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ABA induction of corn flooding tolerance through root lignification: Physiological study and molecular cloning of a peroxidase gene

Hwang, Shih-Ying, Ph.D.

The Ohio State University, 1993
ABA INDUCTION OF CORN FLOODING TOLERANCE
THROUGH ROOT LIGNIFICATION: PHYSIOLOGICAL STUDY AND
MOLECULAR CLONING OF A PEROXIDASE GENE

Dissertation

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

By

Shih-Ying Hwang, B.S., M.S.

The Ohio State University

1993

Dissertation Committee:
Dr. Tara VanToai
Dr. Steven St. Martin
Dr. Richard Pratt
Dr. Caroline Breitenberger

Approved by
Tara VanToai
Co-Advisor

Co-Advisor

Department of Agronomy
To my Father
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my advisor, Dr. Tara VanToai, for her encouragement, patience, and guidance throughout this study and my graduate program. Thanks go to the members of my advisory committee, Dr. Steven St. Martin, Dr. Richard Pratt, and Dr. Caroline Breitenberger for their comments and suggestions. I would also like to thank Dr. Norman Fausey for his support throughout my graduate program. Assistance from Ms. Ginny Schnipke is also appreciated.

I offer my sincere appreciation to my wife, Shih-Ing Chen, for her support throughout my graduate studies. Also, my daughter Olivia, a joyful company in the final stage of my graduate study.
VITA

June 16, 1958 .......... Born in Taipei, Taiwan, R.O.C.

1982 .................. B.S., National Taiwan University
Department of Agronomy. Taipei, Taiwan, R.O.C.

1989 .................. M.S. Department, The Ohio State
University

1989-1993 .............. Graduate Research Associate,
Agronomy Department, The Ohio
State University

PUBLICATIONS


FIELD OF STUDY

Major Field: Agronomy

Studies in plant molecular biology, Dr. Tara VanToai
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CHAPTER I

INTRODUCTION

Flooding is an important environmental stress affecting agricultural production not only in the humid temperate region of the U.S. but also in many areas around the world. According to Skaggs (1987), about one third of the 321 million acres of the U.S. cropland is prone to flooding. In the midwest, at least 60% of the cropland is classified as too wet for efficient agricultural production (The Soil Survey Group, The Ohio State University). Following heavy rainfalls or furrow irrigation, a large amount of the soil pores of poorly drained soils are saturated with water (Meyers et al., 1987). Organisms quickly consume the dissolved oxygen present in the soil. Since oxygen diffusivity is 2,000 times less in flooded soils than in well drained soils (Armstrong, 1978), soil anaerobiosis occurs rapidly after flooding. Therefore, flooding injury results primarily from the lack of oxygen (i.e. hypoxia or anoxia) (Jackson and Drew, 1984).
Differential Flooding Tolerance in Plants

Plant tolerance to flooding comprises a range of phenotypic expressions from a minimal survival of a few hours of flooding to actual growth under long term stress. Tolerance to short term flooding mostly occurs at the physiological and biochemical levels and is a prerequisite for long term tolerance. Since even hydrophytes do not possess the mechanism to survive complete anoxia, mesophytes that can tolerate flooding must develop mechanisms to transport oxygen from aerial plant parts to the roots (Kawase, 1976). Such mechanisms involve morphological and anatomical changes in the roots and shoots of flooded plants.

In flood susceptible species, root extension and dry-matter accumulation halted immediately upon exposure to anoxia (Jackson and Drew, 1984). Roots of cotton (*Gossypium hirsutum*) and soybean (*Glycine max*), which are not very tolerant to flooding, can survive anaerobiosis only for 0.5 to 14 hours (Huck, 1970; Turner et al., 1983). Corn is more tolerant to flooding and can survive for up to 70 hours (Sachs et al., 1980). Rice (*Oryza sativa*) seminal roots are most tolerant to anoxic stress and can survive as long as 96-120 hours (Bertani et al., 1980). Expansion of coleoptile cells and shoot growth are also observed in flooded

Flooding tolerance also differs among corn and soybean cultivars. For example, Fausey *et al*. (1985) reported that Mo17 corn was much more susceptible to flooding than A632 and H60 corn. Stone (1985) indicated that the soybean cultivar Corsoy was much more tolerant to flooding than Harosoy 63.

**Physiological Responses of Plant Roots to Anaerobic Stress**

Several major physiological changes occur in plant roots in response to anoxic stress:

1. **Anaerobic Respiration**

   Under aerobic conditions, the oxidation of one mole of glucose to \( \text{CO}_2 \) and water yields 38 moles of ATP. The transfer of electrons from cytochrome oxidase to \( \text{O}_2 \) is blocked under anaerobic conditions such that the production of ATP via electron transport system is interrupted. As a consequence, anaerobic metabolism produces only two moles of ATP per mole of glucose consumed (Crawford, 1978). The continuous production of ATP at the substrate level under anaerobiosis requires the presence of NAD'. Under anaerobiosis, the recycling of NAD' from reduced NADH in the
cells is achieved mainly by the enzyme lactate dehydrogenase (LDH) or alcohol dehydrogenase (ADH). These two enzymes convert pyruvate, the product of glycolysis, to lactate and acetaldehyde, respectively. LDH has an alkaline pH optimum, while PDC has a more acid pH optimum (Davies et al., 1974; Leblova et al., 1976). According to Roberts et al. (1984), the cytoplasmic pH of maize under anaerobic conditions is about 0.5 unit lower than the normal cellular pH. Therefore, under anaerobic conditions, PDC is more active than LDH and acetaldehyde is produced which is then converted to ethanol by the enzyme ADH (Crawford, 1978). While ethanol is the main fermentation product in plants (Saglio et al., 1988; Smith and ap Rees, 1979), other products, such as lactate, malate, alanine, and succinate have also been reported to accumulate in anaerobic tissues (Roberts et al., 1984). In rice, the rate of ethanol production is sufficient to generate enough ATP for the growth of rice embryos under anoxia (Pradet et al., 1985).

2. Energy Metabolism

According to Atkinson (1969) the energy status of the cell can be expressed by the ratio [ATP + 0.5 ADP]/ [ATP + ADP + AMP] which is also known as the adenylate energy charge (AEC). AEC is the result of the equilibrium between
ATP-utilizing and ATP-regenerating reactions. Under optimal conditions, tissues maintain a high AEC (0.8-0.9) where most of the adenine nucleotides are in the ATP form. Since a stress can affect either the ATP-utilization and/or ATP-regeneration pathways, AEC is not always correlated with stress. According to Pradet et al. (1985), cold and heat stresses show no effect on AEC in plants because ATP-regeneration and ATP-utilization are affected equally. By contrast, the AEC is sharply reduced under anoxic conditions. Due to the interruption of the oxidative phosphorylation pathway, ATP-regeneration is limited while ATP-utilization is not affected. Tolerance to anoxic stress has been shown to be correlated with high AEC and sustained metabolic activity under anoxic stress (Pradet and Bomsel, 1978; Pradet and Raymond, 1983). After one day of flooding, the AEC of lettuce seeds decreased to 0.25, but the AEC of rice seeds remained as high as 0.8 (Pradet et al., 1985). Embryos and coleoptiles of rice seeds continue to maintain DNA, RNA, and protein synthesis after the onset of flooding (Mocquot et al., 1977 and 1981).
3. Changes in Protein Synthesis

A. Inhibition of Protein Synthesis

Protein synthesis is an energy-dependent process. Environmental stresses, including drought, cold temperature, heat, and anoxia, which interrupt the ATP-regenerating reactions, also inhibit normal protein synthesis (Sachs and Ho, 1986; Rhodes, 1987). Anoxia inhibits the translation of aerobic mRNAs in corn roots (Sachs et al., 1980). However, in vitro translation of mRNAs isolated during the first five hours of anaerobiosis produced both aerobic and anaerobic polypeptides (Sachs et al., 1980). Two dimensional gel electrophoresis of proteins synthesized by aerobic corn roots produces hundred of different polypeptides (Sachs et al., 1980). In contrast, only 22 polypeptides are seen in anaerobic corn roots. Under anoxic conditions, the polyribosomes in soybean roots become disassembled, probably due to a reduction in the AEC (Lin and Key, 1967). Under longer anoxia, when the AEC in rice embryos is reduced to 0.60, normal protein synthesis is interrupted. During the recovery from stress, protein synthesis gradually increases and reaches the maximum level when the AEC is 0.80 (Pradet et al., 1985).
B. Induction of Stress Proteins

Environmental stresses invoke the synthesis of some specific polypeptides, known as stress proteins in plant and animal cells (Webster, 1980). The anaerobic response in corn comprises two phases: "early" and "late" anaerobic protein synthesis. During the first hour of anaerobiosis, four novel polypeptides known as "transitional proteins" are synthesized. After three hours of anoxic treatment, a steady-state synthesis of 22 polypeptides known as "anaerobic proteins (ANPs)" occurs, which lasts until the roots die at about 70 hours (Sachs et al., 1980). The authors also pointed out that about 70% of labeled ³H-leucine was incorporated into these newly synthesized proteins under anaerobic conditions. However, this was only observed in "Berkeley Fast" inbred line. Large differences in the pattern of in vivo protein synthesis were observed in other inbred lines (Freeling and Bennett, 1985).

In wheat, Bertani and Brambilla (1982) observed that ANP production was a function of the O₂ partial pressure. The de novo protein synthesis pattern began to change to ANP production when O₂ partial pressure was at 0.10 atm, and became most pronounced at 0.01 atm. The induction of ANP synthesis results from an increase in steady-state mRNA levels (Gerlach et al., 1982). Both the synthesis of ANP's
and the cessation of aerobic mRNA translation are reversible for up to 60 hours of anaerobiosis in maize roots (Hake et al., 1985).

The enzymes ADH1 and ADH2 are two of the major ANPs in corn roots (Sachs and Freeling, 1978). ADH1 enzyme activity is necessary for seedling survival of anoxia (Schwartz, 1969). It plays a major role in the regeneration of oxidation source (NAD') so that glycolysis can be continued under anoxia. Maize *Adhl* null mutant seedlings died within a few hours of submersion but ADH' wildtype survived (Schwartz, 1969). However, some *Adhl* null mutants able to survive 24 hours of anoxia also exist (Lemke-Keyers and Sachs, 1989). The physiological role of ADH2 is not understood. Other ANP's have been identified as enzymes of the glycolytic and alcohol fermentation pathways: ANP55 is cytosolic glucose phosphate isomerase I (Kelley and Freeling, 1984a). ANP64 is probably pyruvate decarboxylase which catalyzes the first reaction in the alcohol fermentation pathway (Laszlo and St. Lawrence, 1983; Wignarajah and Greenway, 1976). ANP33 and ANP35.5 are fructose-1,6-diphosphate aldolase, the final enzyme in the first stage of glycolysis (Kelley and Freeling, 1984b). Sucrose synthase, responsible for the breakdown of sucrose to UDP-glucose and fructose, was identified by Springer et
al. (1986) as an anaerobic protein. Lactate dehydrogenase (Hoffman et al., 1986) and hydrogenase (Torres et al., 1986) are also inducible by anaerobic stress. Anaerobic stress also induced an increase in activities of neutral proteinases in corn roots. The proteinases may be responsible for the degradation of abnormal proteins produced by the stress (Hwang and VanToai, 1990) that may be toxic to the plants.

**Lignification**

Lignin is an essential component of cell walls in vascular plants. It is a complex polymer consisting of three different monomeric lignols, coumaryl, coniferyl, or sinapyl alcohol, depending on the plant species (Sarkanen and Ludwig, 1971).

The biosynthesis of lignin is a free radical polymerization process which involves three steps (Hwang et al., 1991). 1) initiation: the formation of a monomeric radical by the removal of a phenolic hydrogen catalyzed by peroxidase; 2) propagation: the combination of a free monomeric radical with a monomer or x-mer; 3) termination: the polymer radical reacts with a hydroxyl radical to form the stable polymer. Using mass spectrophotometry to analyze the intermediates of different in vitro lignin biosynthesis
reactions, Hwang et al. (1991), provided evidence to indicate that the main primer for the initiation of monomer radicals is a hydrogen peroxide-peroxidase. The reduction of oxygen peroxidases to produce hydrogen peroxide-peroxidase is catalyzed by an NADH-dependent dehydrogenase. Thus, the mechanism of lignification depends not only on a peroxidase enzyme, but also on a NADH-depending dehydrogenase. The involvement of dehydrogenase, possibly alcohol dehydrogenase, in lignification was also reported by Keevers and Gaspar (1985) and Mader et al. (1980).

The main function of lignin in the xylem and sclerenchyma is to provide mechanical strength and rigidity for the plants (Schuch et al., 1990). In addition, lignin also plays a role in the reduction of water and oxygen permeability through cell walls (Schuch et al., 1990). Lignification has been suggested as a mechanism of fungal disease resistance. Lignified tissues provide an effective barrier against the invasion of pathogens (Ride, 1983). In the pathogenic resistance response, lignin or lignin-like substances are synthesized in the epidermis or parenchyma cells which are not typically lignified (Schuch et al., 1990).
**Peroxidase**

The primary function of peroxidase is to oxidize molecules at the expense of hydrogen peroxide. Linossier was the first to isolate an enzyme from pus and coined the name peroxidase (Gaspar et al., 1982). Multiple functions of peroxidase have been known for many years. The occurrence of isoperoxidases has been under extensive investigation by gel electrophoresis (Hunter and Market, 1957). The purification of isoperoxidases from different plant materials showed they do not differ significantly in size (from 40 to 50 kDa), absorption spectrum, and activity (Gaspar et al., 1982).

