>
<tr>
<td>xan 507</td>
<td>267</td>
<td>290</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Table 3.4.** Concentrations of IAA in shoots of 19 d old seedlings of xan427, xanWT and xan507 plants. Seeds of three genotypes were germinated and grown in OMS medium. Shoots of 19 d old seedlings were harvested and freeze-dried. Endogenous IAA levels were measured using GC-MS-SIM.
Table 3.5. Concentrations of IAA in roots of 19 d old seedlings of xan427, xanWT and xan507 plants. Seeds of three genotypes were germinated and grown in OMS medium. Roots of 19 d old seedlings were harvested and freeze-dried. Endogenous IAA levels were measured using GC-MS-SIM.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total IAA (ng)</th>
<th>Root Dry Wt. (mg)</th>
<th>IAA (ng mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xan 427</td>
<td>33</td>
<td>78</td>
<td>0.4</td>
</tr>
<tr>
<td>xan WT</td>
<td>27</td>
<td>94</td>
<td>0.3</td>
</tr>
<tr>
<td>xan 507</td>
<td>159</td>
<td>93</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*First experiment*

| xan 427  | 349           | 310              | 1.1           |
| xan WT   | 683           | 418              | 1.6           |
| xan 507  | 2436          | 368              | 6.6           |

*Second experiment*

| xan 427  | 319           | 237              | 1.3           |
| xan WT   | 510           | 315              | 1.6           |
| xan 507  | 1629          | 212              | 7.7           |

*Third experiment*
Discussion

A study of the effects of the tobacco anionic peroxidase on plant growth and development through its involvement with \textit{in vivo} IAA metabolism was undertaken to better characterize its potential role in IAA metabolism.

Although more IAAs found in top 3 buds of xan427 plants before decapitation (Table 3.1) is consistent with the hypothesis, their extremely weak apical dominance as indicated in Figs. 2.8 and 2.9 is in sharp contrary to the IAA theory. A large decrease in IAA content was observed in axillary buds of xan427 plants 24h after the decapitation (Table 3.2). Subsequent reduction in IAA concentrations was again prominently displayed on xan427 buds 1 to 3, by 72h after decapitation (Table 3.3). The reduction in IAA content found in xan427 buds may help to explain the increased bud growth shortly following decapitation (Figs. 2.9) as some similar studies suggested (Blazkova et. al., 1999; Chen et. al., 1997). It is difficult, however, to explain why xan427 buds started to elongate before removal of the apex and continued to grow at a faster rate three days after decapitation when their IAA levels dropped even further from day 0. One explanation could be that IAA does not play as a significant role in apical dominance as believed. There may be some other factors that help control apical dominance and determine when to trigger the outgrowth of axillary buds. The other explanation could be that the initial release of IAA suppression from the apex and the initial changes in sensitivity to endogenous IAA few hours following decapitation were the most crucial for their outgrowth. Growth may be initiated by an increase in cell division and some cell elongation. Once the optimal cell numbers are set, buds continue to grow primarily
through cell enlargement and therefore less needs for continuous elevated levels of IAA. There was much less endogenous IAA in intact wild type plants by the same time following decapitation. However, contrary to what the IAA content was detected by 24h following decapitation in xan427 buds, the concentrations of IAA in the first three buds of xanWT plants had actually risen 4-5 fold before decreasing to close to the original level before excision. Xan507 buds of all positions had the least amount of IAAs before and after decapitation.

This is consistent with the physiological processes shown in Figs. 2.8 and 2.9. By 72h following decapitation, the average weight of first two lateral buds of xan427 plants had increased more than 10-fold and the buds had increased in length by about 2.3 fold in length. Bud weight gain was less significant in wild type plants (about 3 times since day 0) and changes in bud weight on xan507 plants were minimal. It was also quite consistent with our daily observation in the greenhouse that intact xan427 plants grew the tallest, followed by the wild type plants whereas xan507 plants were the shortest (Fig. 2.7).

