1995

Molecular and biochemical investigation of the mechanisms of acclimation to chilling stress in maize seedlings

Marc David Anderson
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Botany Commons, and the Molecular Biology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/10878

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Molecular and biochemical investigation of the mechanisms of acclimation to
chilling stress in maize seedlings

by

Marc David Anderson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Botany
Interdepartmental Major: Plant Physiology

Approved:
Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy.

For the Interdepartmental Major
Signature was redacted for privacy.

For the Major Department
Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1995
# TABLE OF CONTENTS

Abbreviations .................................................................................. v
Acknowledgments .............................................................................. vi
Abstract .......................................................................................... vii
Chapter 1. Introduction ..................................................................... 1
  Dissertation Organization ................................................................. 1
  Low Temperature Stress .................................................................. 1
  Effects of Chilling on Plant Metabolism ......................................... 2
    Respiration .................................................................................. 4
    Carbohydrate Metabolism ............................................................... 7
    Desiccation .................................................................................. 8
    Photosynthesis ............................................................................ 9
    Photoinhibition / Oxidative Stress in Chloroplasts ...................... 11
    Active Oxygen Generation in Microbodies, Mitochondria, and the
      Cytosol .................................................................................. 14
  Project Goals .................................................................................. 16

Chapter 2. Differential Gene Expression in Chilling-
Acclimated Maize Seedlings and Evidence for The Involvement of Abscisic Acid in Chilling
Tolerance ....................................................................................... 18
  Abstract ....................................................................................... 18
  Introduction .................................................................................. 20
Ascorbate and Glutathione Analyses............................................. 68
Statistical Analyses................................................................. 69

Results ......................................................................................... 69
Hydrogen Peroxide Levels......................................................... 69
Region of CAT and POX Induction in the Mesocotyl ................. 70
Catalase Isozymes ................................................................. 74
Peroxidase Isozymes ............................................................... 81
Lignin Content.......................................................... 87
Superoxide Dismutase and Ascorbate Peroxidase Isozymes ...... 87
Glutathione Reductase Isozymes ............................................... 90
Ascorbate and Glutathione Levels ............................................... 93
Effects of ABA Treatment ......................................................... 95

Discussion ............................................................................... 104
The Role of Catalase during Acclimation and Chilling .......... 104
The Role of Peroxidase during Acclimation and Chilling .......... 106
The Role of Glutathione Reductase during Acclimation and Chilling ................................................................. 108
Effect of Acclimation and Chilling on the Steady State Pools of Ascorbate and Glutathione ............................................. 110
Effect of ABA on CAT, POX, and GR ............................. 112

Acknowledgments .......................................................... 114

CHAPTER 4. SUMMARY AND CONCLUSIONS ...................... 115

REFERENCES ........................................................................ 123
ABBREVIATIONS

Seedling treatments
ABA .................. 3 d old seedlings treated with ABA of various concentrations
ABACH ............... ABA-treated seedlings subsequently exposed to 4°C (chilling)
ABA@4 .............. Prechilled seedlings treated with ABA
Ac .................... 3 d old seedlings exposed to 14°C (acclimation)
AcABA .............. ABA-treated seedlings subsequently exposed to acclimation, then chilling
AcCh ................. Acclimated seedlings subsequently exposed to chilling
Ch .................... 3 d old seedlings exposed to 4°C (chilling)
Cont, Cont3d ...... 3 d old seedlings (control)
Cont4d .............. 4 d old seedlings

Other abbreviations
APX ................. ascorbate peroxidase
AsA ................. ascorbic acid (reduced form)
AT .................... 3-amino-1,2,4-triazole
car .................. chilling acclimation responsive
CAT .................. catalase
DHA ................. dehydroascorbate
GR ................. glutathione reductase
MTT .................. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT .................. 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'(3,3'-dimethoxy-4,4'-
                    diphenylene)ditetrazolium chloride
POX ................. guaiacol peroxidase
SOD ................. superoxide dismutase
TEMED ............. N,N,N',N'-tetramethylethylenediamine
ACKNOWLEDGMENTS

I would like to express sincere thanks to my advisor, Dr. Cecil Stewart for his guidance during the course of this project. I am especially appreciative of his willingness to share his experience, professional or otherwise, in effort to prepare me for a career in science.

The success of this project is directly attributable to the contributions of Dr. T.K. Prasad. We have worked closely on nearly all aspects of this project and much of what I learned has been from his instruction. Hopefully, we will find the opportunity to work together and continue our friendship in the future.

I am grateful to the members of my dissertation committee, Drs. Jim Colbert, Alan Knapp, Barry Martin, and Richard Shibles for their efforts in reviewing the dissertation.

I am fortunate to have been a member of the botany department and the IPPM program. The many friendships and associations have made this an enjoyable place to live and work.

To my wife, Kim, and my three boys, Wesley, Robert, and Nicholas; I am very grateful for the love and support that you have shared over the years. Please know that you, and our savior, Jesus Christ, will always be first in my life.
ABSTRACT

Seedlings of chilling-sensitive maize inbred G50 (Pioneer) survived poorly when exposed to a chilling stress. Acclimation or ABA treatment caused a dramatic improvement of chilling tolerance. Differences in gene expression between acclimated and unacclimated seedlings were investigated and three cDNA clones (designated car333, car30, and car757) were isolated, representing transcripts that were up-regulated during acclimation. Car30 and car757 were not homologous to any known sequence but car333 was 97.8% homologous to cat3, maize mitochondrial catalase. The up-regulation of CAT3 during acclimation suggested that chilling stress caused the generation of H$_2$O$_2$ in the mitochondria and increased catalase activity was needed to prevent oxidative damage. Measurement of H$_2$O$_2$ revealed a 4-fold increase in the coleoptile+leaf and mesocotyl of chilled seedlings but H$_2$O$_2$ was maintained near unchilled levels in seedlings that were acclimated before chilling. In effort to explain the acclimation-induced protection from oxidative stress, isozyme profiles of catalase, guaiacol peroxidase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase were examined. None of these enzymes responded to acclimation in the coleoptile+leaf. However, examination of ascorbate and glutathione pools revealed an increased synthesis of glutathione, which might be responsible for the protection of the coleoptile+leaf from oxidative stress. In the mesocotyl, superoxide dismutase and ascorbate peroxidase activities were unaffected but those of catalase, peroxidase, and glutathione reductase were altered by acclimation. It was proposed that induction of CAT3 may serve as the
first line of defense against mitochondria-generated H$_2$O$_2$. Induction of cell wall peroxidases may be responsible for the observed increase in lignin content, which would serve to improve the mechanical strength of mesocotyls and allow them to remain intact when exposed to a chilling stress. Cytosolic glutathione reductase may have been shifted to forms that were more active at low temperature. All of these changes illustrated the numerous ways that chilling tolerance was enhanced in the mesocotyl. In contrast to acclimation, the response of antioxidant enzymes to ABA was dramatically different. Despite the fact that both ABA and acclimation induced chilling tolerance, the mechanisms of action were clearly different, at least in terms of antioxidant defenses.
CHAPTER 1. INTRODUCTION

Dissertation Organization

This dissertation consists of a general introduction (Chapter 1), two individual manuscripts (Chapters 2 and 3) and a general conclusion (Chapter 4). Chapter 1 outlines the effects of low temperature stress on plant metabolism and introduces the project goals. The manuscript presented as Chapter 2 documents the existence of a chilling acclimation phenomenon in dark-grown maize seedlings and examines the possibility that ABA mediates the acclimation process. The discovery that catalase transcripts were up-regulated during acclimation initiated studies on the importance of antioxidant defenses in chilling tolerance. This manuscript has been published in the May, 1994 issue of Plant Physiology 105: 331-339. Permission has been granted from the American Society of Plant Physiologists to include this copyrighted material in the dissertation. The manuscript presented as Chapter 3 involves a more detailed look at the antioxidant defenses of maize seedlings and their role in chilling tolerance. This manuscript has been submitted to Plant Physiology. Chapter 4 summarizes the acclimation project as a whole. Literature cited throughout the dissertation has been compiled into a single "References" section.

Low Temperature Stress

With agricultural practices tending toward earlier planting and expanding the geographic range of a crop, the ability to tolerate low temperatures is a valuable agronomic trait. Crops adapted to the temperate climate, such as the small
grains, are generally considered resistant to chilling temperatures (0-10°C, Levitt, 1980). Although agronomic performance may be greatly affected by extended periods of low temperature, irreversible damage only occurs in these crops when they are exposed to freezing temperatures. The ability to tolerate temperatures below freezing in these crops has been attributed to the ability to avoid or confine the damaging effects of ice formation (Guy, 1990). In contrast, crops of tropical origin, such as maize, can be irreversibly damaged by chilling temperatures. The effects of chilling on metabolism in chilling-sensitive crops has been extensively studied (see reviews by Graham and Patterson, 1982; Wang, 1982; Markhart, 1986) and through knowledge of the effects of chilling, it becomes possible to envision strategies to improve chilling tolerance.

**Effects of Chilling on Plant Metabolism**

Levitt (1980) generalized chilling as having three direct effects on sensitive plants; altered reaction kinetics, protein denaturation, and phase transitions of membranes. The rates of all metabolic reactions are temperature-dependent. Differences in the susceptibility of enzymes to low temperature, in terms of activation energies, would result in a disruption of metabolic coordination and would be observed as a shift in metabolite pools. Numerous examples of cold induced inactivation of soluble enzymes exist (Graham and Patterson, 1982). Hydrophobic interactions among subunits of enzymes such as pyruvate P_i dikinase and phosphofructokinase are weakened by low temperature resulting in a loss of quaternary structure. A cold-induced conformational change in rubisco results in its inactivation (Chollet and Anderson, 1977).
Perhaps the most far-reaching effect of low temperature is through its effects on membranes. Membranes exist in a fluid state. Many membrane-associated proteins are dependent on an optimal level of fluidity, both in maintaining conformation and in some cases such as the light harvesting complex in the thylakoids, the ability to migrate along the membrane. At some temperature, membranes begin to solidify which severely disrupts the function of membrane-associated proteins. Armand and Staehelin (1979) observed the lateral and vertical displacement of proteins away from regions of the membrane in the solid phase, using freeze-fracture electron microscopy. The temperature at which the phase transition occurs has been correlated with susceptibility to low temperature. Lyons and Raison (1970) showed that the activation energy of succinate dehydrogenase (association with the mitochondrial inner membrane) was sharply increased at a specific temperature within the chilling range and attributed this to the point at which the membrane underwent a phase transition. Succinate dehydrogenase from chilling resistant species had a constant activation energy. In addition to affecting membrane-associated proteins, a phase change can greatly alter the permeability of the membrane to small molecules (Levitt, 1980). The increased leakiness of membranes leads to a loss of compartmentalization, collapsed gradients, and a disrupted metabolism.

The temperature at which a phase transition occurs appears to be dependent on the lipid composition, with greater unsaturation promoting a lower phase transition temperature. Numerous examples exist where chilling tolerance is correlated with the extent of lipid unsaturation (e.g. Roughan, 1985; Hugly and Somerville, 1992; Riken et al., 1993) and efforts to alter the degree of
unsaturation of membrane lipids have resulted in improved chilling tolerance (Murata et al., 1992; Kodama et al., 1994). However, some have disputed the idea that the degree of lipid unsaturation in a membrane is the sole determinant of the phase transition temperature. Kenrick and Bishop (1986) failed to find a correlation between the extent of membrane lipid unsaturation and chilling tolerance among plant species of differing chilling tolerance. Webb et al. (1992) demonstrated that reconstituted thylakoid lipid vesicles did not exhibit a phase change at any temperature above 0°C, even when the proportion of saturated lipids greatly exceeded that found in thylakoids of chilling sensitive plants. Thus, there are likely other membrane qualities that contribute to the sensitivity of the membrane to low temperature. Sharom et al. (1994) have even contested the idea that low temperature directly causes the phase change and have suggested that it is the result of biochemical events occurring during rewarming. Although it is clear that low temperature affects the function of membranes and membrane-associated proteins and that a phase change is associated with chilling injury, there remains much to be discovered about the mechanism by which low temperature affects membrane function and about the qualities of the membranes that are important for chilling tolerance.

**Respiration**

Through the direct effects of chilling on proteins and membranes, chilling affects many aspects of primary and secondary metabolism. The effect of chilling on respiration appears to depend on a number of factors including severity and duration of exposure and on the susceptibility of the plant to chilling.
Generally, respiration declines during chilling stress, sharply increases during rewarming, and, if chilling injury is not permanent, declines to prechilled levels after a period of recovery (Lyons, 1973). This increase in respiration has consequently been used as a marker for chilling stress. In plants tolerant to the imposed low temperature treatment, the burst in respiration is not observed (Lyons, 1973). Respiratory rates are usually measured in terms of oxygen consumption and it should be noted that this represents oxygen utilized not only by the terminal oxidase and the alternative oxidase of the mitochondrial electron transport chain but also by other sources (residual). Thus, while the burst in oxygen consumption during rewarming may represent an increased energy need during a recovery process, the residual oxygen consumption (which is not necessarily important for recovery but may instead be a symptom of stress) may contribute substantially to the total oxygen consumption in chilled plants (Prasad et al., 1994b).