Peroxidase is a glycoprotein which contains a brown-red ferriporphyrin. The amino acid sequences of many peroxidases have been characterized: horseradish (*Armoracia rusticana*) peroxidase (Shannon et al., 1966), two Japanese radish (*Rapharus sativus*) peroxidases (Shimizu and Morita, 1966), two wheat peroxidases (Jeanjean et al., 1975), five peroxidases from turnip and horseradish (Welinder and Mazza, 1975) and eight isoperoxidases of tobacco (Kim et al., 1980). Horseradish peroxidase C has been shown to be quantitatively dominant to other horseradish isoperoxidases. Structural analysis showed that this peroxidase consists of a single polypeptide chain of 308 amino acid residues, including four disulfide bridges. It also contains a heme
prosthetic group, two Ca$^{2+}$ and eight neutral carbohydrate side chains (Welinder, 1979). The calcium is significantly important to the properties and activities of isoperoxidases (Haschke and Friedhoff, 1978; Ogawa et al., 1979). It was evidenced that calcium can maintain the conformation of isoperoxidases and the removal of Ca$^{2+}$ by guanidine hydrochloride and EDTA inactivates the enzyme (Haschke and Friedhoff, 1978).

1. Peroxidase and Respiration

Peroxidase has been shown to possess certain oxidative activities (Nicholls, 1965; Aylward and Haisman, 1969). It provides an alternative pathway for the oxidation of NADH (Rubin and Ivanova, 1963). According to Erecinska et al. (1973), electron transport can be carried out through mitochondrial peroxidase without coupling with the third site of oxidative phosphorylation. Rich et al. (1976) found significant peroxidase activity and hydrogen donor ability in the mitochondrial fraction of mung bean hypocotyls. Peroxidase activity in germinating rice seeds was well correlated to the respiration rate and was considered to be the respiratory mechanism of anoxic rice seedlings (Paul and Mukherji, 1972). Recently, a peroxidase has also been shown to be involved in an oxygen uptake process which generates
NAD$^+$ in *Brachypodium pinnatum* (Van der Werf et al., 1991). This report also strongly supports the alternative pathway which carried out by the oxidase competence of peroxidase.

The involvement of peroxidase in the mitochondrial alternative respiration pathway and its relationship to the anoxic tolerance response in plants remains speculative (Drew, 1990). According to Lambers (1980 and 1982), this pathway functions to consume excess carbohydrate: an obviously wasteful respiration process that generates little ATP. More recently, the alternative pathway has been viewed as a transient response which supplements the cytochrome pathway when the demand for energy is high (Bingham and Farrar, 1988).

2. Peroxidase and Lignin Formation and Their Relationships to Anaerobiosis Tolerance

Stress conditions alter peroxidase activity and its isozyme pattern. Peroxidase activity increases in response to stresses such as ozone (Castillo and Greppin, 1986; Castillo et al., 1984), pollution (Heath, 1980), radiation (Bednar et al., 1976), nutritional disorder (Leidi et al., 1987), wounding (Lagrimini and Rothstein, 1987; Espelie et al., 1986), infections (Lagrimini and Rothstein, 1987;
Parent et al., 1985), salinity (Chang et al., 1981), aging (Hazell and Murray, 1982), cold (Gerloff et al., 1967), and anaerobiosis (Siegel et al., 1966). The increase in peroxidase activity in stressed plants is correlated with lignin formation (Campa, 1990).

The peroxidase from different plant tissues can be separated by electrophoresis, ion exchange chromatography, or isoelectric focusing into either basic or acidic groups. These basic and acidic peroxidases are involved in many growth and developmental processes such as root formation (Gaspar, 1981; Druart et al., 1982), flower initiation (Gaspar and Greppin, 1975), abscission (Gaspar et al., 1978), thigmomorphogenesis (Boyer et al., 1983), and vitrification (Kevers et al., 1984) as well as responses to wounding (Desbiez et al., 1981) and to polluting agent (Castillo et al., 1984). Gaspar et al. (1985) proposed a two-step response of basic and acidic peroxidases to environmental stresses. Environmental stress serves as a stimulant which triggers the induction of basic peroxidase activity. The induced basic peroxidase catalyzes the synthesis of ethylene and the release of Ca$^{2+}$ from the Golgi body or endoplasmic reticulum to the cytosol. The increase in calcium concentration, in turn, induces or
activates the acidic peroxidases, which then move to the cell walls to catalyze the lignification reaction.

The reduced elongation of *Bryonia dioica* is due to the changes in cell wall mechanical properties resulting from accelerated lignification (DeJaegher et al., 1985). Basic isoperoxidase activity was shown to increase within minutes after stress treatment and was followed by an increase in acidic peroxidase after several hours of stress. There is also evidence which suggests that basic peroxidases serve as IAA oxidases when they are present in the cell wall.

When carnation tissues are exposed to excess water, they become succulent and clear, a phenomenon known as vitrification (Kevers et al., 1984). Vitrification apparently results from a deficiency in lignification and is correlated with an initial increase in basic peroxidase followed by a decrease in acidic peroxidase (Kevers and Gaspar, 1985).

Plant roots lacking the flooding tolerance response appear vitrified (i.e. soaking and clear) soon after the stress (Reid and Bradford, 1984; Hook and Brown, 1973) and die if the stress persists. It remains to be demonstrated if root thigmomorphogenesis is related to the flooding tolerance response. Protein synthesis inhibitors do not suppress the rapid increase in activity of basic peroxidases
and the incorporation of labeled protein precursors (Castillo, 1984). The rapid increase in basic peroxidase activity induced by the stress is, therefore, probably not a result of de novo protein synthesis but due to the activation of existing enzymes in the tissue. In contrast to the basic peroxidases, the incorporation of labeled amino acids in acidic peroxidase is very active in response to stress (Castillo, 1984). The induction of acidic peroxidase by stress and its role in the lignification process is therefore genetically regulated and may be related to the anaerobic tolerance response.

**Peroxidase Genes in Plant Growth and Development**

Peroxidase is involved in many biological processes in plants, including respiration, IAA oxidation (Schneider and Wightman, 1974), lignification and stress tolerance (Van Huystee, 1990). The isozyme pattern resolved by electrophoresis is inadequate to determine the function of the different isoperoxidases. Molecular cloning of the different peroxidase genes and their use in transformation experiments may provide an understanding of the function of the individual isoperoxidase. These isoperoxidases may respond differently in such ways as tissue-specificity, developmental regulation, and modulation by environmental
stress. The nucleotide sequences of many plant cDNA peroxidase clones have been characterized including a cDNA clone for the acidic, lignin-forming peroxidase from tobacco (Nicotiana tobacum) (Lagrimini et al., 1987), a cDNA clone from suberizing potato (Solanum tuberosum) tubers (Roberts et al., 1988), a gene for suberization-associated peroxidase in tomato (Lycopersicon esculentum) (Roberts and Kolattukudy, 1989), two putative ethylene-induced peroxidases cDNA clones from cucumber (Cucumis sativus) (Morgens et al., 1990), two cationic peroxidase cDNA clones from cultured peanut (Arachis hypogaea) cells (Buffard et al., 1990), two genomic DNAs of horseradish peroxidase genes (Fujiyama et al., 1990), two genomic DNAs encoding peroxidase of Arabidopsis thaliana (Intapruk et al., 1991), one cDNA clone encoding barley (Hordeum vulgare) endosperm-specific peroxidase (Rasmussen et al., 1991), one peroxidase gene was cloned from wheat (Triticum aestivum L.) that was pathogenically inducible (Rebmann et al., 1991), one pathogen-induced cDNA clone of peroxidase from rice (Reimmann et al., 1992). More recently, one barley peroxidase genomic DNA has been characterized and the chromosomal localization has been mapped (Theilade and Rasmussen, 1992). Amino acid deduced from cDNA clones indicated that a stretch of amino acid (Arg-Leu-His-Phe-
His-Asp-Cys-Phe-Val) is conserved in all of these peroxidase cDNA clones.

**Involvement of Phytohormones in Anoxic Stress**

Changes in metabolic and physiological conditions in plants under anoxia have been shown to be regulated by gene expression (Sachs and Ho, 1986), which, in turn, is mediated probably by certain translocatable substance(s) (Hanson and Hitz, 1982). In response to anaerobiosis, the concentrations of ABA (Zhang and Davies, 1987) and of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Bradford and Yang, 1980) increased, while the concentrations of auxin, gibberellins and cytokinins declined (Reid and Bradford, 1984). The mechanism which regulates these changes remains mostly unclear, except in the case of ACC, whose biosynthesis has been shown to require oxygen. The role of phytohormones in the anoxic tolerance response also remains to be elucidated.

Jackson and Campbell (1975a) observed a higher level of ethylene in flooded plants than in control plants. Ethylene, however, could have been originated from exogenous microbial sources rather than produced endogenously in flooded plants (Jackson and Campbell, 1975b). According to Kawase (1976), ethylene was synthesized by submerged roots and transported to the shoots through the intercellular spaces. However,
since the synthesis of ethylene requires oxygen, which is limited under flooded conditions, the large accumulation of ethylene levels in the shoots cannot be explained by the passive transport of oxygen from aerial parts to the roots (Bradford and Dilley, 1978; Bradford et al., 1982).

In 1979, Adams and Yang identified the ethylene biosynthesis pathway from methionine to ethylene. Two of the intermediates in that pathway are S-adenosylmethionine (SAM) and ACC. The conversion of ACC, the immediate precursor of ethylene, to ethylene requires oxygen. ACC is accumulated in anaerobic apple tissues and is rapidly converted to ethylene when the tissues are returned to air. According to Jackson et al., (1978) while anoxia inhibits ethylene production in tomato roots, ethylene is produced when the roots are returned to air. Thus, ACC accumulates in flooded roots and is transported to the shoots in the transpiration stream. Although the pathway of ethylene formation in anoxic plants is clear, the function of ethylene in anaerobiosis tolerance is not fully understood.

Abscisic acid (ABA) has been proposed as a common mediator of stress tolerance in plants (Quarrie and Jones, 1977). There is evidence that ABA plays a role in the acclimation of plants to environmental stresses including cold, drought and salinity (Singh et al., 1987; Mohapatra et
al., 1988; Davies et al., 1980). However, the role of ABA in the anoxic stress tolerance response has not been reported.

The evidence has shown that the synthesis of ABA in plants is through the "indirect pathway" which produces ABA by the oxidation of epoxy-carotenoids to xanthoxin. Xanthoxin is then metabolized to ABA-aldehyde and which is oxidized to ABA (Zeevaart and Creelman (1988). The viviparous mutants of maize are blocked in the early stages of carotenoid biosynthesis and are ABA deficient (Moore and Smith, 1985; Neill et al., 1986).

The topic of how plant cells detect changes in the external environment and transduce them into a physiological response is a very attractive subject. The mechanism of how cellular events occur in response to ABA is still in the early stage of investigation. The mechanisms of stimulus-response coupling have been extensively studied in animal systems, allowing many of the components of the signal transduction pathway to be identified (Berridge and Irvine, 1989). It has been proposed that Ca$^{2+}$ acts as a second messenger during ABA-stimulated stomatal closure (De Silva et al., 1985). Ca$^{2+}$ influx has been demonstrated in stomatal guard cell (McRobbie, 1989). However, attempts to
determine whether ABA stimulates a change in $[Ca^{2+}]_{cyt}$ have proved inconclusive (MacRobbie, 1989).

The concentration of ABA has been shown to increase during environmental stresses (Bray, 1989). Gene expression has been shown to be inducible by ABA treatment (Singh et al., 1987; Mohapatra et al., 1988; Davies et al., 1980). Some genes that expressed under those stress conditions are also inducible by ABA treatment. It suggests that ABA may induce some gene expression that are required for the plants to be tolerant under stress.

This research investigates the effects of ABA treatment on the anoxic tolerance of corn seedlings.

The objectives of this study were:

1. To demonstrate that exogenous ABA treatment increases the tolerance of corn seedlings to anaerobiosis.

2. To examine the de novo protein synthesis in corn roots induced by ABA treatment.

3. To show that ABA treatment induces lignification of corn roots.

4. To clone and sequence a peroxidase gene which might be involved in root lignification and the anaerobic stress tolerance response.

The information obtained from this research will provide the foundation for future studies of:
1. The role of calcium in the activation/induction of peroxidase genes.

2. The sequence of the promoter and intron regions involved in the ABA response process.

3. The role of root lignification in the anaerobiosis tolerance responses.
CHAPTER II

ABSCISIC ACID INDUCES ANAEROBIOsis TOLERANCE IN CORN

INTRODUCTION

Anoxic stress caused by excessively wet soil is detrimental to plant growth and productivity. Anoxic corn roots have been shown to synthesize some 20 polypeptides known as "anaerobic proteins" (Sachs et al., 1980). Many of these anaerobic proteins have been identified as enzymes of the glycolytic and alcohol fermentation pathways (Kelley and Freeling, 1984a; Kelley and Freeling, 1984b; LaRosa et al., 1987; Springer et al., 1986). However, the significance of anaerobic proteins in the root's adaptation to anoxic stress remains unclear. ADH1 and ADH2 are two of the anaerobic proteins whose synthesis is induced by anoxic stress (Sachs et al., 1980). According to Crawford (1967), activities of ADH enzymes were much lower in plant species that were tolerant to anoxia than in species that were susceptible.
The author proposed that ethanol was phytotoxic and its accumulation in root tissues was the determinant of anoxic susceptibility. The difference in ADH activities, however, was not observed in flood-tolerant and -susceptible corn genotypes (Lemke-Keyes and Sachs, 1989; VanToai et al., 1985). Schwartz (1969) reported that the expression of the Adhl gene was necessary for corn seedlings to survive anoxic stress. However, according to Lemke-Keyes and Sachs (1989), Adhl null mutants having some flooding tolerance also existed. The inheritance of these mutants indicated the involvement of another gene, different from Adhl, in the mechanism of anoxic tolerance in corn.