Quantitative analysis in 19 d seedlings revealed that IAA levels were high in shoots and low in roots of xan427 plants, but reversed in xan507 seedlings (Tables 3.4, 3.5). These results, in part, contradict my hypothesis that the levels of auxin should be high in both shoots and roots of xan427 plants. Likewise, less IAAs should be found in xan507 shoots as well as roots. There could be pH or structural changes in cells of transgenic seedlings as results of altered TobAnPOD activity that lead to more transport or accumulations of IAA in xan507 roots than in the underproducers. IAA levels are distributed more evenly in shoots and roots of xanWT seedlings (Tables 3.4, 3.5). There
may be other peroxidase isoenzymes that are capable of degrading auxins in roots when the availability of the TobAnPOD is scarce such as in xan427 root tissues. It is also possible that peroxidase has little effects in IAA metabolism and that other factors such as hormone interactions dictates auxin synthesis and degradation.

Quantitative analysis on these seedlings also found that xanWT and xan427 roots have almost the same amount of IAA (Table 3.5). This is inconsistent with the results shown in Figs. 2.1 and 2.2 in which more adventitious roots and longer roots were observed in xan427 shoot cuttings. These inconsistent findings may be explained by changes in the activity of cell wall cross-linking or lignification as a result of altered peroxidase activity in transgenic plants. There have studies suggest that TobAnPOD is responsible for lignification and is involved in cell wall strengthening through cross-linking (Fry, 1986; Graham and Graham, 1991; Klotz, 1995). Therefore, even though xan427 roots have same amount of IAAs as in xanWT roots, a decrease in cross-linking in cell wall and lignification processes in xan427 roots may help promote their growth and development.

When looking at changes in inter-bud IAA levels of the same genotype pre- and post-decapitation, a gradual descending pattern in IAA distribution from the first to the fifth bud was displayed in both intact and decapitated xan427 plants. Similar pattern pre- and post-decapitation was observed in comparable buds of wild type plants. No significant changes in IAA levels in xan507 buds of different positions was observed. IAA concentrations remained low and relatively unchanged prior to, and after, the release of apical dominance.
Studies on differential behavior of lateral buds due to their position had been investigated and confirmed in some studies (Blazkova et al., 1999), our results added additional support to the theory. A strong positional effects on endogenous IAA levels within the buds of the same genotype was observed. Our results also suggest that the growth rates of the lateral buds were affected by their genetic composition (Fig. 2.9).

Therefore, we can conclude that it is unlikely that peroxidase plays a major role in \textit{in vivo} oxidation of IAA. The interrelationship between the two along with the subsequent biological responses that were being brought out during the process may be very complex and further investigation is needed.
CHAPTER 4

CONCLUSION

Peroxidases (EC 1.11.1.7) have been implicated in several physiological processes including auxin metabolism (Grambow and Langenbook-Schwich, 1983), lignification (Müder and Füssl, 1982), wound healing and pathogen defense (Espelic et al., 1986; Lagrimini, 1991; Lagrimini et al., 1993). However, it has been difficult to directly determine the role peroxidases play in these important physiological processes. By utilizing transgenic plants with specific overexpression or suppression of individual peroxidase isoenzyme it may be possible to elucidate and compare the effects of these enzymes on auxin metabolism by looking at differences in auxin-mediated physiological responses instead of through pure biochemical reactions in vitro.

Since peroxidase has been shown to metabolize auxins in vitro, it is reasonable to hypothesize that peroxidase can regulate IAA levels in vivo. Studies into the effects of altered TobAnPOD activity on: 1) auxin-mediated growth and development; 2) free endogenous IAA levels in axillary buds, young root and shoot tissues; and 3) expression of an auxin-inducible gene in xanWT and transgenic plants were undertaken to provide clues to its function in IAA metabolism and to test the validity of this hypothesis. If the hypothesis is true, the transgenic plants should have similar phenotypes as those normal
plants treated with exogenous auxin. In other words, transgenic plants with elevated auxin levels as result of the suppression of the TobAnPOD gene should have more adventitious root formation, inhibited root growth and stronger apical dominance as those in normal plants treated with exogenous auxins. Also, results of the quantitative analysis to measure free endogenous IAA levels should be consistent with our prediction which assumes the underproducers have more IAAAs than the wild type plants and the overproducers have the least amount of free IAAAs.

My results showed that the physiological data of plants with altered peroxidase activity were, in general, consistent with the IAA theory. The underproducer shoot tip explants had more adventitious root formation (Figs. 2.1, 2.3). Tobacco plants under-producing the TobAnPOD grew the tallest, while the overproducers were the shortest (Fig. 2.7). The quantitative data showing xan427 shoots contained the most IAAs (Table 3.4) is also in agreement with the IAA theory and my original hypothesis. Although greater curvature changes after gravi-stimulation were expected in xan427 roots (Fig. 2.13), the presence of low IAA levels in the roots (Table 3.5) were not.