Chilling causes a decline in the activity of the terminal oxidase and an increase in the activity of the alternative oxidase (Leopold and Musgrave, 1979; Kiener and Bramlage, 1981; Van de Venter, 1985; Prasad et al., 1994b). The deleterious effects of chilling on membrane-associated proteins has been suggested as the cause of the decline in cyanide-sensitive electron transport (Kiener and Bramlage, 1981; Purvis and Shewfelt, 1993). This may lead to an increased generation of superoxide as electrons are transferred to ground state oxygen in the matrix (potentially a source of "residual" oxygen uptake). In addition, protein levels of the terminal oxidase decline in response to chilling (Prasad et al., 1994b) leading to a decreased capacity of cyanide-sensitive
electron transport. The increase in the alternative oxidase capacity has been shown to be due to an increase in the levels of alternative oxidase protein (Stewart et al., 1990b; Vanlerbergh and McIntosh, 1992). However, when a relatively chilling tolerant maize line (B73) was exposed to nonchilling low temperatures (14°C), no change in the alternative oxidase capacity was observed unless the low temperature treatment was applied during early seedling growth (Stewart et al., 1990a). If the complete mitochondrial complement has been established at normal growth temperatures, subsequent exposure to low temperature does not affect the alternative oxidase capacity. When a chilling sensitive maize line (G50) was exposed to a chilling temperature (4°C), the alternative oxidase capacity was significantly elevated (Prasad et al., 1994b). The role of the alternative oxidase during chilling is unknown but its capacity has been shown to be higher in plants with a greater degree of chilling tolerance (Van de Venter, 1985; Stewart et al., 1990b). It has been suggested that it may serve as a nondestructive outlet for electrons during conditions when the supply of respiratory electrons exceeds the capacity of the terminal oxidase (Purvis and Shewfelt, 1993). This was supported by the work of Vanlerberghe et al. (1994) in that aox1 antisense mutants of tobacco were unable to survive antimycin A treatment (inhibits the terminal oxidase) while the wild type survived.

In addition to effects on the terminal oxidase and alternative oxidase, another component of the electron transport chain that is severely affected by chilling is succinate dehydrogenase. Lyons and Raison (1970) have demonstrated that in chilling sensitive plants, succinate dehydrogenase was inactivated by chilling temperatures, possibly due to a disruption in its function as the mitochondrial
inner membrane underwent a phase change. Another Krebs cycle enzyme, isocitrate dehydrogenase was inactivated by chilling while malate dehydrogenase wasn't greatly affected in germinating soybean embryonic axes. Further, glutamate dehydrogenase was also inhibited, restricting the flow of \( \alpha \)-ketoglutarate from the cycle (Duke et al., 1977). Buescher (1975) observed an increase in citrate and a decrease in malate in tomato pericarp tissue in response to low temperature, which would be consistent with the effects of chilling on Krebs cycle enzymes. Two enzymes of glycolysis, phosphofructokinase (Graham and Patterson, 1982) and glyceraldehyde-3-phosphate dehydrogenase (Guy, 1990) and one enzyme of the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase (Duke et al., 1977) are also known to be inactivated by chilling. Along with the reduction in the terminal oxidase, all of these changes illustrate how respiration is greatly reduced by chilling, and how greater susceptibility of some respiratory components to chilling relative to others can lead to dramatic changes in the pools of various metabolites.

**Carbohydrate Metabolism**

The inactivation of phosphofructokinase has been suggested as contributing to the observed increase in simple sugars, especially sucrose (Graham and Patterson, 1982). Increases in sucrose phosphate synthase (Guy et al., 1992), invertase (Graham and Patterson, 1982), and a decrease in starch synthetase (Guy, 1990) all contribute in the direction of carbohydrate metabolism toward the accumulation of sucrose. Rather than as a symptom of chilling injury, this is
usually thought of as a response by the plant to protect itself during exposure to low temperature. It has been suggested that sucrose may function as a cryoprotectant during freezing injury or as an osmolyte, to help maintain cell turgor during the desiccation associated with low temperature stress (Guy et al., 1992).

**Desiccation**

That low temperature causes desiccation in some plants has been well established (Emaus et al., 1983; Capell and Dorffling, 1989; Pardossi et al., 1992) and it likely contributes to chilling injury. While freezing-induced desiccation is more severe and involves loss of available water as ice is formed (Guy, 1990), chilling-induced desiccation is more likely due to a decreased hydraulic conductance of the roots (Markhart, 1984; Bassiri Rad and Radin, 1992) and by interference with stomatal closure (Emaus et al., 1983; Zhang and Davies, 1990). Chilling tolerance has been associated with the ability to synthesize ABA resulting in a resistance to water stress (Capell and Dorffling, 1989). Exogenous application of ABA (Pardossi et al., 1992) or artificial induction of ABA with mefluidide (Zhang et al., 1986) resulted in resistance to both chilling and desiccation. However, numerous examples exist where chilling tolerance was promoted by ABA despite the fact that, in these cases, chilling had no effect on water potential (Daie and Campbell, 1981; Lalk and Dorffling, 1985; Lang et al., 1994). Thus, in some, if not all cases, ABA promotes chilling tolerance by mechanisms beyond that of maintenance of water potential. There have been suggestions that ABA has a stabilizing effect on membranes.
which would prevent many of the symptoms associated with chilling injury. There are numerous examples of common induction of genes by both chilling and ABA (e.g. Kurkela and Franck, 1990; Guo et al., 1992; Lee and Chen, 1993) thus, ABA may also be acting as a factor in signal transduction. However, the mechanism by which ABA induces chilling tolerance, beyond that of regulating water status, has yet to be completely defined.

Photosynthesis

A marked decline in photosynthesis is observed in response to chilling stress. It has been suggested that limited CO₂ availability, a result of chilling-induced water stress and stomatal closure, is partly responsible for the decline in photosynthesis (Markhart, 1986). Many of the enzymes involved in photosynthetic carbon fixation are affected by low temperature. As previously stated, rubisco conformation is altered by low temperature, resulting in its inactivation (Chollet and Anderson, 1977). There is also evidence that since rubisco activase requires an association with thylakoid membranes, chilling-induced damage to thylakoid membranes would prevent stimulation of rubisco activase and consequently, prevent activation of rubisco (Byrd et al., 1995). Further, since rubisco requires a stromal pH of about 8.0 for activation, a loss of compartmentation due to chilling could potentially collapse the pH gradient and prevent rubisco activation. Even though rubisco activity declines in response to chilling, the pool size of RuBP also declines and the reason for this is due to the chilling sensitivity of the two bisphosphatases, fructose-1,6-bisphosphatase and
sedoheptulose-1,7-bisphosphatase, in the regenerative phase of the Calvin cycle (Sassenrath et al., 1990). In addition, four of the enzymes of the Calvin cycle (triose phosphate dehydrogenase, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulose kinase) are regulated by a thioredoxin mediated control of their redox state. It is possible that the increased oxidative state of the stroma during chilling (see following discussion) may serve to inactivate these enzymes. Lastly, several enzymes important in the CO2 concentrating system of C4 plants are inactivated by chilling, including NADP-malate dehydrogenase and pyruvate Pi dikinase (Taylor et al., 1974). PEP carboxylase is particularly susceptible to low temperature (Uedan and Sugiyama, 1976). Inhibition of these enzymes would greatly diminish the availability of CO2 to the bundle sheath cells, limiting photosynthesis. Overall, the effect of these changes in enzyme activities in response to chilling is a dramatic reduction in the ability of the plant to fix carbon.

While chilling certainly affects the fixation of carbon, perhaps the most devastating effect of chilling is on the photosystems. Chilling injury is dependent on light intensity and is manifested as an increase in fluorescence, destruction of chlorophyll, increased lipid peroxidation, ultrastructural damage to the thylakoids, a disruption in electron transfer, and a reduction in photosynthetic rate (Wise and Naylor, 1987a; Peeler and Naylor, 1988). Thus, the damage caused by chilling is manifested as photoinhibition, probably by a mechanism similar to that caused by excessive light (Krause, 1988). Photoinhibition is the decline in photosynthesis due to light-dependent generation of active oxygen species.
(\textsuperscript{1}O_2, O_2^-, H_2O_2, and OH\textsuperscript{-}) which are responsible for the observed damage symptoms.

**Photoinhibition / Oxidative Stress in Chloroplasts**

Chilling likely promotes the generation of active oxygen by causing a phase change in the thylakoid membrane or by altering the characteristics of the component proteins. The result is a restriction in the flow of electrons through the photosystems. When the light energy captured by the light harvesting complex exceeds the flow of electrons through the photosystems, it can be dissipated either as heat, radiationless transfers, fluorescence, or by transfer to carotenoids. Excess light energy can also be transferred to molecular oxygen to form singlet oxygen (\textsuperscript{1}O_2), a highly reactive and destructive active oxygen species (Salin, 1988). Carotenoids and \(\alpha\)-tocopherol within the thylakoid membrane and ascorbate in the stroma serve to quench \(\textsuperscript{1}O_2\) as it is formed (Knox and Dodge, 1985). Superoxide (O_2\textsuperscript{-}) is another active oxygen species that can arise as electrons are donated to molecular oxygen from reduced ferridoxin, and possibly other components of photosystem I (Halliwell, 1987). A copper / zinc superoxide dismutase is present in the stroma to react with O_2\textsuperscript{-} to form hydrogen peroxide (H_2O_2). When O_2\textsuperscript{-} and H_2O_2 are present in high levels in the same compartment, they can react with each other to form hydroxyl radical (OH\textsuperscript{-}, Haber and Weiss, 1934), another highly destructive active oxygen species. To prevent this reaction from occurring, H_2O_2 scavenged by a system in which electrons from NADPH are shuttled through glutathione and ascorbate to reduce H_2O_2 to water (Foyer and Halliwell, 1976). Ascorbate peroxidase,
dehydroascorbate reductase, and glutathione reductase are the enzymes responsible for catalyzing this electron transfer system. During photoinhibition, the generation of active oxygen species exceeds the capacity of the scavenging systems and causes destruction of chlorophyll, lipid peroxidation, and other oxidative damage within the chloroplast. Lipid peroxidation is a particularly damaging process in which active oxygen species initiate a chain reaction of oxidative events within a membrane. This results in the release of toxic organic peroxides and aldehydes such as malondialdehyde (McKersie et al., 1990). In addition, the damage to the membrane results in increased permeability to small molecules and thereby exacerbates the effects of chilling on membranes. In addition to rapid active oxygen generation, chilling also causes rapid depletion of ascorbate, carotenoids, and α-tocopherol (Wise and Naylor, 1987b) as well as inactivation of several antioxidant enzymes such as dehydroascorbate reductase, glutathione reductase (Jahnke et al., 1991), and catalase (Feierabend and Engel, 1986). This would compound the problem of oxidative stress in chilling-sensitive plants.

In addition to causing oxidative stress, chilling has also been observed to cause a state transition, so as to favor photosystem I over photosystem II (Baker et al., 1983). This would promote cyclic over non-cyclic electron transport resulting in a decreased quantum yield. It could also further decrease electron flow relative to photon capture and contribute to oxidative stress. This state transition may also be a result of a chilling-induced phase change of the thylakoid membrane, which could cause displacement of the photosystems relative to each other. Evidence for this was provided by Murata et al. (1975)
when they showed that the shift from state 1 to state 2, induced by varying the light quality to favor one or the other state, was prevented at temperatures at and below the phase transition temperature of the membrane. This result suggested that the pigment systems were unable to interact, due to the condition of the membrane. On the other hand, thylakoid membranes from chilling resistant plants showed the expected state transitions.

Differences in the susceptibility to photoinhibition among plants have been observed (e.g. Hetherington et al., 1989; Hodgson and Raison, 1991) but the factors responsible for increased tolerance have not been demonstrated in most cases. Gombos et al. (1994) showed that the extent of unsaturation in membrane lipids did not affect the development of chilling-induced photoinhibition but instead, may enhance recovery. Jimenez and Pick (1993) showed that differences in carotenoid content had little effect on the tolerance to photoinhibition. However, enhancement of antioxidants may be a primary mechanism of photoinhibition tolerance at low temperature. Sen Gupta et al. (1993) demonstrated that chilling-induced photoinhibition was reduced in transgenic tobacco that overexpress pea Cu/Zn superoxide dismutase. Likewise, Foyer et al. (1991) were able to improve tolerance to oxidative stress in transgenic tobacco that overexpress a bacterial glutathione reductase. Artificial elevation of \( \alpha \)-tocopherol and ascorbate using the fungicide, uniconizole, resulted in a reduction in the symptoms of photoinhibition (Seneratna et al., 1988). Numerous examples exist where chilling tolerance was correlated with the levels of antioxidants (e.g. Clare et al., 1984; Jahnke et al., 1991; Feierabend
et al., 1992; Walker and McKersie, 1993). Thus, it appears that antioxidants play a key role in the protection of chloroplasts from oxidative stress during chilling.

**Active Oxygen Generation in Microbodies, Mitochondria, and the Cytosol**

Chilling-induced active oxygen generation is not limited to the chloroplasts but likely arises from a number of sources within the cell. H$_2$O$_2$ is generated as a by-product of normal metabolic processes such as photorespiration and $\beta$-oxidation of lipids and these reactions are housed in the microbodies (Tolbert, 1971). Various oxidases are also present in the microbodies that generate either O$_2^-$ or H$_2$O$_2$ (Elstner, 1991). The O$_2^-$ that is formed is reduced to H$_2$O$_2$ by either an iron or a manganese superoxide dismutase and the high level of H$_2$O$_2$ produced from all of these sources in the microbodies is detoxified by catalase. Presumably, the isolation of all of these reactions into one compartment along with high levels of catalase is an efficient way to detoxify H$_2$O$_2$ while minimizing exposure to other cellular components. Chilling has been shown to cause a net reduction in peroxisomal catalase activity in the light, both through an oxidative destruction of catalase (Feierabend and Engel, 1986) and by depressing catalase synthesis (Feierabend et al., 1992). The loss of catalase in the peroxisome would make this organelle a prime source of oxidative stress during chilling in the light.