Corn roots are more tolerant to anaerobiosis if they are preexposed to a period of hypoxia (Saglio et al., 1988; Johnson et al., 1989). Corn roots which were exposed to a hypoxic pretreatment prior to anoxic stress survived 96 hours of anoxia. However, seedlings with no hypoxic pretreatment lost their viability in less than 24 hours. The authors reported this phenomenon as "acclimation" to anoxic stress.

ABA was first discovered as a naturally occurring growth inhibitor (Creelman, 1989; Pilet and Chanson, 1981) that induced leaf abscission and bud dormancy. As an inhibitory phytohormone, ABA regulates some key biochemical and physiological processes in plants including seed
maturation, embryo dormancy, and stomatal opening. There is evidence that ABA also plays an important role in mediating plant responses to environmental stress (Davies and Mansfield, 1983; Quarrie and Jones, 1977). ABA has been shown to induce plant tolerance to environmental stresses including drought (Hiron and Wright, 1973, Mundy and Chua, 1988), cold (Mohapatra et al., 1988), and salinity (LaRosa et al., 1987).

The level of ABA was reported to increase in flooded bean (Hiron and Wright, 1973) and pea (Pisum sativum) roots (Zhang and Davies, 1987). Boussiba et al. (1975) proposed a hypothesis of cross adaptation ability of ABA imposed on plants. Plant mutants lacking the ability to produce or respond to ABA are far less capable of surviving environmental stress (Zeevaart and Creelman, 1988). It is probable that ABA regulates a mechanism is common to many stresses and promotes stress adaptation. However, the role of ABA in plant tolerance to anoxic stress remains unknown. This study characterized the effects of exogenous ABA on ADH activities and anoxic tolerance in corn.
MATERIALS AND METHODS

Plant Materials

Corn (Zea mays L., inbred line H60) seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 15 min, rinsed with distilled water three times, and allowed to imbibe for 15 min in distilled water. The seeds were then rolled in germination papers (Anchor Paper, St. Paul, MN) soaked with 2 mM captan ([N-trichloromethylthio]-4-cyclohexene-1,2-dicarboximide) and allowed to germinate in the dark at constant temperature (27±1°C) for 3 d. Healthy, unblemished seedlings were sorted for uniformity in size before being used in the experiments.

Treatment with ABA

The aqueous solution of (±) ABA (Sigma Chemical Co., St. Louis, MO) was obtained by adjusting the pH of the suspension to 7.0 with potassium hydroxide. Three-day-old corn seedlings were pretreated with 100 μM ABA or with deionized distilled water in the dark and at room temperature for 24 h. The seedlings were placed in plastic trays (45 x 35 cm) such that only the root tips were submerged in water with or without ABA. In the dose-response experiment, ABA concentrations of 0, 10, 25, 50, 75, and 100 μM were used. In the ABA treatment duration study, the root
tips were treated with 100 μM ABA for 0, 2, 4 ,8 and 12 h. In studies with cycloheximide (CHM), a protein synthesis inhibitor, 5 mg/L CHM was used alone or with ABA. For the no-pretreatment, seedlings were rolled in moist germination papers. After the pretreatment, the roots were rinsed with distilled water before the seedlings were subjected to anoxic stress. Each treatment of 50 seedlings was replicated three times.

Anoxic Treatment

Anoxic stress was imposed in the dark at 27±1°C by complete submersion of 50 seedlings (4-d-old) in closed 950-mL glass jars containing degassed, distilled water for 1, 2, or 3 d. In the aerobic control treatment, the seedlings were rolled in moist germination papers. After the treatment, stressed and control seedlings were planted in vermiculite and grown in the growth chamber at 27±1°C with 16 h of photoperiod at 800 μE m²s⁻¹ light intensity. The appearance of fleshy white roots and green leaves after 7 d of growth was used as the criterion for seedling viability. Each treatment of 50 seedlings was replicated three times.
Extraction and Determination of ABA

ABA was extracted from 4-, 5-, 6-, and 7-day old roots of corn H60 and rice M202 including control roots which were not stressed. Root tissues were frozen in liquid nitrogen and homogenized in a chilled (4°C) mortar with a buffer containing 90% methanol and 10 mg/L butylated hydroxytoluene at the ratio of 20 mL/G tissue. The homogenate was extracted for ABA as described by Raikhel et al. (1987). The suspension was filtered through Whatman No. 1 filter paper. The cleared supernatant was vacuum dried at 30°C and resuspended in 1 mL TBS (25 mM Tris[hydroxymethyl]aminomethane-HCl (Tris-HCl), 100 mM NaCl, 1 mM MgCl₂, 0.1% (w/v) sodium azide, pH 8.5). The whole procedure was carried out in the dark or under dim light.

ABA concentration was determined by ELISA assay using a Phytodetek-ABA kit (Idetek, San Bruno, CA). One hundred μL of ABA standards or samples were added to each antibody-coated well followed by 100 μL of Phytodetek alkaline phosphatase tracer. The plate was covered with a plate sealer and incubated at 4°C for 3 h. At the end of incubation period, the solution was decanted and the wells were washed with 200 μL of wash buffer (0.85% (w/v) NaCl, 0.05% (v/v) polyoxyethylene sorbitan (Tween 20), 0.1% (w/v) sodium azide, pH 7.0) three times. Excess wash buffer was removed by patting the plate on paper towels before 200 μL
of substrate (1 mg/mL p-nitrophenyl phosphate in 9.6% (v/v) diethanolamine, 0.5 mM MgCl₂, pH 9.6) were added to each well. The wells were covered with a plate sealer and incubated at 37°C for 60 min. The reaction was stopped by adding 100 μL 1 N NaOH to each well. After 5 min, the intensity of the color developed was read at 405 nm with an ELISA plate reader.

**Assay of Alcohol Dehydrogenase Activity**

Root tissues (0.2 g) were homogenized in a chilled mortar with 1 mL cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged at 14,000 g and 4°C for 15 min. The soluble protein content in the extract was determined using the BCA (a Trademark of Pierce for protein assays using bicinchomericin acid) protein reagent (Pierce, Rockford, IL) as described by Hwang and VanToai (1990). The specific activity of ADH in the extract was assayed by the procedure of Irish and Schwartz (1969).
RESULTS

Effect of ABA Treatment on Anoxic Tolerance in Corn

Figure 1 shows the dose-response curve of the anoxic survivability of corn seedlings to exogenous ABA. Corn seedlings stressed anoxically for 1 d without any pretreatment showed 8% anoxic survivability when planted in vermiculite. Control seedlings, which were not stressed anoxically, showed 100% survival rate after being transplanted. The survival rate of seedlings pretreated with ABA increased with increasing ABA concentrations. Seedlings pretreated with water had a 47% survival rate, while seedlings pretreated with 100 μM ABA showed an 87% survival rate. In addition, the surviving seedlings that were pretreated with ABA grew taller and more normal (surviving seedlings of other treatments showed wrinkled leaf after stress) than seedlings that received no pretreatment or were pretreated with water only (Figure 2). When corn seedlings were treated with 100 μM ABA for 2, 4, 8, 12 h and stressed anoxically for 1 d, their survivability increased progressively with the duration of the pretreatment (Figure 3). Since pretreatment with 100 μM ABA for 24 h allowed optimal seedling survivability, this treatment was used throughout the study.
Effects of ABA Treatment on Anoxic Tolerance in Corn and Rice

In this experiment, corn and rice seedlings were pretreated with 0 and 100 μM of ABA or 100 μM ABA together with 5 mg/L cycloheximide for 24 hours before being stressed in degassed, distilled water for 1, 2, and 3 days (Figures 4 and 5). While 8% of the corn seedlings with no pretreatment survived one day of stress, acclimation by pretreatment with water increased the survival rate to 47%. However, ABA pretreatment increase the survival rate to 87%. When cycloheximide (a protein synthesis inhibitor used to show that the anoxic survivability requires protein synthesis) was added together with ABA, the increase in survival rate was reduced to 71%. After three days of anoxic stress, the survival rate of corn in the no-pretreatment, water pretreatment, ABA pretreatment and ABA/CHM pretreatment was 1, 3, 34, and 9%, respectively.

Rice seedlings survived anoxic stress much better than corn seedlings (Figure 4). After one day of stress, the survival rate of rice seedlings with no pretreatment was 82% and which did not decline even after three days of stress. Pretreatment with water increased the survival rate of rice seedlings to 95%, while pretreatment with ABA or ABA/CHM showed no further effects. Pretreatment with 100 μM ABA increased the survival rate to 98%.
ABA Concentrations in Corn and Rice Roots under Anoxic Stress

ABA concentration in control corn and rice roots fluctuated with seedling age but was lower in 4-day-old seedlings than in older seedlings (Figures 6 and 7). ABA concentration of control four-day-old corn and rice roots was 4.88 and 6.97 ng/g fresh weight, respectively. Pretreatment with water increased the ABA level in corn and rice roots to 11.8 and 13.08 ng/g fresh weight, respectively. Pretreatment with ABA resulted in much higher ABA concentrations, 18.47 ng/g fresh weight in corn roots and 40.76 ng/g fresh weight in rice roots. In corn roots with no pretreatment, the ABA concentration after one day of anoxic stress was 6.8 ng/g fresh weight. This level declined to 1.59 ng/g fresh weight after 3 days of stress. In rice roots with no pretreatment, the ABA concentration was 16.17 ng/g fresh weight after one day of anoxic stress and remained as high as 14.23 ng/g fresh weight after three days of stress. However, ABA concentration during anoxic stress of the water-pretreated corn roots was higher (7.57 and 6.50 ng/g fresh weight after one and three days of anoxia, respectively) than of the water-pretreated rice roots (3.96 and 5.67 ng/g fresh weight after one and three days of anoxic stress, respectively). ABA concentration remained
very high after three days of anoxic stress of corn (23.15 ng/g fresh weight) and rice (45.83 ng/g fresh weight) roots which were pretreated with ABA.

**Effects of ABA Treatment on Alcohol Dehydrogenase Activity**

Specific activity of ADH in control corn roots was 2.2 units/min/mg protein (Figure 8). While pretreatment of corn roots with water for 24 h induced a 50% increase in ADH activity, a three-fold increase in ADH activity was detected with the ABA pretreatment. After 1 d of stress, ADH activity in the non-pretreated corn roots increased to 7.0 units/min/mg protein. This level was not significantly different from the 8.3 ADH units/min/mg protein in the water pretreated roots and 8.7 ADH units/min/mg protein in the ABA pretreated roots.
Figure 1. Effects of ABA concentration on the survival rate of corn seedlings. Three-day-old corn seedlings were pretreated with different concentration of ABA from 0 to 100 μM for 24 hours followed by exposure to anoxic stress in degassed, distilled water for one day. The seedlings were planted in vermiculite in the growth chamber for seven days before the survival rate was determined. NP is four-day-old seedlings stressed in degassed, distilled water for one day without any pretreatment. Data shown are the mean±SD of three replicates of 50 seedlings per treatment.
Figure 2. Seven day growth of seedlings after 24 h of anoxic stress. Three-d-old seedlings were pretreated with water, 100 μM ABA for 24 h. The seedlings were stressed anoxically for 1 d, planted in vermiculite, and placed in the growth chamber for 7 d.
Figure 3. Effect of ABA pretreatment duration on the survival rate of corn seedlings. Three-day-old seedlings were pretreated with 100 μM ABA for 0 to 24 hours, followed by anoxic stress in degassed, distilled water for one day. The seedlings were planted in vermiculite in the growth chamber for seven days before the survival rate was determined. Data shown are the mean of three replicates of 50 seedlings per treatment.
Figure 4. Anoxic Survivability of Corn Seedlings. Three-day-old seedlings were pretreated with water, 100 μM ABA, 5 mg/L CHM, 100 μM ABA plus 5 mg/L CHM, or wrapped in moist germination papers (NP) for 24 h. The seedlings were stressed anoxically for 1, 2, or 3 d, planted in vermiculite, and placed in the growth chamber for 7 d before their survivability was determined. Data shown are the mean of three replicates of 50 seedlings per treatment.
Figure 5. Anoxic Survivability of Rice Seedlings. Three-day-old seedlings were pretreated with water, 100 μM ABA, 5 mg/L CHM, 100 μM ABA plus 5 mg/L CHM, or wrapped in moist germination papers (NP) for 24 h. The seedlings were stressed anoxically for 1, 2, or 3 d, planted in vermiculite, and placed in the growth chamber for 7 d before their survivability was determined. Data shown are the mean of three replicates of 50 seedlings per treatment.
Figure 6. ABA concentration in corn roots. Three-day-old seedlings were pretreated with 100 μM ABA or water, or were wrapped in moist germination papers (NP) for 24 h. The seedlings were stressed anoxically for 0, 1, 2, or 3 d. At the end of the anoxic stress, ABA in seedling roots was extracted and determined as described in the text. Each analysis was replicated three times. Points without error bars indicate that the SD is smaller than the symbol.
Figure 7. ABA concentration in rice roots. Three-day-old seedlings were pretreated with 100 μM ABA or water, or were wrapped in moist germination papers (NP) for 24 h. The seedlings were stressed anoxically for 0, 1, 2, or 3 d. At the end of the anoxic stress, ABA in seedling roots was extracted and determined as described in the text. Each analysis was replicated three times. Points without error bars indicate that the SD is smaller than the symbol.
Figure 8. ADH activity in corn roots. Three-day-old seedlings were pretreated with 100 μM ABA or water, or were wrapped in moist germination papers (NP) for 24 h. ADH activity in seedling roots was determined at the end of the pretreatment (before anoxia) and also 1 d after anoxic stress. Control seedlings 4 and 5 d old, respectively, were not stressed. Data shown are the mean±SD of three replicates.
DISCUSSION

To eliminate the effects of developmental stage on the response of corn seedlings to ABA, seedlings of the same age were used in this study. Three-day-old seedlings were pretreated with ABA or water, or were wrapped in moist germination papers (control) for 24 h. At the beginning of anoxic stress, all seedlings, whether pretreated or not, were 4 d old. The duration of anoxic stress lasted for 1, 2 or 3 d depending on the experiment. In experiment where anoxic stress lasted for 1 d, seedlings were 5 d old when they were planted in vermiculite for survivability determination (Figures 1, 4, and 5), or processed for ABA and ADH determination (Figures 6, 7, and 8). Five-day-old seedlings, neither pretreated with ABA nor stressed anoxically, were used as the control in these experiments.