Increased elongation in roots of xan427 and syl427 shoot tip explants is in contrary with the IAA theory and the original hypothesis (Figs. 2.2, 2.4). Quantitative data revealed that much lower IAAs were in these roots than hypothesized (Table 3.5), suggesting that peroxidase may not play a direct role in IAA metabolism in roots as expected. Also, the increase in IAA levels as a result of the overexpression of the TobAnPOD gene in the transgenic plants did not result in the strong suppression in the outgrowth of the lateral buds (Figs. 2.8 and 2.9). In contrary, the outgrowth of the lateral
buds were stimulated in the underproducer plants suggesting that IAA is not be the factor that suppresses bud growth.

Although the data obtained from various physiological studies had generally provided a good indication that peroxidase may play a role in auxin metabolism, it was not sufficient to directly link their ability to oxidize IAA in vitro. I then decided to examine these plants further to determine whether or not IAA was involved in the process, and thus might have caused these transgenic plants to behave differently in growth than the wild type plants. Two types of studies were conducted. The first one was designed to study changes in concentrations of IAA in the axillary buds of wild type and transgenic plants before and following decapitation, and to correlate them with data obtained from the earlier growth studies performed on the buds of the same plants.

The second one was designed to investigate changes in the expression levels of IAA inducible gene in the lateral buds of xanthi wild type and transgenic plants before and following decapitation, and to associate them with the data obtained from the growth and IAA quantification studies combined to better understand the overall effects of tobacco anionic peroxidases to the endogenous IAA levels and IAA inducible gene expression.

Quantitative IAA analysis in axillary buds of 3 genotypes revealed inconsistent results between IAA and apical dominance, especially in the top 3 buds of xan427 plants before and after decapitation. The concentration of IAA in those buds fell substantially just 24h following decapitation and continued to drop even though the axillary buds had grown much larger by then. However, results from other studies are not consistent with
these results in that a rise in auxin level due to decapitation was often observed (Gocal et al., 1991; Pearce et al., 1995). Nonetheless, a significant difference in IAA levels was observed in transgenic plants, especially in xan427 line when compared to those in wild type plants, indicating that under- and over-expression of the TobAnPOD in transformed plants results in changes in IAA levels in vivo. Changes of IAA levels might have caused differential growth observed in xan427 and xan507 plants upon the release of apical dominance, also as seen in the whole plant growth studies (Fig 2.7).

No conclusion could be drawn in the attempt to compare the differences in the expression level of auxin inducible gene, pCNT103, between the transgenic and wild type plants due to incomplete dataset obtained from one single Northern hybridization analysis. Furthermore, pCNT103 may not be the best target gene to be used in our study to investigate the relationship between peroxidase activity, IAA content, and auxin-inducible gene expression before and after the release of apical dominance since it is primarily expressed in root tips of young seedlings under inductive condition. The expression of this gene remained low and sometimes undetectable in root tips grown with the induction of exogenous auxins. A repeated Northern blot analysis using shoot-tip specific cDNA probes is needed for more conclusive interpretation on the effects of peroxidases in IAA regulation in vitro.

Finally, the results of this research can be summarized as follows: 1) although some physiological data is consistent with my hypothesis, little connection between IAA levels and peroxidase expression was observed in quantitative analysis studies; 2) the effects of peroxidase on IAA metabolism may be greater in shoots than in roots as shown
in Tables 3.4 and 3.5; 3) peroxidase may have an opposite effects on IAA metabolism in young roots which exhibited accelerated elongation rate as result of reduced free endogenous IAA levels as seen in Fig. 2.2 and Table 3.5; and 4) the apical dominance data suggests that IAA may not be the major factor that suppresses axillary bud growth. In fact, it appears to have opposite effects on apical dominance in this study.

In summary, these data showed that TobAnPOD may not be directly involved with IAA catabolism and that trying to explain all growth and developmental processes with this simple hypothesis proved to be insufficient and unsuccessful. It is likely that there are developmental changes or hormone-interaction patterns with complex consequences rendering some interpretations based on simple one-dimensional changes in auxin level an over-simplification as indicated by some of my results.
LITERATURE CITED