O$_2^-$ generation from sites of rapid electron transport appears to be particularly prominent during chilling. The electron transport chain of the mitochondria may be a significant source of O$_2^-$ production as electron flow is restricted by the effects of chilling. The site of O$_2^-$ production appears to be from
the NADH dehydrogenases and from the ubiquinone pool (Rich and Bonner, 1978; Freeman and Crapo, 1982). \(O_2^-\) generated in the mitochondria is scavenged by a manganese superoxide dismutase, producing \(H_2O_2\). The \(H_2O_2\) scavenging system in mitochondria is not well known, but catalase may be involved, at least in maize (Scandalios et al., 1980). Glutathione reductase (Edwards et al., 1990) and a cytochrome c peroxidase (T.K. Prasad, M.D. Anderson, C.R. Stewart, manuscript submitted) have also been localized in mitochondria and may contribute to the scavenging of \(H_2O_2\). Also, as previously stated, the alternative oxidase may utilize excess electrons in a nondestructive manner, decreasing the likelihood of \(O_2^-\) production.

Another site of electron flow is the cytochrome \(P450\) complex on the endoplasmic reticulum which serves as a mixed-function oxidase in various hydroxylation reactions. It has also been suggested as a source of \(O_2^-\) generation (Freeman and Crapo, 1982; Elstner, 1991). Other sources of active oxygen in the cytosol are not well known but it is thought that an NADH oxidase on the plasma membrane may generate \(O_2^-\) (Elstner, 1991). The function of this enzyme in unstressed plants is unknown. It has also been suggested that much of the hydrogen peroxide in the cytosol emanates from the organelles (Puntarulo et al., 1991). The hydrogen peroxide scavenging system in the cytosol is also not well known but there is increasing evidence that an ascorbate-glutathione cycle, similar to that in the chloroplasts is in operation (Alscher, 1989). In addition, it has been suggested that \(H_2O_2\) may diffuse into the vacuole to be detoxified by a guaiacol peroxidase, using any of various phenylpropanoids as the electron donor (Takahama, 1991).
Overall, there are many potential sources of active oxygen which may be affected by chilling. As in the chloroplasts, if the generation of active oxygen from any of these sources exceeds the capacity of the scavenging systems, oxidative stress can result. The diversity of active oxygen sources throughout the cell illustrates the need for a broad range of antioxidant protection mechanisms and each may potentially contribute to the protection of the cell from chilling-induced oxidative stress.

Project Goals

Susceptibility to low temperature is certainly a limiting factor in the stand establishment of maize. Pioneer inbred G50 is particularly susceptible. While the production qualities of G50 are otherwise satisfactory, poor stands after exposure to low temperature have limited its usefulness as a marketable line. However, it has been observed that exposure of G50 to a noninjurious low temperature induces a degree of chilling tolerance, allowing it to survive subsequent exposure to more severe low temperatures. This acclimation phenomenon provided an opportunity to examine the molecular basis for this improvement in chilling tolerance. Mechanisms of chilling tolerance are often studied by comparing tolerant with susceptible plants of different genetic origin. By utilizing acclimation, differential chilling tolerance is observed in plants of identical genetic origin, allowing the use of subtraction methodologies to identify transcripts that may be important in chilling tolerance. Therefore, it was the goal of this project, first to document the existence of an acclimation phenomenon, then to determine the molecular basis for acclimation-induced chilling tolerance.
This might then lead to identification of the genes and their associated metabolic processes that are responsible for chilling tolerance, possibly providing candidates for genetic enhancement of chilling tolerance in maize seedlings.
CHAPTER 2. DIFFERENTIAL GENE EXPRESSION IN CHILLING ACCLIMATED MAIZE SEEDLINGS AND EVIDENCE FOR THE INVOLVEMENT OF ABSCISIC ACID IN CHILLING TOLERANCE

Manuscript published in Plant Physiology 105: 331-339

Marc D. Anderson, Tottempudi K. Prasad, Barry A. Martin and Cecil R. Stewart

Abstract

An acclimation phenomenon was characterized in seedlings of chilling sensitive maize (Zea mays L.) inbred G50 (Pioneer). Seedlings were germinated at 27°C for 3 d, then exposed to chilling treatments of 4°C, 5°C, or 6°C for 2 d, 4 d, 7 d, or 10 d in darkness. Damage symptoms in the more severe treatments included a waterlogged appearance and a discoloration of the tissue. The symptoms were most obvious in the mesocotyl. After a 10 d grow-out period in

1This research was supported by grants from Pioneer Hi-Bred International (C.R.S.) and USDA NRICGP #92-37100-7646 (C.R.S., T.K.P.).

2Corresponding author

Authors' contributions: The cDNA library was constructed by Prasad. Isolation and characterization of differentially expressed transcripts were conducted by Anderson and Prasad in conjunction. All other experiments, statistical analyses, and manuscript preparation were conducted by Anderson. All authors participated in the conception and planning of the research.
the greenhouse, moderately damaged seedlings exhibited chlorotic areas, an occasional disruption in leaf expansion, and a constriction of the mesocotyl. Growth and survival were improved by first exposing seedlings to a 14°C acclimation treatment for 3 d before applying the chilling treatment. After chilling at 5°C for 7 d, 79% of the acclimated seedlings survived whereas only 22% of the unacclimated seedlings survived.

Differences in gene expression between acclimated and control seedlings were investigated using subtraction and differential screening techniques. Transcripts corresponding to three genes; car333, car30, and car757 (chilling acclimation responsive); were present in higher levels in seedlings after acclimation. Sequence analysis identified car333 as cat3, which encodes maize mitochondrial catalase. Characterization of these three clones revealed that all corresponding transcripts were elevated in acclimated seedlings in a manner that depended on the organ; coleoptile+leaf, mesocotyl, or root. While transcripts were elevated in all three organs in response to acclimation, car30 was most abundant in the coleoptile+leaf and root, whereas cat3 and car757 were most abundant in the coleoptile+leaf and mesocotyl. Catalase activity followed the same general trend as cat3 transcript levels.

Exogenous treatment with ABA resulted in an improvement in growth and survival of unacclimated, chilled seedlings. Inhibition of ABA biosynthesis with fluridone abolished acclimation-induced chilling tolerance and exogenous application of ABA to fluridone-treated seedlings restored chilling tolerance. Exogenous ABA treatment also resulted in increases in cat3, car30, and car757 transcript levels and catalase activity in the same organ-specific manner as in
acclimated seedlings. These results indicate that ABA synthesis is essential for chilling tolerance. However, measurement of ABA levels in mesocotyls during acclimation and chilling revealed only a marginal increase during acclimation and a dramatic increase during chilling, regardless of whether or not seedlings were acclimated. Thus, although ABA may be required for chilling tolerance, we have no conclusive evidence that the acclimation process is mediated by ABA.

Introduction

Chilling damage results from exposure of a chilling-sensitive plant to low, nonfreezing temperatures. Visual symptoms of chilling damage in vegetative tissue often include surface lesions and necrotic areas, a waterlogged appearance, internal discoloration, wilting, acceleration of senescence, a reduction in growth, and death (Saltveit and Morris, 1990). On the cellular and molecular levels, Levitt (1980) generalized chilling stress as having three direct effects; reduction of metabolic rates, reduction of membrane fluidity, and denaturation of proteins. Overall, the documented effects of chilling on metabolism are numerous (see reviews by Graham and Patterson, 1982; Wang, 1982; Markhart, 1986) and have been reported as affecting respiration, photosynthesis, phenolic metabolism, sugar metabolism, redox regulation, and others. It is the means by which some plants are able to survive these effects that is of interest, especially in the development of chilling tolerance in agriculturally important plants.

Many plants acclimate to low temperatures. Exposure to noninjurious low temperatures allows plants to acquire the capability to survive damaging
temperatures. Changes in gene expression have been observed during acclimation to freezing in *Arabidopsis* and wheat (Lin et al., 1990), alfalfa (Mohapatra et al., 1987), and spinach (Guy et al., 1985) and to chilling in rice (Hahn and Walbot, 1989). Biochemically, most theories on the mechanism of acclimation involve the maintenance of membrane fluidity by higher lipid unsaturation, the maintenance of enzymes that would otherwise be altered in nonacclimated plants, or increases in protective substances (Levitt, 1980; Guy, 1990). Much remains to be discovered about how these metabolic changes coordinate to produce a level of low temperature tolerance during acclimation.

There has been considerable interest in the role of ABA in mediating tolerance to low temperature (either chilling or freezing, depending on the plant species under study). Elevated ABA levels have been observed in plants exposed to low, nonfreezing temperatures (Riken et al., 1976; Daie and Campbell, 1981; Lalk and Dorffling, 1985) and exogenous application of ABA has been shown to confer freezing tolerance to alfalfa (Mohapatra et al., 1988), and to *Arabidopsis* (Lang et al., 1989) and chilling tolerance to maize cell suspension cultures (Xin and Li, 1992). Heino et al. (1990) demonstrated that ABA deficient *Arabidopsis* has low tolerance to freezing while Li (1991) showed that artificial induction of endogenous ABA by mefluidide conferred chilling tolerance to maize, cucumber, and pepper and freezing tolerance to wheat, lemon, and crabapple but not potato which is incapable of being acclimated. Lee et al. (1993) attributed the difference between chilling sensitive and chilling resistant rice to the ability to increase ABA levels in response to chilling stress. Even though the evidence is strong that ABA is involved in low temperature
tolerance, the means by which ABA acts to produce tolerance is poorly understood. Numerous studies have shown common induction of genes (Kurkela and Franck, 1990; Guo et al., 1992; Lee and Chen, 1993) and proteins (Lang et al., 1989; Xin and Li, 1993) by both ABA and low temperature, thus it is likely that ABA and low temperature utilize some of the same mechanisms in producing their effect.

In this study, we have described chilling acclimation in seedlings of chilling sensitive maize inbred G50 (Pioneer). The existence of an acclimation phenomenon provided a means to compare genetically identical plants, differing only in the level of chilling tolerance, for differences in gene expression. We have identified transcripts from three car (chilling acclimation responsive) genes and have characterized their expression. We have also explored the effect of ABA on expression of these three car genes and the role of ABA in chilling tolerance and during chilling acclimation in maize seedlings.

Materials and Methods

Seedling Growth and Survival

Chilling sensitive maize inbred G50 (Pioneer Hi-Bred International Inc., Johnston, IA) was used to test whether acclimation or ABA treatment could improve chilling tolerance. Seeds were planted in Redi-Earth Peat-Lite Mix (Grace Sierra Horticultural Products Co., Milpitas, CA) and grown at 27°C for 3 d in darkness and seedlings were then acclimated by exposure to 14°C for 3 d in darkness. Control seedlings did not receive a 14°C treatment. Little seedling growth occurred during the acclimation treatment and there was little difference
in development between acclimated and control seedlings despite differences in age. Acclimated or control seedlings were then exposed to chilling treatments of 4°C, 5°C, or 6°C (all incubator temperatures in this and subsequent experiments fluctuate approximately ± 0.5°C) for 2 d, 4 d, 7 d, or 10 d. The number of surviving seedlings were counted and dry weights (five seedlings were sampled when available) were taken both before and after a 10 d grow-out period in the greenhouse (22±3°C, 16:8 (L:D), fertilized once with 20/20/20 (NPK) Peatlite). Unchilled controls were also included. Seedlings with extensive necrosis, a severely constricted mesocotyl, or with no evident growth were considered dead and were not included in the final counts. Only the survivors were included in the final weights.

Acclimation status and chilling duration were replicated 3 or 4 times within a temperature. Percent survival and dry weights were analyzed as a factorial design with acclimation status, chilling temperature, chilling duration, and replicate within temperature as the main effects. Because temperature was not randomized with acclimation status and chilling duration within a replication, replication within temperature was used as the error to test for temperature effects. Unchilled controls were replicated seven times and were analyzed separately.

To determine whether exogenous application of ABA affected chilling tolerance, seedlings were exposed to 0, 100, 333, or 1000 µM ABA (mixed isomers, Sigma). Seedlings were germinated in germination paper at 27°C for 3 d in darkness. The imbibing solution was then replaced with the appropriate ABA solution and seedlings were allowed to grow an additional 24 h. Seedlings
were transplanted into Redi-Earth and allowed to become established at 27°C for 12 h after which they were transferred to 4°C for 7 d. As with the acclimation experiment, seedling counts and dry weights were taken both before and after a 10 d grow-out period in the greenhouse. Treatments were replicated three times and percent survival and dry weights were analyzed as a randomized complete block design. Means were separated using Tukey's studentized range test.

To further investigate the role of ABA in chilling tolerance, fluridone was used to inhibit ABA biosynthesis in attempt to abolish acclimation-induced chilling tolerance and determine whether ABA was required. Fluridone was administered by imbibing seeds in 0.1 mM fluridone/0.1% Tween 20 for 24 h at 27°C in darkness. Fluridone-treated and untreated seeds (imbibed in water) were then planted in Redi-Earth and watered with the same solution with which they were imbibed. The following treatments were then applied:

- Cont........27°C for 2 d
- AcCh........27°C for 2 d, 14°C for 3 d, 4°C for 7 d
- AcABA.....irrigate with 300 μM ABA at planting, 27°C for 3 d, 14°C for 3 d, 4°C for 7 d
- ABACh....irrigate with 300 μM ABA at planting, 27°C for 3 d, 4°C for 7 d
- ABA@4...27°C for 2 d, 4°C for 2 h, irrigate wet media with cold 1 mM ABA (same amount of ABA applied as ABACh), 4°C for 7 d
- Ch..........27°C for 2 d, 4°C for 7 d

Fluridone-treated seeds were not used in the ABA@4 and Ch treatments. Following the above treatments, seedlings were transferred to the greenhouse for a 7 d grow-out period. Since fluridone-treated seedlings contain no
carotenoids, chlorophyll is photooxidized and seedlings survive from their seed reserves. This precluded extension of the grow-out period to 10 d as in the other survival experiments. Seedling counts and dry weights were taken as before. Treatments were replicated three times and percent survival and dry weights were analyzed as a randomized complete block design. Means were separated with Tukey's studentized range test.