Since an aqueous ABA solution was used to pretreat corn seedlings before the anoxic stress, the pretreatment with water was included in the study to separate the effect of ABA from the effect of water. In the water pretreatment, seedling root tips were exposed to water in an open tray. As such, this pretreatment was not anoxic. By exposing the seedlings to a hypoxic condition before the anoxic stress, the water pretreatment improved the survivability of corn seedlings by about 40% over the non-pretreated roots.
(Figure 1). This process, known as acclimation, has been reported by Saglio et al., (1988). According to these authors, the tips of unacclimated corn roots died within 9 h of strict anoxia. However, tips of roots that had been acclimated by pre-exposure to 2 to 4 kPa partial pressure of oxygen for 18 h survived in excess of 22 h of anoxia. Acclimation to cold (cold hardening) or heat stress by pretreatment of the seedlings to nonlethal doses of low or high temperatures has also been documented (Bjorkman et al., 1980; Li, 1984). It is apparent that acclimation induces the expression of the genes necessary for stress tolerance in plants (Key et al., 1987; Mohapatra et al., 1987). Whether the exposure of corn root tips to water induces expression of anoxic tolerance genes remains to be clarified.

The effectiveness of ABA pretreatment in improving anoxic survivability of corn seedlings was much higher than the effects of water pretreatment alone (Figure 1 and 4). ABA has been shown to increase plant tolerance to cold, drought, and salinity stress (Irish and Schwartz, 1987; Lemke-Keyes and Sachs, 1989; Quarrie and Jones, 1977; Raikhel et al., 1987). This study was the first to identify that treatment of corn seedlings with ABA improved their tolerance to anoxic stress by 10-fold over untreated seedlings.
The effects of acclimation were different on the more flood-tolerant rice seedlings. In rice, where the water-pretreated seedlings showed almost 100% survival rate, no further increase in survival rate was possible with ABA-pretreatment (Figure 5).

Contrary to the report that ABA levels increased in anoxic pea roots (Zhang and Davies, 1987), in this study, ABA levels in corn roots declined during anoxia. The differential response may be due to the physiological differences between dicots and monocots. In addition, corn seedlings were etiolated in this study, whereas the pea plants reported by Zhang and Davies (1987) were growing under light.

ABA concentration of corn and rice roots increases with the seedling age until 6 day old and decreases there after (Figures 6 and 7). According to Finkelstein et al., (1985), ABA concentration increased in germinating rape (Brassica napus) seed embryos and decreased in older seedlings. The reason for the decline remains unclear. During anoxic stress, ABA concentration dropped in corn roots but remained high in rice roots. Pretreatment with water increased ABA concentration in both corn and rice roots prior to stress. During stress, ABA concentration in the water-pretreated corn and rice roots declined and is possibly due to vitrification of corn and rice roots that caused the leakage.
of ABA. Even though an absolute correlation between ABA concentration and flooding tolerance did not exist, there was a clear trend that tolerance to anoxic stress is related to relatively high ABA concentrations.

ABA has been shown to induce many genes known as rab (responsive to ABA) genes in plants (Finkelstein et al., 1985; Skriver and Mundy, 1990). Several of these genes have been isolated and characterized. Although these genes are expressed normally in seeds undergoing desiccation, they are induced in tissues subjected to osmotic stress by drought, salt, or cold (Skriver and Mundy, 1990). Since CHM reduced the effects of ABA on anoxic stress survivability of corn seedlings, protein synthesis might be involved in the induction of anoxic tolerance in corn by ABA.

The role of ADH in anoxic tolerance in corn was implicated by Schwartz (1969), but the lack of correlation between anoxic ADH activities of plant roots and their tolerance to anoxia has reported in many studies (Barta, 1986; Lemke-Keyes and Sachs, 1989; VanToai et al., 1985). These investigations, however, have focused mainly on the ADH activities induced by anoxia. This study showed that despite the large differences in anoxic survivability of the ABA pretreated, water pretreated, and non-pretreated corn seedlings, their ADH activities were induced to a similar level after 1 d of anoxic stress. However, ADH activities
were much higher in the ABA pretreated corn roots before the stress. In summary, this study showed that exogenous ABA induced anoxic tolerance in corn seedlings. A strong correlation between ABA levels in anoxic corn roots and their tolerance in corn might be mediated by the synthesis of new protein including ADH.
CHAPTER III

ANAEROBIOsis TOLERANCE IN CORN: ABA INDUCTION OF PROTEIN SYNTHESIS AND LIGNIFICATION

INTRODUCTION

Plants are constantly exposed to adverse environmental conditions such as drought, flooding, extreme temperatures, excessive salts, and infection by pathogenic agents. Plants have to develop indispensable metabolic and structural adjustment to cope with stress conditions. The synthesis of specific proteins which are either structural proteins or enzymes is necessary for the plants to survive such adverse conditions (Smith, 1990).

ABA has been shown to induce the synthesis of new proteins that may be related to the stress tolerance mechanisms for drought (Heikkila et al. 1984), salt (Singh et al., 1987), and low temperature (Robertson et al., 1987;
Robertson et al., 1988). It is not known whether ABA affects gene expression at the transcriptional, post-transcriptional or translational level.

The lignin biosynthetic pathway is well established. It includes nine enzymes: 1) phenylalanine ammonia-lyase (PAL); 2) cinnamate 4-hydroxylase; 3) p-coumarate 3-hydroxylase; 4) catechol 0-methylase; 5) ferulate 5-hydroxylase; 6) hydroxycinnamate: CoA ligase; 7) cinnamoyl: CoA reductase; 8) cinnamoyl alcohol dehydrogenase; 9) peroxidase. PAL is one of the key enzymes in the biosynthesis of lignin (Hahlbrock and Grisebach, 1979). It catalyses the conversion of phenylalanine to cinnamic acid, the first intermediate in lignification. Peroxidase catalyzes the polymerization of monolignols to form lignin and is another key enzyme in the lignification (Keever and Gaspar, 1985; Mader et al., 1980).

Pathogenic infection, wounding, and abiotic stresses including cold temperature and mineral deficiency have been known to trigger the formation of a defensive barrier around many plant organs including leaves, fruits, and tubers (Kolattukudy and Soliday, 1985). These defensive barriers made of either lignin or suberin are synthesized from monolignols by a polymerization process involving peroxidase as a key enzyme (Grisebach, 1981). Up to 29 different peroxidase isozymes have been reported in plants (Chou et
al., 1986). Their expression is highly organ specific, developmentally regulated and influenced by environmental factors (Lagrimini and Rothstein, 1987). The anionic peroxidase isozymes associated with cell walls have been shown to catalyze the polymerization of coumaryl and coniferyl alcohol \textit{in vitro} to form lignin. Roberts and Kolattukudy (1989) isolated an anionic peroxidase gene which appears to be a key component in the regulation of suberization in potato and tomato callus tissues. The suberization-inducing anionic peroxidase was stimulated by exogenous ABA (Cottle and Kolattukudy, 1982).

Roots of many wetland species (e.g. paddy rice) have been reported to develop structural features that permit them to tolerate anoxic environment (Justin and Armstrong, 1987; Armstrong et al., 1988; Lann et al., 1989). Conservation and prevention of radial diffusion of oxygen inside the meristematic zones was facilitated by the deposition of suberized and lignified layers in the epidermal cell walls (Clark and Harris, 1981; Armstrong and Armstrong, 1988). Being impermeable, lignin also regulates the movement of water through cell walls (Schuch et al., 1990). Rice does not require environmental signal transduction to be flooding tolerance due to the nature of its preformed lignified and suberized cells and the process is not controlled by ethylene (Jackson et al., 1985).
DeJaegher et al., (1985) and Boyer et al. (1979) showed that lignification of root tissues inhibits cell division and elongation, while it promotes radial expansion of root cells. This lignification results in short, thick roots with slightly swollen tips, a phenomenon known as root thigmomorphogenesis. The acidic isoperoxidases have been shown to play a role in root thigmomorphogenesis (Gaspar et al., 1985).

Excess-water-induced basic peroxidase activity in cultured carnation (Dianthus caryophyllus) tissues and caused vitrification. A decrease in activity of acidic isoperoxidase was also seen in these tissues (Keevers and Gaspar, 1985). Keevers et al. (1984) verified that the cause of vitrification was derived from a deficiency of lignification. Interestingly, flooded roots also appear "more succulent and clear" as compared to unflooded roots (Hook and Brown, 1973). The phenomenon is similar to vitrification. Plants exhibiting such responses are intolerant to waterlogging and die if flooded conditions persist (Reid and Bradford, 1984). A possible relationship between flooding tolerance and root thigmomorphogenesis, therefore, exists.

This study was attempting to establish the relation of ABA-induced anoxic tolerance with lignin formation in roots of ABA-treated corn seedlings.
MATERIALS AND METHODS

Plant Materials

Corn (Zea mays cv. H60) seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 15 min, rinsed with distilled water 3 times, and imbibed for 15 min in distilled water. The seeds were then rolled in germination paper (Anchor Paper, St. Paul, MN) soaked with 2 mM captan ([N-trichloromethylthio]-4-cyclohexene-1,2-dicarboximide) and allowed to germinate in the dark at (27±1°C) for 3 d. Healthy, unblemished seedlings were sorted for uniformity in size before being used in the experiments.

ABA Treatment and Anoxic Stress

Three-d-old corn seedlings were pretreated with 100 μM (+) ABA (Sigma, St Louis, MO) in the dark and at room temperature for 24 h as described in chapter III.

Anoxic stress was imposed in the dark at 27±1°C by complete submersion of 30 corn seedlings in closed 950 mL glass jars containing degassed, distilled water for 1 or 2 d.

In Vivo Labeling

Intact seedlings were used in the labeling experiment to avoid wound induced changes in protein synthesis (Davies
and Schuster, 1981; Schuster and Davies, 1983). Following ABA treatments, seedlings were placed in an anaerobic chamber (Forma Scientific). Each group of three seedlings was placed in a small petri dish (3.5 cm in diameter) with the roots immersed in 2 mL of the degassed, distilled water. After 3 h, 500 μCi of $[^{35}S]$methionine were added to each petri dish and the labeling continued for 2 h in the dark and at room temperature. The anaerobic atmosphere of the chamber is 5% H$_2$, 15% CO$_2$, and 80% N$_2$.

**Two-dimensional Gel Electrophoresis**

The 1-cm root tips were cut from the labeled seedlings, rinsed three times with degassed, distilled water and frozen in liquid nitrogen before being ground in 0.5 mL protein extraction buffer (50 mM Tris-HCl pH 8.5, 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 1 mM PMSF) with a mortar and pestle. The samples were incubated for 15 min at room temperature and insoluble materials removed by centrifugation at 14,000g for 15 min. Proteins in the supernatant were precipitated by adding 2 volumes of cold acetone and incubating at -20°C for 30 min. After centrifugation at 14,000g for 15 min, the supernatant was discarded and the protein pellet was washed with acetone. Protein pellets were then dissolved in 30 μL lysis buffer (9.5 M urea, 10% (v/v) NP-40, 5% (v/v) 2-mercaptoethanol,
0.16% (v/v) Pharmalyte pH 4-6.5, 0.04% (v/v) Pharmalyte pH 3-10) and stored at -70°C.

Two-dimensional gel electrophoresis was done as described by O'Farrell (1975). Isoelectric focusing gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 60 min. Proteins (200,000 cpm) were loaded in IEF tube gels and ran at 400 V for 18 h. The second dimensional SDS gel were ran at 30 mAmps until the tracking dye mobilize to the bottom of the slab gel. Kodak XAR-5 film was placed in contact with the dried gels and exposed at -70°C for 4 days.

**Determination of Electrolyte Leakage**

The electrolyte leakage from the seedlings was measured as the conductivity of the flooding medium after 1 and 2 d of stress using a Yellow Springs Instrument (YSI) model 33 SCT conductivity meter. The amount of electrolyte leakage is expressed as micromhos (µmhos) per thirty seedlings.