Isolation of Differentially Expressed Transcripts

Total RNA was extracted from Cont and Ac seedlings (Chomczynski and Sacchi, 1987) and poly(A)$^+$ RNA was separated on an oligo dT column (Davis et al., 1986). A cDNA synthesis kit (Amersham) was used to construct a cDNA library in λZAPII (Stratagene) with poly(A)$^+$ RNA from Ac seedlings. Blunt ended cDNA was ligated to EcoRI adapters before cloning into the EcoRI site of the vector. The library was screened for clones that were differentially expressed in Ac seedlings by hybridizing to a subtraction probe. The subtraction probe was formed by hybridizing $^{32}$P-labeled first strand (FS) cDNA from Ac poly(A)$^+$ RNA with biotinylated Cont poly(A)$^+$ RNA, and precipitating all RNA-DNA hybrids with avidin. Putative positives were rescreened by differential hybridization to $^{32}$P-labeled FS cDNA from either Ac or Cont poly(A)$^+$ RNA. Phagemids from three differentially expressed plaques were rescued by in vivo excision according to manufacturer's instructions (Stratagene). Phagemid DNA was digested with EcoRI and the inserts were gel purified. $^{32}$P-labeled DNA probes were synthesized using random hexamer primers and were used to hybridize to RNA gel blots containing poly (A)$^+$ RNA from Cont or Ac seedlings.
The three clones were sequenced in both directions by the di-deoxy chain termination method using the universal and reverse primers of the pBluescript plasmid. ddNTPs, tagged with base-specific fluorescent dyes, were detected with a laser in an automated sequencer.

Characterization of Differentially Expressed Transcripts

Seedlings for RNA isolations were grown in darkness and exposed to the following treatments:

- **Cont**....27°C for 3 d
- **Ac**........27°C for 3 d, then 14°C for 3 d
- **AcCh**.....27°C for 3 d, then 14°C for 3 d, then 4°C for 4 d
- **Ch**.........27°C for 3 d, then 4°C for 4 d
- **ABA**.......27°C for 3 d in the presence of 100 μM ABA
- **ABACH**....27°C for 3 d in the presence of 100 μM ABA, then 4°C for 4 d

Total RNA was isolated from coleoptile+leaf, mesocotyl, and root of seedlings exposed to the above treatments. Total RNA was electrophoresed on 1% formaldehyde agarose gels and transferred to nylon membranes (GeneScreen). 32P-labeled DNA probes were synthesized from the three differentially expressed inserts (as described above) for hybridization to the RNA gel blots.

**Catalase Activity**

Cell-free extracts were prepared from coleoptile+leaf, mesocotyl, and root of Cont, Ac, AcCh, and Ch seedlings, grown as previously described for RNA isolations. Extracts were also prepared from coleoptile+leaf, mesocotyl, and root
of seedlings that had been germinated in germination paper for 3 d at 27°C in
darkness, then exposed to 0, 300, or 1000 μM ABA or 24 h at 27°C in darkness.
An additional set of ABA treatments were transferred to 4°C for 4 d in darkness.
Tissue (0.5 g) was ground in 1 ml of 200 mM sodium phosphate buffer, pH 7.8,
containing 1 mM EDTA, 1 mM PMSF, and 20 mg polyvinylpolypyrrolidone.
Insoluble material was removed by centrifugation at 16000 g for 15 min in a
microcentrifuge. Protein concentration was measured by the Lowry method
(Lowry et al., 1951) using BSA as a standard. Catalase activity was measured
immediately after extraction according to the method of Beers and Sizer (1952).
Cell-free protein extracts (10 μl, 50-100 μg protein) were mixed with 660 μl water
and the reaction was started by adding 330 μl of a 50 mM potassium phosphate
solution, pH 7.5 containing 59 mM H₂O₂. Catalase measurement was made
both with and without 10 mM 3-amino-1,2,4-triazole (AT). At this concentration,
AT inhibits CAT1 by 93%, CAT2 by 98%, and CAT3 by 32% (Chandlee et al.,
1983) allowing differences in CAT3 activity among treatments to be monitored
with little interference from other isozymes. All acclimation and ABA treatments
were replicated three times and activity was analyzed as a factorial design with
acclimation or ABA treatment, chilling, and replication as the factors. Means
were separated with Tukey's studentized range test. The acclimation experiment
was analyzed separately from the ABA experiment.

ABA Analysis

Cont, Ac, Ch, or AcCh seedlings were grown as previously described for RNA
isolations. In addition to the Ac treatment (acclimation at 14°C for 3 d) seedlings
were also exposed to acclimation at 14°C for 2 h, 4 h, 8 h, and 1 d so that changes in ABA levels during acclimation could be determined. Cont and Ac were replicated three times and the remaining six treatments were replicated twice. Mesocotyls from all treatments were excised directly into liquid nitrogen and stored at -70°C until ABA analysis. Approximately 2 g of mesocotyls were analyzed for ABA content by the isotope dilution method of Li et al. (1992). Free ABA was extracted from mesocotyls with acetone in the presence of an internal standard, $^2$H$_3$-ABA. After flash evaporation of the acetone, the pH of the aqueous extract was elevated to 9.5 and partitioned against hexanes to remove hydrophobic solutes. The pH was then adjusted to 2.5 and the free ABA was extracted from the aqueous phase into ethyl acetate. The ethyl acetate was flash evaporated and the residue was taken up in 70% (v/v) methanol, pH 8.5 and passed through a $^{13}$C sep pac cartridge (Waters). The eluate was dried down, taken up in 2 ml of 0.1 M acetic acid:methanol, 80:20, filtered through a 0.45 μm pore size nylon filter (Alltech), and further purified by HPLC.

ABA was separated on a 10 x 250 mm semi-preparative reverse phase $^{18}$C column of 5 μm particle size (Phenomenex) using a two slope solvent program of 20-45% (v/v) methanol in 0.1 M acetic acid over 11.6 min followed by 45-80% (v/v) methanol in 0.1 M acetic acid over 20.2 min and a flow rate of 2.5 ml/min. ABA was detected at 254 nm. The ABA fraction was collected at 27.5-30.8 min and flash evaporated down to approximately 10 μl. This was methylated with diazomethane for at least 1 h, dried down under nitrogen stream, and taken up in 100% methanol (approximately 10 μl final volume). ABA was quantified by electron impact GC-MS using selected ion monitoring. Methylated ABA was
injected (splitless injection) on a 0.25 mm ID x 30 m Econo-Cap SE-54 capillary column (Alltech) with a fused silica stationary phase coated with a 0.25 μm polydiphenyldimethylsiloxane film. A 0.25 mm ID x 1.5 m deactivated fused silica guard column was also used. The temperature program was 100-250°C for 15 min, then isocratic 250°C for 5 min. The carrier gas was helium at 50 ml/min, the injector temperature was 250°C and the interface was 280°C. The MS ion source was operated at 70 eV and 200°C.

ABA levels were calculated by taking the ratio of ion currents at m/z 190 and m/z 193 (IC190/IC193) times the amount of deuterated standard added. All calculations were corrected for the abundance of the m/z 193 fragment in undeuterated standards and of the m/z 190 fragment in the deuterated standard. ABA level was analyzed as a completely randomized design and means were separated using Tukey's studentized range test.

Results

Seedling Growth and Survival

Visual symptoms of chilling damage ranged from a slight reduction in growth to a complete browning and decay of the entire seedling, depending on the severity of the chilling treatment. Visual symptoms in moderately damaged seedlings included chlorotic areas, browning and desiccation of leaf margins, and an occasional disruption in leaf expansion. The mesocotyl was frequently constricted and appeared to be the organ most sensitive to chilling. It is likely that these damage symptoms resulted directly from chilling as well as from photooxidation and secondary pathogen infection after seedlings were exposed.
to greenhouse conditions. Interestingly, chilled seedlings that were previously acclimated or treated with a sufficient dose of ABA exhibited a swelling in the mesocotyl. It is possible that this swelling is indicative of some form of protection in the mesocotyl.

Chilling damage in maize seedlings was found to be dependent on both temperature and duration of exposure. Survival was unaffected by chilling stress less severe than 5°C for 7 d while shoot dry weights were reduced with increasing degree of chilling stress (Table I). Acclimation significantly affected survival and shoot dry weights. The effect on survival was most evident in the 5°C for 7 d treatment where survival in the Ac treatment was 79% while that of the Cont treatment was 22%. In terms of dry weights, acclimated seedlings grew better than control seedlings in treatments where the chilling stress was sufficient to significantly affect the growth.

ABA treatment had a significant effect on growth and survival of seedlings exposed to 4°C for 7 d (Table II). The largest effect was observed at 1000 μM ABA but 333 μM also had a significant effect. The inhibition of ABA by fluridone dramatically affected the ability of acclimated seedlings to tolerate chilling stress as shown in Table III. In unchilled seedlings, fluridone treatment had no effect on survival although seedling weights were greatly reduced. AcCh seedlings showed a dramatic reduction in survival due to fluridone treatment. Exogenous application of ABA to fluridone-treated seedlings restored their ability to survive a chilling stress. ABA applied at 4°C provided some chilling tolerance but less than when it was applied at planting.
Table I. Effect of Acclimation, Chilling Temperature, and Chilling Duration on Survival and Shoot Dry Weight of Chilled Maize Seedlings

<table>
<thead>
<tr>
<th>Temp</th>
<th>Duration</th>
<th>Percent survival</th>
<th>Dry wt / seedling (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ac</td>
<td>Cont</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac</td>
<td>Cont</td>
</tr>
<tr>
<td>4°C^a</td>
<td>2 d</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>4 d</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>68</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>10 d</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>5°C^b</td>
<td>2 d</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>4 d</td>
<td>96</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>79</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>10 d</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>6°C^a</td>
<td>2 d</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>4 d</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>7 d</td>
<td>98</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>10 d</td>
<td>88</td>
<td>48</td>
</tr>
<tr>
<td>Unchilled Controls^c</td>
<td>98</td>
<td>98</td>
<td>195</td>
</tr>
</tbody>
</table>

^aThree replicates. Percent survival: SE = 4% Dry weight: SE = 8
^bFour replicates. Percent survival: SE = 3% Dry weight: SE = 7
^cSeven replicates. Percent survival: SE = 2% Dry weight: SE = 10

Differential Gene Expression

Three differentially expressed clones were isolated from the cDNA library (prepared with poly (A)^+ RNA from Ac seedlings). These were designated car333 (cat3), car30, and car757 and transcript levels are illustrated in Fig. 1. As shown, the full length sizes of cat3, car30, and car757 were 1.8, 1.6, and 1.5 kb respectively. Densitometry showed that transcript levels in Ac seedlings were 2.6-fold higher than in Cont seedlings for cat3, 3.1-fold for car30, and 2.4-fold for car757. Car30 and car757 were not homologous to any sequence in the
Table II. Effect of Exogenous ABA Treatment at Different Concentrations on Survival and Shoot Dry Weight of Chilled Maize Seedlings

<table>
<thead>
<tr>
<th>ABA Concentration</th>
<th>Percent survival&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dry wt / seedling (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 µM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66</td>
<td>80</td>
</tr>
<tr>
<td>333 µM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41</td>
<td>57</td>
</tr>
<tr>
<td>100 µM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>0 µM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>22</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three replicates. Percent survival: SE = 8%
<sup>b</sup>Three replicates. Dry weight: SE = 4
<sup>c</sup>Survivors present only in one replicate. Dry weight: SE = 7

Table III. Effect of Fluridone and ABA Treatment on Survival and Shoot Dry Weight of Chilled Maize Seedlings