**Determination of Peroxidase Activities**

After anaerobic treatment, the 1-cm tips of seedling radicals (0.1 g fresh weight) were homogenized in 2 mL of 10 mM sodium phosphate buffer (pH 6.0) and 1% (w/v) PVPP with a Brinkman polytron homogenizer. The homogenate was centrifuged at 14,000g for 10 min at 4°C and the resulting supernatant was used as crude enzyme extract. The reaction
mixtures contained 550 µL of 10 mM sodium phosphate buffer (pH 6.0), 250 µL of 50 mM guaiacol, 50 µL of 100 mM H₂O₂ and 100 µL of crude enzyme extract. One unit of peroxidase activity was defined as the change of 1 absorbance unit at 470 nm per min per mg protein.

Syringaldazine has been shown to be a substrate specific for lignification and is important to verify the lignification process (Goldberg et al., 1983). The peroxidase assay in this protocol is: syringaldazine (50 mg) was dissolved in 100 mL hot ethanol. The reaction mixture containing 1 mL of syringaldazine, 1 mL of 0.03% H₂O₂, 1 mL of 10 mM sodium phosphate (pH 7.5), and 0.5 mL of enzyme solution. One unit of peroxidase enzyme activity was defined as the change of 1 absorbance unit at 530 nm per min per mg protein.

**Determination of Phenylalanine Ammonia-Lyase Activities**

Crude enzyme extract was obtained by the same extraction procedure as described for the peroxidase assay above except that 0.05 M sodium borate buffer (pH 8.8) was used. The reaction contained 500 µL of enzyme extract, 2,000 µL of 0.05 M sodium borate buffer (pH 8.8), and 500 µL of 0.1 M phenylalanine (in sodium borate buffer). The reaction mixture was incubated at 37°C for 1 h, and 100 µL of 2 M HCl was added to stop the reaction. Absorbance was
read at 290 nm against a blank containing no phenylalanine. One unit of PAL was defined as the change in 1 absorbance unit per h per mg protein.

**Determination of Lignin Content**

Seedling root tips (2.0 g) were ground in liquid nitrogen and homogenized in 20 mL methanol with a Brinkman polytron homogenizer. The homogenate was vacuum filtered through a glass fiber membrane. The residues were dried at 60°C for 24 h before the lignin content was assayed by derivatization with thioglycolic acid as described by Bruce and West (1989).

**Assay of Peroxidase Isozyme Pattern**

The peroxidase isozyme pattern was resolved by native, discontinuous polyacrylamide slab gel electrophoresis. The resolving gel was 10% acrylamide in 0.375 M Tris-HCl (pH 8.8) and the stacking gel was 5% acrylamide in 0.125 M Tris-HCl (pH 6.8). The tips (1 cm) of seedling radicals (3.0 g) were ground with a chilled mortar in 0.1 mL of 10 mM sodium phosphate buffer (pH 6.0) and 1% (w/v) PVPP. The homogenates were centrifuged at 14,000g for 15 min. The protein content of the supernatant was determined using the Pierce BCA protein reagent (Smith et al., 1985) and the same amount of protein was loaded to each lane. The tracking dye
was 1% bromophenol blue. The stacking and separating gels were electrophoresed at 15 and 25 milliamps constant current, respectively.

Peroxidase activity was stained by incubating the gels in 250 mL of 10 mM sodium phosphate buffer (pH 6.0) and 0.1% guaiacol for 30 min. The gel was rinsed with distilled water before being transferred to 0.03% hydrogen peroxide solution.
RESULTS

Induction of Protein Synthesis by ABA

A 24 h treatment with 100 μM ABA of corn roots before anoxic stress causes a change in the pattern of polypeptide synthesis. The thirty one newly synthesized proteins induced by ABA treatment can be divided into groups based on their pIs. Their molecular weight ranged from 10 KD to 86 KD (Table 1). Figure 9 shows the polypeptide profiles of ABA-treated and -untreated roots after 5 h of anoxic stress. Most polypeptides induced by ABA are acidic. Five low molecular weight (10, 11, 12, 13, and 15 KD) and six high molecular weight (40, 59, 61, 71, 72, and 73 KD) polypeptides were found significantly induced by ABA treatment.

ABA Effects on Root Morphology

After 24 h of ABA treatment, corn roots were shorter in length and thicker in diameter than control, untreated roots. ABA treated roots also had significant swelling at the 1-cm root tips (Figure 10). However, the water-pretreated root tips had no observable difference as compared to control, untreated root tips. When corn roots were subjected to 24 h of anoxic stress, the untreated and water pretreated roots showed the soaked, clear appearance
of vitrification, or susceptibility to anoxia. However, most of ABA treated roots remained thick and firm throughout the anoxic stress period.

**Leakage of Electrolytes to the Flooding Medium**

ABA-treated roots leaked 43% and 35% less electrolytes to the flooding medium after 1 and 2 days of flooding, respectively, than untreated roots (Figure 11).

**Effect of ABA on Lignin Content**

Treatment with ABA induced a 33% increase in the lignin content of the 1-cm tips of seedling radicals (Figure 12). The lignin content remained higher in ABA-treated roots than in untreated roots after one and two days.

**Phenylalanine ammonia-Lyase Activities (PAL) and Peroxidase Activities**

PAL activities in untreated roots were $3.1 \pm 0.1$ unit mg$^{-1}$ protein. ABA treatment increased PAL activities in the 1-cm tips of seedling radicals to $5.7 \pm 0.2$ unit mg$^{-1}$ protein (Figure 13). Peroxidase activities assayed by guaiacol as substrate were $5.1 \pm 0.1$ unit mg$^{-1}$ protein in untreated roots and increased 133% to $11.9 \pm 1.7$ unit mg$^{-1}$ protein in response to ABA treatment (Figure 13). When syringaldazine was used as substrate, the peroxidase
activities increased from 12.5 ± 1.5 to 25.3 ± 0.7 unit mg⁻¹ protein (Figure 13).

**Effects of Calcium and ABA on Peroxidase Isozyme Pattern**

Native PAGE resolved root peroxidases into two isozyme bands, A1 and A2, and some minor bands (Figures 14). ABA treatment greatly enhanced the intensity of isozyme band A2 and also to a smaller extent, band A1 (Figure 14). Calcium treatment also increased the intensity of peroxidase isozyme bands A1 and A2 but the effect was less than ABA treatment (Figure 14). Anoxic stress significantly reduced the intensity of both isozyme bands in untreated roots but did not affect the intensity of these isozyme bands in ABA-treated roots. Peroxidase isozyme patterns in corn shoots were not affected by ABA treatment.
Table 1. In vivo protein synthesis induced by ABA. These proteins are not present in ABA-untreated roots and are grouped according to their pI value.

<table>
<thead>
<tr>
<th>Protein groups</th>
<th>pI</th>
<th>MW (KD)</th>
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<tbody>
<tr>
<td>1</td>
<td>5.6</td>
<td>10, 20</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>12, 73</td>
</tr>
<tr>
<td>3</td>
<td>5.8</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>5.9</td>
<td>40, 72</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>15, 46, 66, 72</td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>18, 70, 77</td>
</tr>
<tr>
<td>7</td>
<td>6.2</td>
<td>45, 50</td>
</tr>
<tr>
<td>8</td>
<td>6.3</td>
<td>39, 47, 85</td>
</tr>
<tr>
<td>9</td>
<td>6.4</td>
<td>30, 59, 61</td>
</tr>
<tr>
<td>10</td>
<td>6.5</td>
<td>33</td>
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<td>86</td>
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<tr>
<td>12</td>
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<td>51</td>
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<tr>
<td>14</td>
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<td>17</td>
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</table>
Figure 9. Autoradiogram of in vivo labeled proteins separated by 2-D SDS-PAGE. The seedlings were exposed to an anaerobic gas mixture (5% H₂, 15% CO₂, and 80% N₂) for 3 h before 35S-methionine was added to the roots. Labeling was continued under anaerobic environment for 2 additional h.
Figure 10. Effects of ABA on corn roots after 24 h of treatment. Panel A, whole roots are shown to indicate the root length. Panel B, A zoom in look of swelling corn root tip treated with ABA.
Figure 11. Solutes in the flooding medium of untreated and ABA-treated corn seedlings after 1 and 2 days of anaerobiosis. Data shown are the mean±SD of three replicates. Column without error bars indicate that the SD is smaller than the symbol.
Figure 12. Lignin content in untreated and ABA-treated corn roots after 1 and 2 days of anaerobiosis. Data shown are the mean±SD of three replicates.
Figure 13. Phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activity in untreated and ABA-treated corn roots. POX1 indicated the substrate for enzyme assay is guaiacol. POX2 indicates the substrate for enzyme assay is syringaldazine. Data shown are the mean±SD of three replicates.
Figure 14. Peroxidase isozyme pattern affected by calcium and ABA. Panel A shows the effects of calcium and ABA on peroxidase isozyme pattern. Lane 1: Control, untreated roots; Lane 2: untreated roots after 1 day of anaerobiosis; Lane 3: ABA-treated roots; Lane 4: calcium-treated roots. Panel B shows the effect of ABA on peroxidase isozyme pattern before and after anaerobiosis. Lane 1: Control, untreated roots; Lane 2: ABA-treated roots; Lane 3: untreated shoot; Lane 4: ABA-treated shoot; Lane 5: untreated roots after 1 day of flooding; Lane 6: untreated roots after 2 days of flooding; Lane 7: ABA-treated roots after 1 day of flooding; Lane 8: ABA-treated roots after 2 days of flooding.
DISCUSSION

This study demonstrates that there are many marked changes in the pattern of protein synthesis in anoxic corn roots caused by ABA treatment. While the identification of these proteins may be important in revealing information about the physiological and biochemical changes in ABA-treated roots, this approach may not detect the real role of these proteins in the anaerobic tolerance response. The proteins induced by ABA treatment are similar to the proteins synthesized in response to stress environments such as drought, cold, and salinity (Mundy and Chua, 1988; Mohapatra et al., 1987; and Singh et al., 1987). However, none of these proteins have been shown to be related to any known physiological processes.

Since the morphological changes in ABA-treated corn roots were typically those of thigmomorphogenesis caused by an increase in lignification (Boyer et al., 1979), root lignification might play a role in the anaerobiosis tolerance response of ABA-treated seedlings. The cells of the meristem and elongation zones of control, untreated roots are normally not lignified. In the present study, the elongation zone of ABA treated corn roots contained more lignin than the elongation zone of untreated roots. According to Kannan and Shaikh (1986), the swelling of
sorghum root tips induced by ABA treatment is related to the tolerance of these seedlings to iron deficiency. Whether the iron was related in the heme binding sites of the acidic peroxidases induced by ABA remains to be determined.

In the present study, ABA treatment induced an increase in activities of both PAL and peroxidase. PAL is the first enzyme in the lignin biosynthesis pathway. It catalyzes the synthesis of cinnamic acid, a lignin precursor. Since the availability of lignin precursor is one of the key factors controlling lignin formation (Abeles and Biles, 1991), higher PAL activities would favor lignification in corn roots.

Peroxidase is the last enzyme in the lignin biosynthesis pathway. The increase in peroxidase activities in response to wounding, pathogenic infection and environmental stresses has been shown to be correlated with lignin formation (Campa, 1990). These isozymes can be separated into two distinct groups: acidic and basic peroxidases. Gaspar et al (1985) indicated that stresses, regardless of whether they were chemical, physical or biological, induced a two-step response of basic and acidic peroxidases in plants. Upon the onset of stress, basic peroxidases were released into the inter-cellular space where they acted as IAA or ACC oxidases. This step did not involve gene expression or protein synthesis. The induction
and release of acidic peroxidases, which followed, required protein synthesis and were directly correlated with lignification. In the present study, I showed that in corn roots, ABA induced an increase in the acidic peroxidase isozyme band in corn roots which remained intense under flooding stress. The induction of peroxidase by ABA treatment was also supported by enzyme assays using syringaldazine, a specific substrate for lignifying acid peroxidases (Goldberg et al., 1983).

Peroxidase is a glycoprotein which contains a ferriporphyrin. Due to this heme prosthetic group, peroxidase can assume different reduction stages. Using gas chromatography and mass spectrophotometry to identify the intermediates of the lignin biosynthesis reaction, Hwang et al (1991) showed that H₂O₂-peroxidase was the key enzyme in lignin biosynthesis. The reduction of O₂-peroxidases to H₂O₂-peroxidases required an NADH-dependent dehydrogenase. Thus, lignification depends on activities of not only PAL and acidic peroxidases, but also of NADH-dependent dehydrogenases such as alcohol dehydrogenases (ADH). The involvement of dehydrogenases, possibly ADH, in lignification was also reported by Mader et al (1980).

In addition to the increase in PAL and acidic peroxidase activities reported here, ABA treatment also induced a 300% increase in ADH activities in corn roots.
(Figure 8; Hwang and VanToai, 1991). The increase in PAL, ADH and acidic peroxidase activities in response to ABA treatment, therefore, could positively affect lignification of corn roots. In the flood-tolerant response of ABA-treated corn seedlings, root lignification occurred prior to flooding stress and remained intense during the two-day flooding duration.

In addition to catalyzing the polymerization of monomeric lignols to produce lignin, peroxidases also possess oxidative activities (Nicholls, 1965; Aylward and Haisman, 1969). Peroxidases have been shown to be the primary respiratory mechanism in anoxic rice seedlings (Paul and Mukherji, 1972) where they serve as electron acceptors for the oxidation of NADH. More recently peroxidases have also been shown to mediate oxygen uptake in Brachypodium pinnatum roots (Van der Werf et al., 1991).