Fluridone was administered by imbibing seeds in 0.1 mM fluridone/0.1% Tween 20 for 24 h, followed by planting in Redi-Earth using the same fluridone solution to saturate the medium. Cont - unchilled controls; AcCh - seedlings acclimated at 14°C for 3 d in darkness then chilled at 4°C for 7 d in darkness; AcABA - 300 µM ABA applied at planting along with the fluridone solution, followed by acclimation and chilling; ABAC - 300 µM ABA applied with the fluridone solution, followed by chilling without acclimation; ABA@4 - cold 300 µM ABA applied to seedlings that had been chilled at 4°C for 2 h, then further chilled for 7 d; Ch - seedlings chilled at 4°C for 7 d. Fluridone was not used in the ABA@4 and Ch treatments. Due to the ephemeral nature of fluridone treated seedlings, weights were taken after 7 d in the greenhouse rather than 10 d as in Table I. All treatments were replicated three times. Percent survival: SE = 8% Dry weight: SE = 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent survival &lt;br&gt;No Fluridone</th>
<th>Dry Wt / Seedling (mg) &lt;br&gt;No Fluridone</th>
<th>Fluridone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>98</td>
<td>134</td>
<td>47</td>
</tr>
<tr>
<td>AcCh</td>
<td>79</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>AcABA</td>
<td>88</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>ABAC</td>
<td>56</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>ABA@4</td>
<td>39</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Ch</td>
<td>14</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. RNA gel blots showing car gene transcripts that are present in higher abundance in acclimated seedlings than in control seedlings.
GenBank/EMBL database. Car333 had 97.8% identity in a 230 base overlap with the 3' end of maize cat3, which codes for maize mitochondrial catalase (Redinbaugh et al., 1988). The effect of various low temperature treatments (Fig. 2) and ABA treatments (Fig. 3) on the relative levels of transcripts from these three genes revealed that while the organ response differed among the three, they were induced by the different low temperature treatments and by ABA. In the coleoptile+leaf, cat3 was induced 3.8-fold by Ac, 5.6-fold by AcCh, and 2.5-fold by Ch. In the mesocotyl, cat3 was induced 4.9-fold by Ac and 7.3-fold by AcCh, but only 2.4-fold by Ch. Cat3 was undetectable in the root of all treatments. Car30 was induced 9.6-fold by Ac, 10.4-fold by AcCh, and 4.8-fold by Ch in the coleoptile+leaf. In the root, car30 was induced 2.4-fold by Ac, 2.3-fold by AcCh, and 1.6-fold by Ch. Car30 was undetectable in Cont mesocotyls but was induced by Ac, and more so by AcCh while remaining very low in Ch. Car757 was in lower abundance than cat3 and car30. It was induced 4.7-fold by Ac, 6.8-fold by AcCh, and 5.0-fold by Ch in coleoptile+leaf; 2.2-fold by Ac, 4.1-fold by AcCh, and 4.2-fold by Ch in mesocotyls; and 2.5-fold by Ac, 6.0-fold by AcCh, and 6.1-fold by Ch in roots. Transcripts from all three car genes were induced by ABA and ABAC in a manner similar to the acclimation treatments. Cat3 was induced 3.6-fold by ABA and 3.4-fold by ABAC in coleoptile+leaf, and 3.3-fold by ABA and 4.5-fold by ABAC in mesocotyls. Again, cat3 was undetectable in the roots. Car30 was not appreciably induced by ABA but was induced 2.4-fold by ABAC in the coleoptile+leaf. In the roots, it was induced 4.7-fold by ABA and 6.3-fold by ABAC. As in the acclimation treatments, car30 was undetectable in Cont mesocotyls, but induction was only slight in response
Figure 2. Transcript levels for *cat3*, *car30*, and *car757* in coleoptile+leaf (C), mesocotyl (M), and root (R) of unchilled seedlings (Cont), acclimated at 14°C for 3 d in darkness (Ac), acclimated and chilled at 4°C for 4 d in darkness (AcCh), or chilled without acclimation (Ch).
Figure 3. Transcript levels for cat3, car30, and car757 in coleoptile+leaf (C), mesocotyl (M), and root (R) of unchilled seedlings (Cont), seedlings germinated in 100 μM ABA (ABA), and seedlings germinated in 100 μM ABA followed by chilling at 4°C for 4 d in darkness (ABACCh).
to ABA alone while ABACh caused moderate induction. *Car757* was induced 5.0-fold by ABA and 4.7-fold by ABACh in the coleoptile+leaf, 4.2-fold by ABA and 4.1-fold by ABACh in the mesocotyl, and 2.7-fold by ABA and 3.5-fold by ABACh in the root. In terms of differences in organ response, *cat3* was most abundant in the coleoptile+leaf and mesocotyl, *car30* was most abundant in coleoptile+leaf with somewhat less in the root, and *car757* was induced from a low level in all organs, although to a greater extent in the coleoptile+leaf and mesocotyl than in the root. The different organ responses in the ABA experiment was similar to that of the acclimation experiment.

Catalase activities for the acclimation experiment are given in Fig. 4, and for the ABA experiment in Fig. 5. No differences in total catalase activity were detected among treatments in the coleoptile+leaf for either experiment. In the mesocotyl, total catalase activity was significantly induced by both acclimation and 1000 μM ABA treatment. In the root, total catalase activity was significantly induced by both 300 and 1000 μM ABA but not by acclimation. Chilling had no significant effect on catalase activity in either experiment. When AT was included in the catalase assay, the induction was nearly the same as when uninhibited rates were measured. The root is known to contain primarily CAT1 (Chandlee and Scandalios, 1984) and very little activity was detected in any of the acclimation treatments in the presence of AT. In the ABA experiment, however, there was significantly higher activity in the 1000 μM ABA treatment than the control, and the 77% inhibition by AT in the 1000 μM ABA treatment was significantly lower than the 86% inhibition by AT in the control. Thus, it is possible that ABA induces CAT3 in the root to levels detectable by this assay.
Figure 4. Effect of acclimation on catalase activity. Catalase activity was measured in cell-free extracts of coleoptile+leaf (C), mesocotyl (M), and root (R) of various treatments as described in Fig. 2. Activity was measured immediately after extraction both with and without 10 mM aminotriazole (AT) in the assay. Activity after AT treatment is indicative of relative CAT3 activity among treatments. Treatments were replicated three times. For uninhibited catalase activity; coleoptile+leaf SE = 2.8, mesocotyl SE = 1.6, and root SE = 1.6. For AT-inhibited catalase activity; coleoptile+leaf SE = 1.9, mesocotyl SE = 1.5, and root SE = 0.4.
Figure 5. Effect of ABA treatment on catalase activity. Catalase activity was measured in cell-free extracts of coleoptile+leaf (C), mesocotyl (M) and root (R) of seedlings exposed to 0, 300, or 1000 μM ABA for 24 h. Activity was measured both A) immediately after ABA treatment and B) after chilling at 4°C for 4 d in darkness. Activity was measured both with and without AT. For uninhibited catalase activity; coleoptile+leaf SE = 1.9, mesocotyl SE = 4.0, and root SE = 2.0. For AT-inhibited catalase activity; coleoptile+leaf SE = 0.6, mesocotyl SE = 1.1, and root SE = 1.1.
A
Catalase Activity Before Chilling

B
Catalase Activity After Chilling

Activity (μmol H₂O₂/min/mg protein)

ABA Concentration (μM)
ABA Levels

ABA levels in mesocotyls of Cont, Ac, Ch, and AcCh as well as at various times during acclimation are given in Fig. 6. Chilling caused a large increase in free ABA levels, regardless of acclimation status. However, very little ABA accumulation occurred during acclimation. Marginal increases in ABA may have occurred in the first hours of acclimation followed by a decline to Cont levels but differences among ABA levels at the various times during acclimation were not statistically significant.

Discussion

Acclimation and Differential Gene Expression

We have demonstrated the presence of a chilling acclimation phenomenon in seedlings of chilling sensitive maize inbred G50 (Pioneer). Visually, the mesocotyl is the organ that is most sensitive to chilling. Acclimation and ABA treatment provided some degree of protection from chilling, which was most evident in the mesocotyl.

Exposure of seedlings to the 14°C acclimation temperature likely induces numerous changes that act in concert to increase chilling tolerance. The metabolic systems that are affected by acclimation in maize are unknown at this time. We have isolated cDNAs representing three differentially expressed genes and found that they differed in organ specificity. Thus, even though the mesocotyl was the most sensitive to chilling in nonacclimated seedlings, it is apparent that acclimation causes changes throughout the seedling. Since the functions of car30 and car757 are unknown at this time, we cannot assign a role
Figure 6. ABA levels in mesocotyls of seedlings exposed to various acclimation and chilling treatments. Treatments were as described in Fig. 2 except the Ac treatment where the duration of acclimation was varied. ABA was measured according to Li et al. (1992). Cont and Ac/72 h were replicated three times. SE = 2.8. All other treatments were replicated twice. SE = 3.4.
to their gene products during acclimation or chilling. The fact that car30 transcript abundance (Fig. 2) in the coleoptile+leaf and root of Ac and AcCh seedlings is higher than that of Ch suggests that its corresponding gene product functions during acclimation. The appearance of low levels of car30 in the mesocotyl of Ac seedlings, then higher levels in AcCh but not Ch is of interest. It is as if acclimation initiates the mechanism of induction but high levels of transcript are not observed until chilling stress is imposed. The initiation during acclimation suggests that car30 may function in protecting the mesocotyl during chilling. Car757 abundance (Fig. 2) is higher in coleoptile+leaf and mesocotyl of AcCh and Ch seedlings than in Ac, as if it is responding to the degree of temperature stress to which the seedling is exposed.

Although we do not know the function of car30 or car757, the function of cat3 is clear. Cat3 codes for mitochondrial catalase which catabolizes the conversion of H₂O₂ to H₂O and O₂. In maize, four catalase isozymes are present, which differ both spatially and temporally during development (Chandlee and Scandalios, 1984; Scandalios et al., 1984; Acevedo and Scandalios, 1991). The coleoptile+leaf contains CAT1 and CAT3 and roots contain CAT1. CAT4 is expressed only transiently in the pericarp of germinating seeds. Etiolated leaves contain CAT1 and CAT3 and in the light, CAT1 disappears and CAT2 appears as the seedling matures. In the developing stem, only CAT3 activity is detectable although both cat1 and cat3 transcripts are present. Catalase activity in these stems is located primarily in the sclerenchyma beneath the epidermis and around the vascular bundles (Acevedo and Scandalios, 1991). Tsaftraris et al. (1983) demonstrated that in light-grown maize leaves, CAT2 was present in
the peroxisomes of bundle sheath cells while CAT3 was associated with the mitochondria of the mesophyll cells. We observed the abundance of cat3 (Fig. 2) to be elevated in the coleoptile+leaf and especially in the mesocotyl in response to acclimation and chilling. Catalase activity in the presence of AT (Fig. 4), representing relative CAT3 activity, generally followed the same pattern as cat3 transcripts with two exceptions. These are the low activity in Ch mesocotyls where transcripts showed significant induction and the high activity in Cont coleoptile+leaf which has low transcript levels. These discrepancies may be the result of post-transcriptional events.

Involvement of Catalase during Acclimation

We have previously shown that H$_2$O$_2$ levels are elevated in mesocotyls of chilled seedlings (Prasad et al., 1994a). Elevation of H$_2$O$_2$ is indicative of a state of oxidative stress under which lipid peroxidation and other deleterious effects on membranes (McKersie, 1991) and inactivation of various enzymes in the photosynthetic apparatus (Asada, 1992b) occur. Elevated H$_2$O$_2$ can also result in an increased occurrence of OH$^-$ (from H$_2$O$_2$ and O$_2^-$ in the Haber-Weiss reaction) which is highly reactive and can contribute significantly to cellular damage (Elstner, 1987). The primary means of defense against active oxygen is via production of active oxygen scavengers such as catalase, peroxidase, superoxide dismutase (SOD), glutathione, ascorbate, carotenoids, and various phenolics and alkaloids (Larson, 1988). In chilling-sensitive plants, the ability to defend against oxidative damage has been shown to be affected by the chilling-induced reduction of antioxidants such as ascorbate, glutathione, and
α-tocopherol (Wise and Naylor, 1987b); catalase (Omran, 1980), and SOD (Michalski and Kaniuga, 1982). Chilling tolerance has been enhanced in chilling-sensitive plants by artificially elevating levels of glutathione, peroxidase, and catalase (Upadhyaya et al., 1989) and SOD (Sen Gupta et al., 1993). In our system, we show that chilling-sensitive maize can be made more tolerant by acclimation and this may involve up-regulating catalase. Further, we have evidence that the up-regulation of catalase is the primary means of maintaining low H$_2$O$_2$ levels in acclimated, dark-grown, maize seedlings when subjected to a chilling stress (Prasad et al., 1994a).

The fact that we are dealing with an induction of cat3 suggests that the source of oxidative stress in dark-grown seedlings is the mitochondria. In the light, a major source of oxidative stress is in the chloroplast where the ascorbate/glutathione mechanism, as well as SOD, are most important in dealing with excess H$_2$O$_2$ and O$_2^-$ (Asada and Takahashi, 1987). Catalase undergoes photoinactivation in the light when plants are exposed to low temperature stress, which was correlated with the low temperature sensitivity of the plant species (Feierabend et al., 1992). Sources of oxidative stress in the dark are not as well studied, but it appears likely that if the membrane-associated cytochrome pathway is impaired by chilling, excess electrons may lead to production of H$_2$O$_2$ and O$_2^-$. Indeed, we have evidence that respiratory activity, the cytochrome pathway, and F$_1$-ATPase activity are irreversibly impaired by chilling; but are protected from irreversible damage by acclimation, H$_2$O$_2$ treatment, and ABA treatment (Prasad et al., 1994b). The ability of dark-grown seedlings to tolerate chilling-induced oxidative stress may well depend on the
ability to protect the mitochondria. Induction of CAT3 during acclimation prevents the accumulation of \( \text{H}_2\text{O}_2 \) to damaging levels in the mitochondria.

**Involvement of ABA during Acclimation and in Chilling Tolerance**

In order to further investigate the mechanism of acclimation, we determined the effects of ABA on chilling tolerance and on the induction of catalase. We observed that 1) treatment with exogenous ABA improved the growth and survival of chilled seedlings and 2) fluridone treatment abolished and fluridone treatment in the presence of ABA restored acclimation-induced chilling tolerance. These results indicated that ABA is essential for survival in the face of a chilling stress. Further, the fact that ABA induced the three car genes indicated that ABA and acclimation have common effects. However, this is not conclusive evidence that the acclimation process is mediated by ABA. Even though ABA improved the survival of chilled seedlings, it was not as effective as acclimation in inducing chilling tolerance (Table III). It is likely that ABA independent events occur during acclimation that contribute to the observed level of chilling tolerance. In addition, the fact that fluridone abolished acclimation-induced chilling tolerance does not exclude the possibility that ABA and acclimation operate through separate mechanisms, both of which are required for survival of chilled seedlings. Finally, the fact that ABA levels are, at best, only marginally elevated during acclimation is also evidence against ABA accumulation as an essential component of the acclimation mechanism. Other works have suggested similar conclusions. Nordin et al. (1991) found that an Arabidopsis low temperature induced gene, *Iti40*, was also induced by ABA but low
temperature induction of *Ittil40* was unaffected in ABA-deficient and ABA-insensitive mutants, and after treatment with fluridone. Gilmour and Thomashow (1991) found that ABA-induced expression of three *cor* genes was greatly inhibited in the *abi1* (ABA insensitive) mutant of *Arabidopsis* but cold-induced expression was unaffected. These facts point to the existence of separate mechanisms for cold- and ABA-induced gene expression. Waldman et al. (1975) found no significant increase of ABA levels in acclimated alfalfa and suggested that a decline in gibberellic acid conferred tolerance by increasing the ABA/GA ratio. Mohapatra et al. (1988) concluded that while ABA may play a role in acclimation to freezing tolerance in alfalfa, the acclimation mechanism was not mediated by ABA given differences in protein profiles, transcripts, and the level of freezing tolerance between ABA treated and acclimated plants.

We observed a dramatic elevation in free ABA levels in response to chilling, both in Ch and AcCh treatments. These results also constitute evidence against the direct involvement of ABA during acclimation since it appears that ABA is responding to something other than acclimation. It is as if ABA levels correspond to the level of stress (possibly chilling-induced water stress) experienced by the plant. Capell and Dorfling (1989) concluded that chilling-induced water stress was responsible for the observed elevation of ABA in chilled cucumber. Other works have shown that exogenous ABA treatment alleviated chilling-induced water stress (Markhart, 1984; Pardossi et al., 1992). Thus, in our experiments, it is possible that ABA is responding to the water status of the seedlings and that the ABA-induced chilling tolerance observed in
our survival experiments is due, in part, to alleviation of chilling-induced water stress.

On the other hand, maintenance of low ABA levels during acclimation does not necessarily mean that it has no role in the acclimation process. Perhaps there is a redistribution of ABA toward its site of action or perhaps the sensitivity to ABA is enhanced during acclimation. Thus, although our data point to separate pathways of induction for ABA-induced and acclimation-induced chilling tolerance, we cannot rule out the possibility that ABA plays a role during acclimation.

Acknowledgments

We wish to thank Drs. C.E. LaMotte and X. Li, Plant Hormone Analysis Facility, Iowa State University, for assistance in the quantification of ABA and Dr. J.T. Colbert for helpful suggestions during the construction and subsequent screening of the cDNA library.
CHAPTER 3. CHANGES IN ISOZYME PROFILES OF CATALASE, PEROXIDASE, AND GLUTATHIONE REDUCTASE DURING ACCLIMATION TO CHILLING IN MESOCOTYLS OF MAIZE SEEDLINGS

Manuscript submitted to Plant Physiology

Marc D. Anderson, Tottempudi K. Prasad, and Cecil R. Stewart

Abstract

We have previously demonstrated that exposure of dark-grown maize seedlings to a noninjurious low temperature (acclimation) resulted in an improvement of chilling tolerance. Compared to the unchilled control, the hydrogen peroxide level was greatly elevated in mesocotyls of chilled seedlings but was maintained at low levels in mesocotyls of seedlings that were acclimated before applying the chilling treatment. The induction of mitochondrial catalase was shown to contribute to the protection of acclimated seedlings from oxidative

1 This research was supported by grants from Pioneer Hi-Bred International (C.R.S.) and USDA NRICGP #92-37100-7646 (C.R.S., T.K.P.).

2 Corresponding author

Authors’ contributions: Hydrogen peroxide measurements were made by Prasad. All other experiments, statistical analyses, and manuscript preparation were conducted by Anderson. All authors participated in the conception and planning of the research.
stress. In this study, we examined the isozyme profiles of catalase, guaiacol peroxidase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase in response to acclimation and chilling. Superoxide dismutase and ascorbate peroxidase were unaffected by acclimation or chilling in any of the organs tested (coleoptile+leaf, mesocotyl, and root). In addition, none of the enzymes were significantly affected by acclimation or chilling in the coleoptile+leaf or root. The lack of induction was not surprising for the roots, which were not severely affected by chilling. However, acclimation prevented the chilling-induced accumulation of H$_2$O$_2$ in the coleoptile+leaf, just as previously reported in the mesocotyl. Measurement of ascorbate and glutathione pools revealed that while chilling promoted oxidation of ascorbate and glutathione and a loss in total ascorbate, acclimation resulted in elevated total glutathione, and a maintenance of reduced ascorbate and glutathione near unchilled levels. Thus, the synthesis of glutathione in acclimated seedlings may contribute to the protection of the coleoptile+leaf from oxidative stress. The most significant changes in antioxidant enzymes in response to acclimation occurred in the mesocotyl, the organ that was visibly the most susceptible to chilling damage. CAT3 was found to be highly induced by acclimation and may represent the first line of defense from mitochondria-generated H$_2$O$_2$. Nine of the most prominent peroxidase isozymes were induced by acclimation. Although the functions of these are unknown, two of the isozymes induced by acclimation were located in the cell wall, suggesting a role in lignification. Lignin content was found to be elevated in mesocotyls of acclimated seedlings, likely improving the mechanical strength of the mesocotyl. Acclimation also caused a change in the
isozyme profile of cytosolic glutathione reductase. The activity of one band was greatly decreased while two others were elevated, with little change in the total activity. It is possible that this shift in isozymes results in an improved effectiveness of the enzyme at low temperature. When taken together, these responses to acclimation illustrate the numerous ways that chilling and oxidative stress tolerances can be enhanced throughout the cell.

Introduction

Exposure of chilling sensitive plants to low, nonfreezing temperatures results in numerous biochemical changes (see reviews by Graham and Patterson, 1982; Wang, 1982; Markhart, 1986) which combine to produce the observed damage symptoms. There is increasing evidence that chilling causes elevated levels of active oxygen species (e.g. Omran, 1980; Wise and Naylor, 1987a; Prasad et al., 1994a) which likely contribute significantly to chilling damage. The active oxygen species; H$_2$O$_2$ (hydrogen peroxide), O$_2^-$ (superoxide), OH· (hydroxyl radical), and 1O$_2$ (singlet oxygen) are present in all plants to various degrees. Metabolic pathways such as β-oxidation and photorespiration generate H$_2$O$_2$ as a normal metabolite and O$_2^-$ is generated by various oxidases. They may also arise as a consequence of one electron transfer to molecular oxygen, especially in the photosystems (Mehler, 1951) or the electron transport chain of the mitochondria (Rich and Bonner, 1978) where electron transfer reactions take place at a high rate. When O$_2^-$ and H$_2$O$_2$ are present in the same compartment, they can react with each other in the metal-catalyzed Haber-Weiss reaction, generating OH· (Haber and Weiss, 1934). These active oxygen species, especially OH· and
$^{1}$O$_2$, are highly reactive and can potentially cause damage to cellular components (Elstner, 1987). They may oxidize enzyme sulphhydryl groups thus affecting their function, or they may initiate lipid peroxidation, which greatly affects membrane function and causes the release of potentially toxic organic peroxides and aldehydes such as malondialdehyde (McKersie et al., 1990).

Under normal conditions, plants have active oxygen scavenging systems which keep active oxygen species below damaging levels (Larson, 1988). O$_2^-$ is converted to H$_2$O$_2$ and O$_2$ by the action of superoxide dismutase (SOD), present in the cytosol, chloroplasts, and mitochondria. H$_2$O$_2$ generated in the chloroplasts is reduced to H$_2$O at the expense of NADPH via ascorbate and glutathione (Foyer and Halliwell, 1976). In this system, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase operate in conjunction to maintain ascorbate and glutathione in their reduced form. This system may also be of importance in the cytosol (Alscher, 1989). Catalase is the primary H$_2$O$_2$ scavenger in the peroxisomes and the mitochondria (at least in maize, Scandalios et al., 1980). In dark-grown maize seedlings, we have shown that mitochondrial catalase is of primary importance in scavenging H$_2$O$_2$ (Prasad et al., 1994a). We have also found the presence of a cytochrome c peroxidase in maize mitochondria which may also possess a scavenging role (Prasad TK, Anderson MD, Stewart CR, manuscript submitted). These scavenging systems maintain low active oxygen levels under normal conditions but when the plant is stressed, the production of active oxygen can exceed the capacity of the scavenging systems, resulting in oxidative damage. The ability of a plant to
improve its active oxygen scavenging capacity, so as to maintain low levels of active oxygen, may be a key element in stress tolerance.

We have previously reported an acclimation phenomenon in chilling sensitive maize inbred G50 (Pioneer HiBred International, Johnston, IA). Dark-grown seedlings exposed to an acclimation treatment before applying a chilling stress, exhibited a dramatic improvement in growth and survival when compared to unacclimated seedlings (Anderson et al., 1994). Three cDNAs were identified which were present in greater abundance in acclimated than in control seedlings. One of these was cat3, which encodes maize mitochondrial catalase. Total catalase (CAT) activity was found to be induced by acclimation primarily in the mesocotyl, visibly the organ most sensitive to chilling damage. We also found that exogenous treatment of the seedlings with ABA improved chilling tolerance and induced both cat3 transcripts and CAT activity in maize mesocotyls. We subsequently showed (Prasad et al., 1994a) that hydrogen peroxide accumulated in the mesocotyl of chilled seedlings but not in seedlings that were acclimated before chilling. CAT and guaiacol peroxidase (POX) activities were induced during acclimation, which served to alleviate chilling-induced oxidative stress. We also provided evidence that a transient increase in hydrogen peroxide during acclimation was acting as a signal for cat3 accumulation. The resulting increase in catalase activity allowed the seedlings to tolerate further hydrogen peroxide generation during a subsequent chilling treatment.

Even though we've shown that CAT and POX were induced by acclimation, these measurements were on a total activity level. In maize, CAT is present as four isozymes (Scandalios et al., 1984), only two of which (CAT1 and CAT3) are
present in shoots and roots of dark-grown maize seedlings. In the coleoptile and leaf, CAT1 and CAT3 are present during early growth and upon exposure to light, CAT1 activity declines and CAT2 appears (Tsaftaris et al., 1983) although cat1 transcripts are present throughout early shoot development (Redinbaugh et al., 1990a). In mature leaves, CAT2 is localized in the bundle sheath cells while CAT1 and CAT3 are present in the mesophyll cells (Tsaftaris et al., 1983). Roots contain primarily CAT1 although cat3 transcripts and protein have been detected at low levels (Redinbaugh et al., 1990a). No documentation is available regarding the expression of catalase isozymes in maize mesocotyls.

The specific function of catalase in maize is still in question. The fact that cat2 null mutants of maize are fully viable (Tsaftaris et al., 1983) calls into question the importance of catalase in C4 plants where photorespiratory H2O2 generation is likely so low that it doesn't build to toxic levels. CAT2, being induced by light and expressed in bundle sheath peroxisomes (Tsaftaris et al., 1983) likely does function in scavenging photorespiratory H2O2 even though its presence may not be crucial for survival. CAT3 in the mitochondria likely scavenges H2O2 formed from reduction of O2 by electrons from the electron transport chain. It has been shown to be under circadian regulation, in which the message is controlled to provide the highest CAT3 activity during the dark period (Redinbaugh et al., 1990b). This is consistent with its proposed role in scavenging H2O2 when respiration is at its highest level. As with cat2, a cat3 null mutant of maize was found to be viable (Wadsworth and Scandalios, 1990), indicating that the function of catalase in the mitochondria is not crucial to survival. It was suggested that the importance of catalase is not fully realized
under optimal conditions but may be much more important under conditions that cause oxidative stress (such as chilling). Indeed, we have found that cat3 induction by acclimation is a primary component of chilling tolerance in dark-grown maize seedlings (Prasad et al., 1994a).

Guaiacol peroxidases occur in numerous isoforms in plants and are involved in lignin biosynthesis, suberin biosynthesis, cross-linking of cell wall polymers, IAA oxidation, and ethylene biosynthesis (see reviews by Gaspar et al., 1991; Siegel, 1993). There are numerous examples of the isozyme profiles being altered by various environmental factors (Siegel, 1993). Several other peroxidases (ascorbate peroxidase, glutathione peroxidase, cytochrome c peroxidase, and NADH peroxidase) are thought to function solely in the scavenging of H₂O₂ and are important in the protection of cells against oxidative stress (Asada, 1992a). Even though guaiacol peroxidases are thought to have specific physiological functions (Asada, 1992a), it is possible that their utilization of H₂O₂ contributes to protection from oxidative stress. Takahama (1991) proposed a model in which guaiacol peroxidases in the vacuole utilized phenolic compounds to reduce H₂O₂ imported into the vacuole from the cytosol during stress. In general, however, the specific function of individual POX isozymes is difficult to determine because of their reactivity to a wide range of different substrates (usually phenolic). Functions are suggested by localization of individual isozymes, by correlating the formation of reaction products with changes in POX activity in different treatments, and by determining the affinity of the isozyme for various substrates. None of these conclusively tie an individual isozyme to its physiological function within the cell.
In previous reports, we have demonstrated the importance of CAT and POX in protection of maize mesocotyls from chilling-induced oxidative stress (Anderson et al., 1994; Prasad et al., 1994a, b). In this study, we report the response of CAT and POX isozymes in coleoptile+leaf, mesocotyl, and root to acclimation and chilling. We extended the study to include the responses of SOD, GR, and APX isozymes as well as steady-state pools of ascorbate and glutathione in an effort to more thoroughly describe the antioxidant systems involved in the acquisition of chilling tolerance in dark-grown maize seedlings.