In the present study, peroxidase activities increased at least 2-fold by ABA treatment and remained high in ABA-treated roots during the two-day flooding. Whether their oxidative activities play any role in the flooding-tolerance response of ABA-treated corn requires further investigation.

The increase in lignin content in ABA-treated roots might serve as an impermeable barrier to reduce the leakage of electrolytes, protein, and other cell contents to the flooding medium. Also, the increase in lignin content may
restrict the radial diffusion of oxygen from inside the roots to the flooding medium (Justin and Armstrong, 1987). Root lignification, therefore, might be an important mechanism by which ABA induces the flooding-tolerance response in corn.

In summary, the results presented support the following working hypothesis: root lignification induced by an increase in activities of PAL, peroxidase, and ADH might confer the flooding tolerance of ABA-treated corn through the formation of an impermeable layer around the roots. This impermeable layer can reduce the movement of water in and out of the roots. It can also reduce the radial diffusion of oxygen from inside the roots to the flooding medium as well as the leakage of solutes and cell contents into the flooding medium.
CHAPTER V

MOLECULAR CLONING OF ABA-INDUCED COMPLEMENTARY DNA ENCODING CORN PEROXIDASE

INTRODUCTION

Peroxidases (donor: hydrogen peroxide oxidoreductase EC 1.11.1.7) are involved in many biological processes in plants. This enzyme has many complex isozyme patterns that are tissue-specific, developmentally regulated, and modulated by environmental stress factors (Lagrimini et al., 1987; Rasmussen et al., 1991; Hertig et al., 1991; Reimmann et al., 1992). Peroxidases oxidize hydrogen donors in the presence of H$_2$O$_2$ and have been shown to be involved in the biosynthesis of cell walls, the regulation of auxin level and the responses to microbial attack (Gaspar et al., 1985). However, specific biological functions of distinct isoperoxidase still have to be established. Regulation of isoperoxidase genes by phytohormones has been shown in
callus tissues of potato and tomato in which anionic peroxidases were induced by ABA at the transcriptional level (Roberts and Kolattukudy, 1989). Peroxidase genes were also induced by ethylene in cucumber cotyledons (Morgens et al, 1990). There was evidence that peroxidases play an important role in cell wall lignification (Prasad and Cline, 1987; Sargent et al., 1974). The molecular cloning of peroxidase genes will help clarify the physiological role of each of the isoperoxidases in plant development and stress tolerance response.
MATERIALS AND METHODS

Extraction of RNA

Five-day-old corn H60 roots (20 g of 1-cm root tips) were frozen in liquid nitrogen and ground to powder with dry ice in an electric grinder. The powder was extracted with extraction buffer (5 M guanidine thiocyanate, 0.05 M Tris-HCl pH 7.5, 2 mM Na₂EDTA, 0.7 M 2-mercaptoethanol) at the ratio of 3 mL/g tissue (Chirgwin et al., 1979). Sarkosyl was added to a final sarkosyl concentration of 5 g/L and the suspension was mixed gently for 20 min at room temperature followed by centrifugation at 25,000 g and 10°C for 30 min. The supernatant was filtered through RNase-free cheese cloth and transferred to 39 mL ultracentrifuge tubes where an 8 mL pad of CsCl solution (5.7 M CsCl, 10 mM Tris-HCl pH 7.5, 10 mM Na₂EDTA) was positioned beneath it using a syringe with a long needle. The tubes were centrifuged at 36,000 rpm and 20°C for 18 h in a Beckman LS ultracentrifuge. The RNA pellets were resuspended (0.1 mL/g tissue extracted) in 10 mM Tris-HCl pH 7.5, 1 mM Na₂EDTA, 50% sarkosyl and 5% phenol. The suspension was extracted with an equal volume of concentrated phenol for 5 min, followed by an equal volume of chloroform for 2 min. The aqueous phase was transferred to a fresh tube and the RNA was ethanol-precipitated at
-20°C overnight followed by centrifugation at 8,000 g for 15 min. The RNA pellet was dissolved in RNase free water.

**mRNA Isolation**

The poly(A⁺) RNA was isolated from total RNA of ABA-treated roots using a PolyATract mRNA isolation kit (Promega). This mRNA isolation system uses a biotinylated oligonucleotide (dT) to hybridize to the 3' poly(A⁺) region of mRNA. The hybrids were then captured and washed at high stringency using streptavidin-bound MagneSphere paramagnetic particles. The mRNA was eluted with RNase-free water.

**DNA isolation**

Twenty grams of corn H60 seeds were ground in an electric grinder with dry ice. The powder was saturated with 100 mL of extraction buffer (0.1 M Tris-HCl pH 7.5, 100 mM EDTA pH 8.0, 250 mM NaCl) containing 10 mg proteinase K. Sarkosyl (10%) was added and the solution was incubated at 55°C for 2 hours with gentle shaking. DNA solution was collected by centrifuge at 6,000 g for 10 min. Final concentration of 10% CTAB and 0.7 M NaCl were added to extract carbohydrate by incubating at 65°C for 20 min. DNA was extracted three times with 120 mL of chloroform: isoamyl alcohol (29:1). The aqueous phase containing DNA was obtained by centrifuge at 6,000 g and 10°C for 10 min. DNA
was then precipitated by adding 0.6 volume of isopropanol at -20°C for 30 min. DNA was pelleted and subjected to CsCl banding at 45,000 rpm for 36 hours in a Beckman ultracentrifuge.

**cDNA Library Construction**

In a sterile RNase-free microfuge tube, 1.5 µg NotI-primer adaptor was added to 5 µg of mRNA (isolated from ABA-treated corn roots) in a total volume of 15 µL. The solution was heated to 70°C for 5 min and cooled to room temperature. First strand cDNA was synthesized by adding 1st strand 5X buffer and RNAsin inhibitor. The solution was heated to 42°C for 5 min, and reverse transcriptase and sodium pyrophosphate were added to synthesize the first strand cDNA from mRNA at 42°C for 60 min. The second strand cDNA was synthesized with the addition of 2nd 10X buffer, *E.coli* DNA polymerase, and *E.coli* RNase H at 14°C for 3 h. The T4 DNA polymerase was added to fill the end of double-stranded cDNA. The double-stranded cDNA was extracted with Tris-EDTA-saturated phenol:chloroform and precipitated by cold ethanol at -20°C overnight.

*EcoRI*-adaptor was ligated to the other end of cDNA before the cDNA was ligated to the lamda arms of the lamda gt11 phage. Lamda gt11 phage DNA containing the cDNA insert was then packaged into its coat proteins. The phages were
then plated on a lawn of E. coli strain Y1090 and the cDNA library size calculated.

**cDNA Library Screening**

Phage plaques were lifted in duplicate with nitrocellulose membranes and screened with a wheat peroxidase cDNA probe (POX375) (Rebmann et al., 1991). Hybridization was done as described later in the Northern and Southern blot hybridization section. After hybridization, the membranes were washed twice with 6X SSC and 0.05% sodium pyrophosphate for 30 min, once with 1X SSC for 30 min, and once with water for 30 min at 25°C. The membranes were wrapped in Saran plastic and placed in contact with Kodak X-ray film at room temperature overnight. Positive peroxidase clones were isolated after three rounds of screening.

**Analysis of Recombinant Phage DNAs**

Phage DNAs were purified according to the Promega's protocol (Protocols and Application Guide, Promega). Phage lysates were obtained by liquid culture and treated with 100 µg/mL of DNase I and RNase A at 37°C for 30 min to remove bacterial DNA and RNA residues. Equal volume of 20% PEG and 2 M NaCl were added to the phage lysates to precipitate phage particles from bacterial debris and collected by
centrifugation at 10,000g and 4°C for 20 min. Phage particles were resuspended in phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgSO₄) at the ratio of 1 mL phage buffer per 10 mL initial lysate. Tris-EDTA (TE)-saturated phenol/chloroform was used to extract phage DNA from phage particles. Phage DNAs were precipitated with the addition of isopropanol in equal volume with the phage DNA solution at -70°C for 20 min. The pelleted phage DNAs were washed with 70% ethanol, dried, and dissolved in 1000 μL of TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Residual bacterial RNA was removed by incubating with RNase (100 μg/mL) at 37°C for 30 min, phage DNAs (different clones) were repurifying with phenol/chloroform extraction and ethanol precipitation.

The purified phage DNAs were digested with NotI and EcoRI. The digested DNAs were separated on a 1% agarose gel, blotted to nylon membrane and subsequently hybridized with either a wheat peroxidase cDNA probe (POX375) or an oligonucleotide probe (peroxidase conserve sequence, 17 mer with degeneracy).

Subcloning

Putative peroxidase cDNA clones were isolated and cleaved with EcoRI and NotI. The cDNA inserts were band-isolated from agarose gel by freeze-fracture and phenol extraction followed by ethanol precipitation (Bewsey et al.,
Plasmid pBlueScript II SK- was cleaved with the same enzymes (EcoRI and NotI) and was purified by the freeze-fracture method described above. The cDNA inserts were ligated with plasmid DNA. The ligation was done with cDNA insert and vector DNA at the ratio of 1:3 using DNA ligase (Promega) at room temperature for 3 h. Transformation was done with XL1-Blue (Stratagene) cells using the protocol supplied by the manufacturer.

Northern and Southern Blot Hybridization

Total RNA (15 μg) was separated by formaldehyde agarose (1.3%) gel containing 0.1 μg/mL ethidium bromide in 3-[
N-Morpholino]propane sulfonic acid (MOPS) (Sigma, St. Louis, MO) buffer for 2.5 h at 5 V cm⁻¹ as described by Maniatis et al. (1982). Separated RNA was transferred to a Zeta probe nylon membrane (Bio-Rad, Richmond, CA) by capillary blotting in 20x sodium chloride-sodium phosphate-EDTA buffer (SSPE) (20x sodium chloride-sodium citrate buffer (SSC) is 3 M NaCl and 0.3 M Trisodium citrate) overnight and at room temperature. The membranes were air-dried and baked in a vacuum oven at 80°C for 2 h. The probe was a wheat peroxidase cDNA (Rebmman et al., 1991) radiolabeled by random priming (Promega, Madison, WI) to a specific activity of 1 to 2x 10⁹ cpm/μg DNA. Pre-hybridization was in 5 x
SSPE, 50% (v:v) formamide, 100 μg/mL fish DNA, and 1 % (w/v) SDS at 37°C overnight. Hybridization was done at 37°C for 24 h with 6 x10⁹ cpm/ng probe. After hybridization, the membranes were rinsed twice with 2 x SSPE, followed by two 30-min washes in 2 x SSPE and 0.2 % SDS at 55°C and a final wash with 0.2 x SSPE at room temperature. The membranes were autoradiographed at -70°C for 1 d. Southern blot hybridization was done as described above except the transfer buffer is 0.4M NaOH, hybridization temperature was 37°C and the wash was at 40°C. Oligonucleotide was labeled by T4 DNA kinase with γ-³²p-dATP.

DNA Sequencing

DNA sequencing primers were synthesized by the Biochemical Instrumental Center (The Ohio State University). The primers were deblocked by incubation at 55°C overnight and cool to room temperature. The deblocked primers were then separated from unincorporated nucleotides using Sephadex G-50 column saturated with DNase-free water. Fractions of 1 mL were collected into 5 separated microfuge tubes and their absorbance read at 260 nm.

DNA sequencing was performed using a Sequenase Kit (Version 2.0, US Biochemicals). Template DNA was denatured in 0.2 M NaOH and 0.2 mM EDTA solution at 37°C for 30 min. Neutralization of DNA was done by adding 0.1 volume of 3 M
sodium acetate (pH 5.4) and the DNA was precipitated by adding 2 volumes of absolute cold ethanol and incubated at -70°C for 15 min. Precipitated DNA was centrifuged at 12,000 g for 15 min, washed with 70% ethanol and dried in vacuum. The DNA pellet was resuspended in water. The primer was added at the same molar ratio with template along with 10% (v/v) DMSO and 0.2 volume of sequencing reaction buffer. The whole solution was heated to 65°C for 2 min and cool to 30°C for the annealing of the template and primer. Labeling reaction was done by adding DTT, labeling mix, 35S-γ-ATP and sequenase buffer to the annealed template-primer solution. The labeling reaction was done at room temperature for 5 min. An aliquot of the labeling reaction was added subsequently to microfuge tubes containing ddATP, ddCTP, ddGTP, and ddTTP individually for the termination reaction. Band compressions in the sequencing gel were resolved by substituting 7-deaza-dGTP for dGTP in the reaction mixture in the presence of DMSO. The concentrations of the labeling mix and sequenase were higher than suggested by manufacturer. The termination reaction was performed for 5 min at 39°C instead of 37°C as suggested by manufacturer. DNA was separated on 6% denaturing polyacrylamide gel in 1X TBE (Tris-Boric-EDTA) buffer.

Electrophoresis was done at constant 60 watts. Three to four loads were electrophoresed from each sample. The gel
was vacuum dried at 80°C and exposed to X-ray film for approximately 36 hours. DNA sequences were analyzed by DNASIS software (Pharmacia).
RESULTS

Establishment of an ABA Treated Corn Root cDNA Library and Isolation of Peroxidase cDNA Clone

A cDNA library of 4-day-old ABA-treated roots was constructed as described in the materials and methods. This cDNA library consisted of 17,600 recombinant clones. The whole cDNA library was plated on a LB-agar plate (150 mm diameter). Twelve positive clones were picked after first round of screening. These twelve putative clones were plated at 5,000 plaques per plate and were rescreened for the peroxidase gene. Nine plaques were picked and plated at 200 plaques per plate for the final screen. Three cDNA clones were isolated.

Sequence Determination of Peroxidase cDNA Clones

DNA sequencing from both 5' and 3' end of cDNA inserts revealed that only one of the three putative clones showed homology with the conserved DNA and amino acid sequences of known peroxidase genes. Further DNA sequencing of the peroxidase cDNA was done using oligonucleotide primers. This peroxidase cDNA was named pCPOX1. The poly(A') tail of the peroxidase cDNA is located on the EcoRI end of insert and has 15 adenine nucleotides. DNA sequence of the 5' end (NotI end) revealed the peroxidase conserve sequence. The
1083 base nucleic acid sequence and 297 predicted amino acid sequence of the pCPOX1 clone is shown in Table 2. There is a potential polyadenylation signal AATTA at 134 bases upstream the poly (A') tail. The other potential polyadenylation signal AAGTA is located at 20 bases upstream the poly(A') tail. When the nucleic acid sequence of this clone is compared with other known peroxidase nucleic acid sequences, the homology is highest (38.0%) with the rice peroxidase cDNA and lowest (29.3%) with the tobacco peroxidase cDNA (Reimmann et al., 1992; Lagrimini et al., 1987). pCPOX1 has 58.1% G+C content. More interestingly, all the peroxidase genes which have been sequenced can be distinguished into two categories according to their G+C%. The peroxidase genes from monocotyledonous plants has 62.2% G+C on average, the peroxidase genes isolated from dicotyledonous plants has 41.7% G+C on average (see Table 3 for references). No dicot peroxidase gene has more than 45% G+C while no monocot peroxidase gene has less than 57% G+C (Figure 15).

**Comparisons of Deduced Amino Acid Sequences of pCPOX1 with Known Plant Peroxidase Sequences**

The 297 amino acid sequence of pCPOX1 clone is predicted according to the location of the conserved sequence. The 5' end of pCPOX1 is missing. Table 3 shows the
alignment of the amino acids of known plant peroxidases. The conserved amino acid stretch is Arg-Leu-His-Phe-His-Asp-Cys-Phe-Val. In pCPOX1 the first histidine is substituted by phenylalanine. Turnip is the other plant that also has substitution at the same amino acid position. The percentage of similarities in amino acid sequences between pCPOX1 and other plant peroxidases are: barley 27.8%; wheat 34.2%; rice 36.0%; Arabidopsis 32.5%; cucumber 31.6%; horseradish 32.7%; peanut 32.2%; potato 29.0%; tobacco 30.0.0%; tomato 30.0%; turnip 38.0% (Figure 16). However, certain portions of the amino acid sequence of the known peroxidase genes are more conserved than other. When the amino acid sequence of pCPOX1 is divided into 3 stretches: N-terminal (1-132), middle (133-275), and C-terminal (276-297), the average homology of the N-terminal stretch to other peroxidase N-terminal stretches is 47.9%; the homology of the middle stretches is only 12.8%, while the homology of the C-terminal stretch is highest at 60.3% (Figure 17). It is interesting that the corn pCPOX1 clone has highest N-terminal stretch homology to turnip (60.2%) and C-terminal stretch to Arabidopsis (72.7%) than to other monocot peroxidase genes.

Eight cysteines and thirteen histidines residues were found (Table 2). Four out of the eight cysteines are located at the same positions compared with all other plant peroxidase (Table 3). Two histidine at residues 45 and 172
of pCPOX1 are also present in all the known peroxidases. One N-glycosylation site (Asn-X-Thr/Ser) is found at residue 73. The unique Cys-Cys-Cys stretch located at amino acid 55-56-57 is not seen in any other peroxidase.

**RNA Blot Analysis**

RNA was extracted from 4-day control roots, 3-day-old roots flooded for 1 day, ABA-treated non-flooded roots, and ABA-treated and flooded roots. Northern analysis shows a strong hybridization of the clone pCPOX1 to RNA from ABA-treated roots at about 1.1 kb. However, this message was diminished in ABA-treated and flooded roots. The 1.1 kb message was not observed in both control and flooded roots. Therefore, the Northern blot analysis shows that pCPOX1 is induced by ABA treatment (Figure 19).

**Genomic Southern Analysis**

Genomic DNA was cleaved with restriction enzymes *BamHI*, *BglII*, and *HindIII*. The restriction with *BamHI* showed one strongly hybridized band and one weakly hybridized band. The restriction with *BglII* showed two very strongly hybridized bands and one weakly hybridized band. The restriction with *HindIII* showed only several weakly hybridized bands (Figure 20). The results indicates that only one to two isoperoxidase genes correspond to the isoperoxidase clone
(pCPOX1) isolated. Other isoperoxidase genes may have different specificity in nucleotide composition and may not be detected.
Table 2. Nucleotide and deduced amino acid sequences of the corn pCPOX1 clone. The amino acid sequence of the predicted translation product is shown below the nucleotide sequence. The stop codon is marked by bold letters and the apparent signal peptide is underlined. Conserved amino acid regions as compared to other known plant peroxidases are listed in bold letters and underlined.

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Table 3. Alignment of 1) corn pCPOX1 amino acid sequence with that of 2) barley BP1 (Rasmussen et al., 1991); 3) barley BP2A (Theilade and Rasmussen, 1992); 4) wheat POX1 (Hertig et al., 1991); 5) wheat POX375 (Rebman et al., 1991); 6) rice pPIR3 (Reimmann et al., 1992); 7) Arabidopsis prxCa; 8) Arabidopsis prxEa (Intapruk et al., 1991); 9) cucumber CuPer2 (Morgens et al., 1990); 10) horseradish HRC (Fujiyama et al., 1990); 11) peanut prxNC1; 12) peanut prxNC2 (Buffard et al., 1990); 13) potato (Roberts et al., 1988); 14) tobacco (Lagrimini et al., 1987); 15) tomato (Roberts and Kolattukudy, 1989); 16) turnip (Mazza and Welinder, 1980). Conserved Cys residues are in bold letters. Stretch of three Cys is underlined. Conserved amino acid sequences are underlined.

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<tr>
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<td>NFSNCTAAADPAIDPSVSNCPSLCNPNTGAAANRLVALTIGQFFK---</td>
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<tr>
<td>-------CTGNSVNPQNLQA-CNCSATLTDSEQLDTTPM---</td>
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<tr>
<td>NFNGSNGPDPTTRANQRICPQGG-NNNTFTNLDISTPND---</td>
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<td>NFNGSNGPDPTTRANQRICPQGNNNTFTNLDISTPND---</td>
</tr>
<tr>
<td>-------NETNIAAFFATLQR-SCAPAKGSDANLFDINSATS---</td>
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Table 3 (continued)

1  -DCLHEGAPEGAPGLRPGALQYRSHRRAGPVLRQYPERGLLGLC
2  -FDNKYYIDLVNREGFVDQDLEFTNAITRPIVERFARSQQDFF
3  -FDNKFVNLQOEGLFVDQDQKYTNAITQPIVESFARSQGDFF
4  -FDNYTNNLLSLQKGLLHSDQVLFNNEPTDTNTRNFSNAAAFS
5  -FDNAYTNLMSQKGLLHSDQVLFNNDTTDNTRRNFSNAAAFS
6  -FDNAYSNLSN-KGLLHSDQVLFNGG-SADNTNTRNFSNAAAFS
7  -FDNNVYNLKEQKGLIQQSDLFSAT-DTIPLVRSPNAYADGTTF
8  -FDNYYTNLRTNQKGLIQQSDLFSAD-DTIPLVNPQYSSDMSVFF
9  -FDNYTTLQSPNLLPLTSQVHLSTP-GBDTOVIVNLFHQASSQNF
10 IFDNKYVNLHEDQKGLIQSDLFSSTPNTNATDITPLVRSFANSTQTF
11 -FDNAYINLNNKGGHSDQQLFNGV-STDSDLTATSSNAATF
12 -FDTSFNSNLRRRGVLQDSALYNDD-PSTKSVQRLGLGFLGLTFN
13 -FDKVYYDNXNNQQMLFSDLQVLTGDA-TTAYGFDVTSNDVSVF
14 -FDNDYFTNLQSNQGLLQTDSQELFSTS-GSAATIAVNYAGSSTQF
15 -FDNDYFTNLQSNQGLLQTDSQELFSTS-AIVNYAGSSTQF
16 -FDNSYFKNLQARGGLLHSDQVLFNDT-IPSIVGGRGYSNSPS-SFN

1  DGHGE---MGDITPLTGSGQIRKNCRRVN
2  EQFGVSIKGQMQRVTSDQGEVRNCSV
3  DQFGVSTGEDQIRVLTDQGVRNCAV
4  SAFTTAMIKMGAPIPLGTQGQLRSISKV
5  SAFTTAMIKMGAPIPTGTQGQLRSISKV
6  SAFTTAMIKMGAPIPLGTQGQLRSISKV
7  NAFVEMNRMGRAPPTGTQGQLRSIKR
8  RAPIDAMIRMGNRPLGTQGEIRQNCRRV
9  LASFGQSMNMLQFPLTGQGERQNCRR
10 NAFVEMDRAMGNLSTPLTGQQLRNCRRV
11 TDFGNAMIKMGNLSPLTGQQITRCRTK
12 VEFGKSMVQSMNNVGTKTGGQIKSCAPN
13 GDFGKSMIKMGDLPSAGAGLIERDVCSSRVN
14 DDFVSMIKLINGSPLGTGQQIRTDCKR
15 DDFVSMIKLINGSPLGTGQQIRTDCKR
16 SDFAAAMIKMGDLPSLGSGERIRKVCGR
Figure 15. The (G+C)% of known plant peroxidase genes. Names of the peroxidase genes of each plant species are listed as follows: corn, pCPOX1; barley 1, BP1; barley 2, BP2A; rice, pPIR3; Arabidopsis 1, prxCA; Arabidopsis 2, prxEa; cucumber, Cuper2; horseradish, prxC; peanut 1, prxPNC1; peanut 2, prxPNC2; potato, tobacco, and tomato have no clone names. See Table 3 for references.
Figure 16. Homology comparison of corn peroxidase amino acid sequence with other plant peroxidase amino acid sequences
Figure 17. The similarity of three different stretches of amino acid sequence of corn pCPOX1 clone to other known plant peroxidase amino acid sequences.
Table 4. Comparison of the number of glycosylation sites in plant peroxidases. Names of the peroxidase genes of each plant species are listed as follows: corn, pCPOX1; barley 1, BP1; barley 2, BP2A; rice, pPIR3; Arabidopsis 1, prxCA; Arabidopsis 2, prxEa; cucumber, Cuper2; horseradish, prxC; peanut 1, prxPNC1; peanut 2, prxPNC2; potato, tobacco, tomato, and turnip have no clone names. See Table 3 for references.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Number of N-glycosylation sites</th>
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</thead>
<tbody>
<tr>
<td>Corn</td>
<td>1</td>
</tr>
<tr>
<td>Barley 1</td>
<td>1</td>
</tr>
<tr>
<td>Barley 2</td>
<td>2</td>
</tr>
<tr>
<td>Wheat 1</td>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>Rice</td>
<td>4</td>
</tr>
<tr>
<td>Arabidopsis 1</td>
<td>6</td>
</tr>
<tr>
<td>Arabidopsis 2</td>
<td>4</td>
</tr>
<tr>
<td>Cucumber</td>
<td>3</td>
</tr>
<tr>
<td>Horseradish</td>
<td>8</td>
</tr>
<tr>
<td>Peanut 1</td>
<td>5</td>
</tr>
<tr>
<td>Peanut 2</td>
<td>1</td>
</tr>
<tr>
<td>Potato</td>
<td>8</td>
</tr>
<tr>
<td>Tobacco</td>
<td>4</td>
</tr>
<tr>
<td>Tomato</td>
<td>8</td>
</tr>
<tr>
<td>Turnip</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 18. The hydropathy profile (Kyte and Doolittle, 1982) for the corn peroxidase protein encoded by pCPOX1 gene.
Figure 19. Accumulation of peroxidase RNA in corn following ABA treatment. Lane A, control 4 d roots; Lane B, untreated flooded roots; Lane C, ABA treated roots; Lane D, ABA-treated flooded roots. The probe was the peroxidase pCPOX1 cDNA. The transcript size was determined from the Lamda ladder resolved on the same gel.
Figure 20. Southern analysis of corn DNA using the coding region of pCPOX1 clone as the probe. Ten micrograms of total corn DNA was digested with BamHI (lane A), BglII (lane B), and HindIII (lane C). Digested DNA was resolved on 0.7% agarose gel and transferred to a nylon membrane.
I have constructed a cDNA library from ABA-treated corn roots and isolated three cDNA clones that hybridized to the wheat peroxidase pPOX375 clone. The whole cDNA library (17,600 clones) was plated on 150 mm LB-plate on a lawn of bacterial strain Y1090. Twelve plaques that hybridized to the wheat pPOX375 probe were obtained. The twelve plaques were plated and rescreened with the same probe. After three rounds of screening, 3 putative peroxidase clones were selected. The synthetic oligonucleotide of the peroxidase gene conserved sequence hybridized with only two of the three clones. DNA sequencing verified that one of these two clones was the peroxidase gene. The clone was named pCPOX1.