Materials and Methods

Plant Material

Chilling sensitive maize (Zea mays L.) inbred G50 (Pioneer Hi-Bred International Inc., Johnston, IA) was used for all experiments. For the acclimation experiments, seeds were planted in Redi-Earth Peat-Lite Mix (Grace Sierra Horticultural Products Co., Milpitas, CA) and grown in darkness at 27°C for 3 d. Seedlings exposed to the acclimated/chilled (AcCh) treatment were then exposed to an acclimation temperature of 14°C for 3 d in darkness, followed by a chilling temperature of 4°C for 4 d in darkness. Seedlings of the chilled (Ch) treatment were transferred directly to the chilling temperature without acclimation. Seedlings of the acclimation (Ac) treatment were acclimated but not chilled. Control (Cont) seedlings received neither the acclimation nor the chilling treatments. For the ABA experiment, seeds were surface sterilized in 1% sodium hypochlorite, imbibed between paper towels saturated with autoclaved 10 mM MOPS, pH 7.0, for 1 d at 27°C, then rolled in germination paper and
placed vertically in the MOPS buffer to keep the germination paper saturated. Seeds were allowed to grow at 27°C for 3 d in darkness. The 3 d control (Cont 3d) received no further treatment. For the ABA treatment (ABA), the MOPS buffer was replaced by 1 mM ABA (mixed isomers, Sigma) in autoclaved 10 mM MOPS, pH 7.0, and allowed to incubate for 1 d at 27°C in darkness. For the ABA/chilled treatment (ABACH), ABA-treated seedlings were subsequently exposed to a chilling temperature of 4°C for 4 d in darkness. For the prechilled ABA treatment (ABA@4), 3 d old seedlings were transferred to 4°C for 2 d, then treated with cold (4°C) 1 mM ABA in MOPS for 1 d at 4°C, then chilled at 4°C for an additional day. Although seedling growth is retarded by the ABA treatment and seedlings are not much larger than the Cont 3d treatment, a second control grown at 27°C for 4 d in darkness (Cont 4d) was included to distinguish ABA effects from developmental effects.

**Hydrogen Peroxide Measurement**

Coleoptile+leaf, mesocotyl, and roots from Cont, Ch, Ac, and AcCh treatments were analyzed for H$_2$O$_2$ levels by the method of Brennan and Frenkel (1977). Fresh tissue (5 g) was ground in 10 ml cold acetone, filtered through Whatman #1 filter paper, and diluted to 15 ml with water. H$_2$O$_2$ was complexed with 20% (v/v) titanium tetrachloride in 12.1 M HCl, and 3 ml concentrated ammonium hydroxide was added to precipitate the complex. This was centrifuged at 10000g for 5 min and the pellet was redissolved in 8 ml of 2 M sulfuric acid. The solution was washed three times with 2 ml acetone and then
diluted to 12 ml with water. H$_2$O$_2$ was quantified by measurement of absorbance at 415 nm and compared to a standard curve.

**Protein Extraction**

Cell-free extracts of the various low temperature and ABA treatments were analyzed for catalase (CAT), guaiacol peroxidase (POX), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) both spectrophotometrically and on nondenaturing gels. Plant material was harvested directly into liquid nitrogen and 0.5 g of frozen tissue was ground in 0.75 ml of 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA, 1 mM PMSF, and 20 mg polyvinylpolypyrrolidone. For analysis of APX, the extraction buffer also contained 5 mM ascorbate. Insoluble material was removed by centrifugation at 16000 g for 15 min at 4°C, and the supernatant was immediately made to 40\% (v/v) glycerol. Since maintenance of consistent CAT electrophoretic mobility and maintenance of GR activity was found to require the presence of DTT, an aliquot of each sample was made to 10 mM DTT to be used for CAT and GR zymograms and GR spectrophotometric assays (Guy and Carter, 1984, protected GR with 5 mM \(\beta\)-mercaptoethanol). Addition of DTT was within 0.5 h from the time of maceration. The remainder of each extract was used for POX and SOD zymograms, for CAT and POX spectrophotometric assays and for determination of total protein content by the Lowry method (Lowry et al., 1951) with BSA as the standard. For the spectrophotometric assay of SOD, extracts were passed through Sephadex G-25 columns (1 ml bed volume) at 4°C using 0.1 M sodium phosphate, pH 7.8, as the elution buffer. This effectively removed
low molecular weight compounds which interfered with the assay. Extracts were then made to 40% (v/v) glycerol. All samples were stored at -20°C until enzyme analysis. Routinely, CAT and APX gels were run immediately after extraction while the stability of POX, SOD, and GR during storage was sufficient so that immediate analysis was not necessary. All spectrophotometric assays were run on the day following extraction. CAT, in particular, noticeably lost activity during storage for two or more days. It is known that the plastidic isoform of APX is extremely labile (Asada, 1992a; Mittler and Zilinskas, 1993) and it is possible that recovery of all APX isozymes was not accomplished in these extractions.

In order to determine which POX isozymes were localized in the cell wall and therefore, potentially involved in lignification, ionically bound cell wall proteins were separated from protoplasmic proteins by the method of Prasad and Cline (1987). Mesocotyls (4 g) from Ac and AcCh treatments were ground in 7 ml of cold (4°C) 0.1 M potassium phosphate, pH 6.4 and 32 ml of cold acetone was added to precipitate proteins. Samples were stored at -20°C overnight, then centrifuged at 5000 g for 5 min. The pellet was suspended in 5 ml of cold buffer and shaken at 4°C for 1 h. This was centrifuged at 5000 g for 5 min and the supernatant was taken as the protoplasmic fraction. The pellet was washed repeatedly with buffer until peroxidase activity in the wash was negligible. The pellet was then extracted with 5 ml of buffer containing 1 mM sodium chloride to release ionically bound proteins from the cell wall material. After centrifugation at 5000 g for 5 min, the supernatant was desalted and concentrated on a Centricon microconcentrator (Amicon, Danvers, MA) with a 10000 molecular weight exclusion limit, according to manufacturer's instructions.
Cell fractionation was conducted for the purpose of localizing GR isozymes. Mesocotyls (10 g) from Ac seedlings were harvested and ground in 20 ml of a solution of 0.4 M sucrose, 165 mM tricine, 10 mM KCl, 10 mM MgCl₂, 10 mM EDTA, and 10 mM DTT, pH 7.5. Macerated material was passed through four layers of cheesecloth and centrifuged at 2000 g for 5 min. The plastid-containing pellet was resuspended in 0.4 M sucrose, 10 mM tricine, 1 mM EDTA, 10 mM DTT, pH 7.2. The supernatant was centrifuged at 12000 g for 10 min and the supernatant was retained as the cytosolic fraction. The mitochondria-containing pellet was resuspended in 0.4 M sucrose and mitochondria were further purified on a 0.6 M sucrose cushion, centrifuging at 10000 g for 20 min. The mitochondrial pellet was then resuspended in resuspending media. Mitochondria and plastids were lysed by repeated freeze/thaw cycles and samples were centrifuged at 10000 g to remove particulates.

**Enzyme Analyses**

Total CAT activity was determined spectrophotometrically by following the decline in A₂₄₀ as hydrogen peroxide (ε = 36 M⁻¹cm⁻¹) is catabolized, according to the method of Beers and Sizer (1952) and as described in more detail in Chapter 2. CAT isozymes were separated on nondenaturing polyacrylamide gels (7% T, 3% C, notation of Righetti, 1983, p 174) at 80 V for 22 h at 4°C using the procedures of Laemmli (1970). The 3X loading buffer contained no β-mercaptoethanol (found to inhibit CAT3) but was made to 60 mM DTT. DTT was required to prevent oxidative inactivation of CAT1 on the gel. Gels were then soaked in 3.27 mM H₂O₂ for 25 min, rinsed in water, and stained in a solution of
1% (w/v) potassium ferricyanide, 1% (w/v) ferric chloride (equal volumes of 2% (w/v) solutions of each component, added sequentially) in a method similar to that of Woodbury et al. (1971).

Total POX activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol ($e = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$) from guaiacol at $A_{470}$ in the presence of $\text{H}_2\text{O}_2$ by the method of Chance and Maehly (1955). Crude protein extracts (5 µl, 25-50 µg protein) were mixed with 975 µl of 0.1 M potassium phosphate, pH 6.4, and 8 µl 1 M guaiacol (in 50% [v/v] ethanol) was added. The reaction was started by adding 12 µl of 25 mM $\text{H}_2\text{O}_2$. POX isozymes were separated by IEF on a flat bed electrophoresis apparatus (LKB Multiphor 2117). The 2 mm gel (5% T, 3% C) contained 10% (v/v) glycerol, 3% (v/v) Pharmalytes (Sigma) pH 3-10, 1% (v/v) Pharmalytes pH 5-8, and 1% (v/v) Pharmalytes pH 8-10.5 and was supported by GelBond PAG film (Pharmecia). The anode strip was saturated with 1 M phosphoric acid and the cathode strip with 1 N sodium hydroxide. Gels were run at 20 W constant power for 5 h at 10°C, with sample applicators removed after 45 min. Following electrophoresis, the gel was soaked in PBS (10 mM sodium phosphate, pH 6.0, 150 mM sodium chloride) for 45 min to equalize pH throughout the gel. The gel was then stained in a solution of 0.1 M sodium citrate, pH 5.0, 9.25 mM p-phenylenediamine, and 3.92 mM $\text{H}_2\text{O}_2$ for 10-15 min in a method used for staining histological sections (Olson and Varner, 1993). Photographs were taken immediately after staining since considerable diffusion of the bands occurred within 0.5 h. In order to determine whether all bands observed using p-phenylenediamine were also reactive with guaiacol, a gel was also stained in a solution containing 0.1 M potassium phosphate, pH 6.4,
20 mM guaiacol, and 5.55 mM H₂O₂ for ~30 min. Since considerable POX activity was observed at the cathode strip, a gel was sectioned and run for various lengths of time ranging from 1 to 2 h. Bands were underfocused but isozymes with pls greater than the pH range of the gel were visible, allowing comparison of activity levels among treatments.

Total SOD activity was measured spectrophotometrically by measuring the inhibition of the O₂•-dependent reduction of cyt c at A₅₅₀, according to the method of McCord and Fridovich (1969). Crude protein extracts (20 μl, 100-200 μg protein) were mixed with 430 μl of water and 530 μl of 100 mM potassium phosphate, pH 7.8, containing 2 mM EDTA, 0.2 mM xanthine, and 0.5 mg/ml cyt c. The reaction was initiated with 20 μl of 0.3 mg/ml xanthine oxidase (in 0.1 mM EDTA, pH 7.5). One unit of SOD is defined as the quantity of enzyme required to inhibit the reduction of cyt c by 50% in a 1 ml reaction volume. Isozymes of SOD were separated on nondenaturing polyacrylamide gels (10% T, 3% C) at 80 V for 19.5 h at 4°C according to the method of Laemmli (1970). β-mercaptoethanol was withheld from the loading buffer. After electrophoresis, gels were stained with a method similar to Beauchamp and Fridovich (1971). Gels were soaked in 50 mM sodium phosphate, pH 7.5, containing 2.45 mM NBT in darkness for 20 min, followed by soaking in 50 mM sodium phosphate, pH 7.5, containing 26.5 mM TEMED and 26.5 μM riboflavin in darkness for 40 min. Gels were then exposed to low light (9 μmol m⁻²sec⁻¹) for ~50 min and transferred to 1% (v/v) acetic acid to stop the reaction.

Total GR activity was measured spectrophotometrically by measuring the decline in A₃₄₀ as NADPH (ε = 6.22 mM⁻¹cm⁻¹) was oxidized, as described by
Edwards et al. (1990). Crude protein extracts (20 μl, 100-200 μg protein) were mixed with 410 μl of water and 500 μl of 0.2 M HEPES, pH 7.8, and 20 μl 10 mM NADPH was added. The reaction was initiated with 50 μl 10 mM GSSG. The DTT present in the crude extracts, which was required to maintain GR activity, was found not to interfere with the assay. Instead, it eliminated the GSSG-independent oxidation of NADPH, thereby improving the accuracy of the assay. GR isozymes were separated on nondenaturing polyacrylamide gels (7% T, 3% C) at 80 V for 17 h and 4°C using the procedures of Laemmli (1970). No loading buffer was necessary. Gels were stained in a solution of 0.25 M Tris, pH 7.8, containing 0.24 mM MTT, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol, and 3.6 mM GSSG in darkness for 1 h (similar to Anderson et al., 1990). Duplicate gels were also stained in the absence of GSSG to distinguish GR from other sources of MTT reduction.

Total APX activity was measured spectrophotometrically by monitoring the decline in A290 as ascorbate (ε = 2.8 mM⁻¹cm⁻¹) was oxidized, using the method of Nakano and Asada (1981). Crude protein extracts (3 μl, 15-30 μg protein) were mixed with 469 μl of water and 500 μl of 0.1 M potassium phosphate, pH 7.0, containing 0.2 mM EDTA and 20 μl 25 mM ascorbate was added. The reaction was initiated with 8 μl 25 mM H₂O₂. Samples run in the presence of 1 mM hydroxylamine hydrochloride (which inhibits APX, Chen and Asada, 1990) did not appreciably oxidize ascorbate in the assay. Thus, APX was the predominant source of ascorbate oxidation in the samples. Removal of low molecular weight compounds from the extract on Sephadex G-25 columns, as was done for samples from which SOD activity was measured, resulted in a
small loss of activity but did not alter the relative differences among treatments. Thus, subsequent replicates of the APX assay were conducted without column purification. APX isozymes were separated on nondenaturing polyacrylamide gels (10% T, 3% C) supported by 10% (v/v) glycerol and stained according to Mittler and Zilinskas (1993). The running buffer contained 2 mM ascorbate and the gels were run without samples for 0.5 h to allow ascorbate to enter the gel. Samples were loaded without loading buffer and the gels were run at 80 V for 6 h at 4°C. Gels were soaked in 50 mM sodium phosphate, pH 7.0, containing 2 mM ascorbate for 30 min to equalize ascorbate concentration throughout the gel, then in 50 mM sodium phosphate, pH 7.0, containing 4 mM ascorbate and 2 mM H₂O₂ for 20 min, then in 50 mM sodium phosphate, pH 7.8, containing 1.22 mM NBT and 14 mM TEMED for 15 min. Gels were stored in 10% (v/v) acetic acid.