The homology of the deduced amino acid sequence of the pCPOX1 clone with other known plant peroxidases is very low, mainly due to the differences of the stretch of amino acid residues number 134 to 275. Cross reaction of peroxidase antiserum derived from petunia with many plant species except Brassica napus, Tagetes cresta, and Zea mays has been reported (Hendriks et al., 1990).

Two regions of the amino acid residues are highly conserved in all known plant peroxidases. The first one is 41Arg-Leu-Phe-Phe-His-Asp-Cys-Phe-Val48. The first phenylalanine found in the pCPOX1 is also present in turnip
peroxidase. In all other plant peroxidases, it is replaced by His. This conserved amino acid sequence belongs to the acid/base catalysis region which includes the functional histidine\(^{45}\) residue. The second conserved amino acid residue is \(97\text{Val-Ser-Val-Ala-Asp-Ile-Leu}^{103}\). The second valine is replaced by cysteine in other known plant peroxidases. The hydropathic plot derived from windows of 7 amino acid residues shown peroxidase pCPOX1 is a secreted protein with a potential of highly hydrophilic 3' end (Figure 18). Only one glycosylation site is found at \(73\text{Asn-Gly-Ser}^{75}\) (Asn-Xaa-Ser/Thr) residues. This glycosylation site is specific for corn peroxidase and not found in others (Table 3). The number of N-glycosylation is variable from 0 in wheat POX1 to 8 in horseradish, tomato, and potato (Table 4).

Four out of the eight cysteine residues are at conserved locations. The possible disulfide bridges are 47/55, 51/54, 56/293, 232/269, derived by analogy with horseradish and turnip peroxidase amino acid sequences (Okamuro and Goldberg 1985; Welinder and Norskov-Lauritsen, 1986). Two histidine residues His\(^{45}\) and His\(^{172}\) are highly conserved. His\(^{42}\) is believed to act in acid/base catalysis and His\(^{172}\) is probably bound to heme iron (Welinder, 1986). One feature of this corn peroxidase that should be noted is the high histidine number in its amino acid sequence (13
histidines) compare to others. The possible functions of high number of histidines is unknown.

The southern analysis detects more than one sequences are hybridized to the pCPOX1 clone. The number of sequences is probably 1 to 2 and is corresponded to the isozyme gel results that show two major isozyme bands (Figure 14). Northern analysis indicated that the pCPOX1 is indeed induced by ABA treatment (Figure 19). The transcriptional mechanism is depleted by the anaerobiosis and is the reason for the failure in the isolation of isoperoxidase gene from an ABA-treated and -flooded corn roots cDNA library (Data not shown).

The isolation of this putative peroxidase encoding cDNA should aid in deciphering the role of this enzyme in the root lignification and the anaerobiosis tolerance mechanism. One genomic peroxidase clone isolated from barley seeds has the DNA sequence GTACGTGTC (Theilade and Rasmussen, 1992) that may be related to the ABA-responsive element GTACGTGGC (Skriver et al., 1991) and the Em gene enhancer ACACGTGGC (Guiltinan et al., 1990). Therefore, the isolation of the genomic clone corresponding to pCPOX1 will provide information about ABA regulation of peroxidase gene expression.
CHAPTER V

SUMMARY AND CONCLUSION

The function of many ANPs (Sachs and Freeling, 1980; Kelley and Freeling, 1984a; Laszlo and St. Lawrence, 1983; Wingnarajah and Greenway, 1976; Kelley and Freeling, 1984b; Springer et al., 1986; Hoffman et al., 1986; Torres et al., 1986) in the anoxic tolerance mechanism remains speculative. In 1978, Crawford presented evidence that ADH activities were inversely correlated with anoxic stress tolerance. However, according to Schwartz (1969), ADH null mutant was very susceptible to anoxic stress. ADH plays a role in the recycling of NDA from NADH that allows the continuation of glycolysis and ATP synthesis at the substrate level. No physiological evidence has been presented to support the function of other ANPs in the anoxic tolerance mechanism.

I have found that ABA treatment induced a 10-fold increase in anoxic survival of corn (Figure 1). Although acclimation by exposure of seedling root tips to water
induced some level of anoxic tolerance, the 48% anoxic survival by acclimation was much lower than the 87% anoxic survival of ABA-treated seedlings. The ABA-induced anoxic tolerance may be co-ordinated by the proteins synthesized in response to ABA treatment.

Two-dimensional gel electrophoresis showed that many proteins are induced by ABA treatment. These proteins can be isolated and N-terminal microsequenced. The oligonucleotides can be synthesized and used as probes to clone the ABA-inducible genes. However, this approach is not feasible in identifying the functions of these proteins.

I have observed that ABA-treated corn roots are shorter and thicker than control roots. ABA-treated roots also showed a swelling at the 1-cm root tip that persisted after flooding (Figure 10). This phenomenon is known as thigmomorphogenesis. However, flooded roots which were not treated with ABA, appeared soaked and clear, a phenomenon known as vitrification. If the morphological changes in ABA-treated roots are related to anoxic tolerance, it should be feasible to determine the role of thigmomorphogenesis in the anoxic tolerance response.

According to Gaspar et al. (1985), the swelling of root tips was probably due to lignification induced by acidic peroxidases. Goldberg et al. (1983) indicated that
syringaldazine was a specific substrate for lignin peroxidases. I have found that ABA treatment induced an increase in phenylalanine ammonia-lyase (PAL) and peroxidase activities in corn roots. A 2-fold increase in acidic peroxidase activities was detected in ABA-treated roots using syringaldazine as a substrate (Figure 13). Root lignin content also increased 33% with the ABA treatment. Differences in acidic peroxidase activities in ABA-treated and -untreated roots were also detected by native polyacrylamide gel electrophoresis (Figure 14). PAGE showed the persistence of peroxidase activities in ABA-treated anoxic roots. Calcium, the ABA activation factor, also showed inductive effects on the same isoperoxidase (Figure 14). The induction effect of calcium on peroxidase indicated a possible relationship of the signal transduction pathway in ABA action. According to this model, ABA acts as a stimulus that activates a sequence of changes in membrane proteins that, in turn, activate the release of calcium ions in the cytoplasm to activate the peroxidase genes of lignification.

A corn peroxidase gene was cloned and its DNA sequence was obtained. The deduced amino acid sequence of this corn isoperoxidase is very different from other plant peroxidases. Amino acid residues 134-275 have only an average of 12% similarity with other known plant
peroxidase amino acid sequences. One N-glycosylation site was found (Table 2). This clone, pCPOX1, is induced significantly by ABA treatment, but the accumulation of its transcript is depleted under anoxia.
SIGNIFICANCE OF THE ABA-INDUCED ANOXIC TOLERANCE

Although ABA has been known as the inducing factor of many stress adaptation mechanisms (Singh et al., 1987; Mohapatra et al., 1988; Davies et al., 1980), ABA concentration has always increased in these stresses. The role of ABA in anoxic stress tolerance has not been reported. In this study, ABA concentration decreased in anoxic corn roots but remained unchanged in rice roots. Since rice is more tolerant to anoxic stress than corn, the maintenance of ABA concentration in anoxic roots may be necessary for anoxic tolerance.

The low ABA concentration in anoxic corn roots could have been due to a reduction in ABA synthesis that requires oxygen (Rock et al., 1991) or could have been due to an increase in ABA metabolism/leakage to the medium. Anoxic rice roots that show high ABA concentration might be able to continue synthesizing ABA under anoxia due to a special mechanism. On the other hand, anoxic rice roots could have also controlled the ABA metabolism and/or its leakage better than the less tolerant corn roots.
SIGNIFICANCE OF THE ROLE OF LIGNIFICATION IN ANOXIC TOLERANCE

The importance of lignification in pathogenic tolerance has been very well documented (Kolattukudy and Soliday, 1985). Most research has focused on the induction of peroxidase by ABA treatment or the isolation of peroxidase genes. This study was first in demonstrating that the induction of lignification by ABA might be involved in anoxic tolerance.

In this study, the acidic peroxidase isozyme band of ABA-treated roots resolved on native polyacrylamide gels remained active under anoxic stress (Figure 14b); However, the northern blot analysis showed that the transcripts of the cloned ABA-inducible peroxidase gene were depleted (Figure 19). The increase in lignification originated by ABA might involve not only the induction of peroxidase genes, but also the maintenance of peroxidase enzyme activities (Theilade and Rasmussen, 1992). While ABA has been shown to stabilize transcripts of water stress-responsive genes (Gomez et al., 1988; Mortenson and Dreyfuss, 1989), whether it also stabilizes peroxidase activities will require further investigation.

This study was first to show the correlation of PAL, POX, and ADH enzyme activities, morphological changes due
to lignification and anoxic tolerance in corn. In this study, I have shown the induction of ADH and peroxidase activity by ABA in support of the hypothesis that both dehydrogenase and peroxidase activities are required for lignification (Hwang et al., 1991).

The ABA-inducible root lignification model of anoxic tolerance proposed in this study will contribute to the understanding of the anaerobic tolerance mechanism in plants.
SIGNIFICANCE OF THE ISOLATION OF AN ACIDIC ISOPEROXIDASE GENE

The isolation of pCPOX1 peroxidase gene will lead to the isolation of its genomic DNA clone. Since an ABA responsive element has been found in a barley peroxidase genomic DNA clone (Skriver et al., 1991), it is possible that the same element might also be present in the promoter of the peroxidase gene isolated from corn roots. The promoter region of pCPOX1 may be used in transformation studies to determine the mechanism of ABA induction. In addition, it could also be used to verify that ABA-inducible root lignification by acidic peroxidases as a model of anoxic tolerance in corn.
APPENDIX

TRANSIENT GUS GENE EXPRESSION IN CORN ROOTS

With the isolation of the corn peroxidase pCPOX1 cDNA clone and its genomic DNA clone, I am also interested in investigating the transient expression of this gene using the particle bombardment technique. The promoter region of this gene can be placed in front of a GUS DNA and other DNA sequences that may serve as buffer for the GUS gene expression to form a plasmid DNA construction. The construct can be used in transformation studies to investigate the effects of ABA on the expression of this gene. Stable transformation could be used to verify the mechanism of anoxic tolerance by ABA-inducible root lignification.

Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells. According to Klein et al. (1987) tungsten particles could be used to introduce macromolecules such as DNA and RNA into epidermal cells of onion (Allium cepa)
with subsequent transient expression of enzymes encoded by these compounds. Cristou et al. (1988) demonstrated that the process could be used to deliver foreign DNA into living cells and result in their stable transformation. The concept has been described in detail by Sanford (1988).

To replace other DNA delivery devices which are complex and expensive, Finer et al., (1992) developed a simple and inexpensive particle bombardment device which was constructed for the delivery of DNA into plant cells. This device is called the Particle Inflow Gun (PIG). The PIG uses helium gas to accelerate the DNA-coated tungsten particles in the presence of a partial vacuum. The pressurized helium stream is the carrier for the tungsten particles instead of macrocarriers used by other device. This device has been tested on several plant tissues including embryogenic suspension cultures of corn and soybean, and leaf tissue of cowpea (Vigna unguiculata) (Finer and McMullen, 1990; Finer et al., 1992). Stable transformation of embryogenic tissue of soybean has been obtained using this bombardment apparatus. However, this device has not been tested on plant roots. The purpose of this study is to develop a suitable protocol for the transfer of foreign gene into corn roots by PIG, especially the transfer of pCPOX1 DNA into corn roots to study the regulation of corn peroxidase in response to ABA treatment.

Particle Bombardment with PIG: Tungsten particles (M10; provided by Sylvania Chemicals/Metals, Towanda, PA) were sterilized in ethanol and the DNA was precipitated according to Vain et al (1992), 20L of DNA (20g), 25 L of tungsten (1 mg), 25 L of 2.5 M CaCl2, and 10 L of 100 mM spermidine (free base) were mixed and placed on ice. After 5 minutes, 70 L of supernatant were removed and discarded.

For bombardment, 5 L of the particle suspension was placed in the center of the screen in a syringe filter unit. The syringe filter unit was assembled and then screwed into the needle adaptor. Corn H60 seedlings, in Petri dishes, were placed on a adjustable shelves 17 cm from the screen. The roots were bombarded with the prechamber (chamber used to hold helium gas) at 100 psi helium pressure.

Post bombardment: seedlings were placed back in the growth chamber for another day to recover from physical bombardment.
Enzyme Assay: Histochemical localization of GUS activity in primary roots was performed as described by Jefferson et al. (1987).

In this study, I have developed a bombardment protocol that consistently provide at least 20 blue foci (blue color spots on the plant tissue where GUS gene expressed) in each root bombardment. Increasing the number of times of bombardment did not show any increase in transient gene expression. Other research on GUS gene expression on corn roots had an average of only 5 blue foci with three bombardments using a BioRad gene transfer device (Lu and Ferl, 1992). Originally, 2 L of precipitated DNA was used for bombardment in cowpea and soybean embryogenic tissue. However, the number of blue foci increased with the increase in DNA concentration up to 5 L per bombardment. While the transient GUS gene expression in cowpea leaves requires gentle shaking of these tissues during the enzyme assay (Finer et al., 1992), I have found that corn roots are damaged by shaking. The results in this study were obtained without shaking of tissues during enzyme assays.
Figure 21. Transient expression of the GUS gene in corn root bombarded with baffle (100 PSI, tissue 17 cm from syringe filter). Plasmid DNA used was pGB5.
Figure 22. Transient expression of the GUS gene in corn root bombarded with baffle (100 PSI, tissue 17 cm from syringe filter). Plasmid DNA used was pBARGUS.
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