**Lignin Analysis**

Lignin was quantified by a modification of the method of Liyama and Wallis (1990). Fresh mesocotyls (~0.15 g) were sectioned into ~3 mm segments, weighed, and placed in test tubes with 3 ml of 80% (v/v) ethanol. Segments were extracted with three successive 1.5 h extractions in 3 ml 80% (v/v) ethanol at 80°C, followed by a 1 h extraction in 3 ml chloroform at 62°C. Segments were then dried for at least 2 d at 50°C. Dried segments were digested in 2.6 ml of a solution of 25% (v/v) acetyl bromide in acetic acid, containing 2.7% (v/v) perchloric acid. After exactly 1 h, 100 μl of each sample was added to 580 μl of a solution of 17.24% (v/v) 2 N sodium hydroxide, 82.76% (v/v) acetic acid and 20 μl of 7.5 M hydroxylamine hydrochloride was added to ensure termination of the
reaction. The volume was made to 2 ml with acetic acid and A_{280} was measured. Lignin was quantified with a standard curve using milled Douglas Fir (containing 31.6% lignin, Musha and Goring, 1974) as the standard.

**Ascorbate and Glutathione Analyses**

Coleoptile+leaf, mesocotyl, and root from Cont, Ch, Ac, and AcCh seedlings were harvested into liquid nitrogen. Extraction of ascorbate was conducted according to Walker and McKersie (1993). Frozen tissue (1 g) was ground to a powder and extracted in 5 ml of 10% (w/v) TCA. Solid material was pelleted by centrifugation at 18000g for 15 min. Quantities of reduced ascorbate (AsA) and dehydroascorbate (DHA) were determined according to Law et al. (1983). For AsA, 200 µl of the supernatant was added to 200 µl water and 200 µl 150 mM sodium phosphate, pH 7.4. For total ascorbate (AsA + DHA), 200 µl of supernatant was added to 200 µl buffer and 100 µl 10 mM DTT and incubated at room temperature for 15 min. 100 µl of 40 mM N-ethylmaleimide was added and incubated at room temperature for 30 min to remove excess DTT. To all samples, 400 µl 10% (w/v) TCA, 400 µl 44% (v/v) phosphoric acid, 400 µl 4% (w/v) bipyridyl (in 70% [v/v] ethanol), and 200 µl 3% (w/v) ferric chloride was added and incubated for exactly 60 min at 37°C. AsA was quantified by measuring the absorbance at 525 nm and comparing to a standard curve. The DHA content was obtained by the difference between total ascorbate and AsA.

For measurement of glutathione, frozen tissue was ground to a powder and extracted (Walker and McKersie, 1993) in 8 ml 5% (w/v) TCA. Solid material was pelleted by centrifugation at 9000g. Quantities of GSH and GSSG were
determined according to Hissin and Hilf (1976). For measurement of GSSG, 100 
μl of the supernatant was incubated for 30 min at room temperature with 40 μl of 
40 mM N-ethylmaleimide and 860 μl 0.1 N sodium hydroxide was subsequently 
added. A 100 μl aliquot was then further diluted to 1.9 ml with 0.1 N sodium 
hydroxide. For measurement of GSH, 100 μl of the supernatant was added to 
900 μl of 0.1 M sodium phosphate, pH 8.0, and a 100 μl aliquot was further 
diluted to 1.9 ml with buffer. To all samples, 100 μl of 7.5 mM o- 
phthaldialdehyde (in 100% methanol) was added and incubated for exactly 15 
min. GSH and GSSG were quantified by measuring the fluorescence at 420 nm 
with excitation at 350 nm and comparing to a standard curve.

Statistical Analyses

H₂O₂, lignin, AsA, DHA, total ascorbate, GSH, GSSG, and total glutathione 
contents, as well as total activities of CAT, POX, SOD, APX, and GR were all 
analyzed as a randomized complete block design. Analyses were conducted 
separately for each tissue type. Means were separated with Tukey's studentized 
range test.

Results

Hydrogen Peroxide Levels

We have previously reported (Prasad et al., 1994a) that hydrogen peroxide 
levels were elevated over 4-fold in mesocotyls of maize seedlings exposed to a 
chilling stress but were maintained at low levels in mesocotyls from seedlings 
that were acclimated before applying the chilling stress. These measurements
indicated that chilling caused an increased generation of H$_2$O$_2$ but acclimation brought about changes that protected the seedling from oxidative stress. These changes were the basis for this study. Hydrogen peroxide levels in the coleoptile+leaf and root under various low temperature treatments are presented in Table 1 along with previously reported values for the mesocotyl (Prasad et al., 1994a). In the coleoptile+leaf, the same pattern was observed as in the mesocotyl, where H$_2$O$_2$ levels were elevated nearly 4-fold in the chilled treatment but were maintained at lower levels in the AcCh treatment. In the root, H$_2$O$_2$ levels were elevated to a lesser extent by all low temperature treatments, although these differences were not statistically significant. Either chilling does not cause as much active oxygen generation, or an adequate antioxidant system is already in place in the roots. We have reported that CAT and POX were induced in mesocotyls during acclimation, consistent with the maintenance of low H$_2$O$_2$ levels in the AcCh treatment (Prasad et al., 1994a). However, CAT (measured on a total activity level) was not induced by acclimation in the coleoptile+leaf (Anderson et al., 1994). Thus, CAT was not responsible for the maintenance of low H$_2$O$_2$ levels in AcCh coleoptile+leaf. Some other antioxidant mechanism must be induced by acclimation in these tissues.

**Region of CAT and POX Induction in the Mesocotyl**

We have established that CAT and POX were induced in maize mesocotyls. In Fig. 1, we have measured total CAT and POX activities in various regions of the mesocotyl, in an effort to more precisely locate the primary site of induction. In Cont seedlings, CAT activity was similar throughout the mesocotyl. However,
Table I. Hydrogen peroxide contents in various tissues of maize seedlings exposed to acclimation and chilling

Seedlings were grown in potting medium at 27°C for 3 d in darkness. AcCh seedlings were then exposed to an acclimation treatment of 14°C for 3 d, followed by a chilling treatment of 4°C for 4 d in darkness. Ac seedlings did not receive the chilling treatment, Ch seedlings did not receive the acclimation treatment, Cont seedlings did not receive either the chilling or the acclimation treatment. Treatments were replicated twice. Hydrogen peroxide contents are expressed as μmol H₂O₂ / g fresh weight. Hydrogen peroxide contents in mesocotyls were previously reported by Prasad et al. (1994a).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Coleoptile+leaf</th>
<th>Mesocotyl</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>238</td>
<td>177</td>
<td>179</td>
</tr>
<tr>
<td>Ch</td>
<td>801</td>
<td>745</td>
<td>351</td>
</tr>
<tr>
<td>Ac</td>
<td>264</td>
<td>250</td>
<td>313</td>
</tr>
<tr>
<td>AcCh</td>
<td>470</td>
<td>364</td>
<td>343</td>
</tr>
<tr>
<td>SE</td>
<td>69</td>
<td>42</td>
<td>89</td>
</tr>
</tbody>
</table>

while acclimation induced CAT to some extent in the apical three segments of the mesocotyl, the site of the greatest induction was in the middle to basipetal (segments 5 and 8) region of the mesocotyl. Compared to apical segments, POX activity was higher in the basipetal portion of Cont mesocotyls, possibly as a function of tissue maturity. Like CAT, the site of the greatest induction of POX by acclimation was in the middle to basipetal portion of the mesocotyl. Coincidentally, this was the region of the mesocotyl that visibly was the most sensitive to chilling damage. In accordance with these results, the apical 1 cm of the mesocotyl and the region 1.5-2.5 cm from the coleoptilar node of the mesocotyl were sampled separately in all subsequent acclimation experiments.
Figure 1. Catalase (CAT) and guaiacol peroxidase (POX) activities in various regions of the maize mesocotyl. Mesocotyls from Cont, Ch, Ac, and AcCh treatments were sectioned into 3 mm segments and segments 1, 2, 3, 5, 8, and the penultimate segment (P) were assayed for CAT and POX. Units for both enzymes were μmol H₂O₂ / min / mg protein. Treatments were replicated three times. CAT and POX activities were analyzed as a randomized complete block design within each segment. For CAT: segment 1, SE=0.68; segment 2, SE=1.36; segment 3, SE=1.48; segment 5, SE=2.09; segment 8, SE=1.85; penultimate segment, SE=2.12. For POX: segment 1, SE=0.15; segment 2, SE=0.22; segment 3, SE=0.22; segment 5, SE=0.10; segment 8, SE=0.25; penultimate segment, SE=0.26.
Catalase Isozymes

In shoots and roots of dark-grown maize seedlings, CAT is present as two isozymes, CAT1 and CAT3. During the separation of these isozymes on nondenaturing polyacrylamide gels, we observed that CAT1, but not CAT3, was very susceptible to oxidation on the gel. Inclusion of β-mercaptoethanol in the loading buffer protected CAT1 from oxidation but caused significant inactivation of CAT3 at concentrations of 10 mM (30 mM in the 3X loading buffer) and above. Lower concentrations did not fully protect CAT1 (data not shown). DTT was found to protect CAT1 but had no deleterious effect on CAT3 activity. The concentration adopted was 20 mM DTT (60 mM in the 3X loading buffer). In addition to requiring DTT in the loading buffer, it was found that even though CAT1 activity was maintained in extracts (as long as glycerol was present), it was still susceptible to oxidation (presumably), manifested as an increase in electrophoretic mobility. This effect is illustrated in Fig. 2, where extracts were left unprotected at 4°C for various lengths of time before adding glycerol and 10 mM ascorbate. Left unprotected, CAT1 lost considerable activity after 2 h and was undetectable after 8 h. Corresponding to the loss of activity was an increase in electrophoretic mobility with increasing oxidation. Morikofe-Zwez et al. (1969) observed this effect in erythrocyte catalase and suggested that these variants arose as a result of irreversible oxidation of sulphydryl groups. Interestingly, addition of glycerol without ascorbate resulted in a maintenance of activity but did not prevent the occurrence of the mobility shift (samples were ~9 h old when loaded on the gel). We envision two types of oxidation, one that affects electrophoretic mobility but does not affect activity, and another that
Figure 2. Effect of various levels of oxidation on catalase (CAT) activity and mobility. Cell-free extracts (containing no added reducing agent) were allowed to slowly oxidize in air at 4°C for various lengths of time, followed by addition of glycerol and 10 mM ascorbate or glycerol alone. Lanes 1 and 2, sample allowed to oxidize 4 h and 0.5 h respectively followed by addition of glycerol but no ascorbate. Lane 4, sample ground in 20 mM ascorbate and glycerol was added immediately after extraction. Lanes 3, 5-9, samples allowed to oxidize 0.5 h, 2 h, 4 h, 8 h, 16 h, 24 h, respectively followed by addition of both glycerol and ascorbate. All samples were loaded (50 µg total protein) on a 7% nondenaturing polyacrylamide gel and stained for CAT activity.
involves direct inactivation. It was also noted that CAT3 was somewhat susceptible to oxidation in terms of the mobility shift, albeit not as susceptible as CAT1. Addition of 10 mM DTT to samples no more than 30 min from the time of tissue maceration was routinely used to maintain consistent electrophoretic mobility among treatments, as has been done by other workers (e.g. Kunce and Trelease, 1986; Mullen and Gifford, 1993).

The response of CAT isozymes to acclimation and chilling is shown in Fig. 3 and corresponding quantification of total activity is given in Table II. The data in Table II shows trends similar to those previously reported (Anderson et al., 1994). We observed no significant differences in total CAT activity among treatments in either the coleoptile+leaf or the root. CAT activity was significantly higher than Cont in the Ac and AcCh treatments in both the top and bottom of the mesocotyl. In contrast to data previously reported, however, we saw a small, but significant, decline in total CAT activity in the Ch treatment. The contributions of CAT1 and CAT3, shown in Fig. 3, closely reflect the total activity data. We observed that both CAT1 and CAT3 contribute prominently to the total activity in coleoptile+leaf and mesocotyls while CAT1 dominates in roots. A faint CAT3 band was observed in roots and it was induced slightly by acclimation but CAT1 was unaffected by any of the treatments. Neither CAT1 nor CAT3 were affected by any of the treatments in the coleoptile+leaf. In the mesocotyl, the increased total CAT activity in the Ac and AcCh treatments appear to be primarily due to an increase in CAT3. Induction of CAT3 by acclimation was visible in both top and bottom portions of the mesocotyl with the bottom having generally greater activities than the top for both CAT1 and CAT3. The small
Figure 3. Separation of catalase (CAT) isozymes from seedlings exposed to acclimation and chilling. Cell-free extracts from coleoptile+leaf, mesocotyl top, mesocotyl bottom, and root of Cont (1), Ch (2), Ac (3), and AcCh (4) treatments (see Table I for treatment description) were loaded at a 30 μg total protein level and stained for CAT activity.
Table II. Lignin content and total activities of CAT, POX, SOD, GR, and APX in various tissues of maize seedlings exposed to acclimation and chilling.

Lignin is expressed as mg lignin / g dry weight. Enzyme units are: CAT and POX, μmol H₂O₂ / min / mg protein; GR, nmol NADPH / min / mg protein; APX, μmol ascorbate / min / mg protein; SOD, the quantity of enzyme required to inhibit the reduction of cyt c by 50% in a 1 ml reaction volume. CAT and POX were replicated nine times, SOD, GR, APX, and lignin were replicated three times. See Table I for treatment descriptions. Mesocotyl Top = a 1 cm segment immediately below the coleoptilar node. Mesocotyl bottom = a 1 cm segment 1.5-2.5 cm below the coleoptilar node.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>CAT</th>
<th>POX</th>
<th>Lignin</th>
<th>SOD</th>
<th>APX</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptile+leaf</td>
<td>Cont</td>
<td>35.50</td>
<td>1.43</td>
<td></td>
<td>4.17</td>
<td>1.43</td>
<td>50.97</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>32.36</td>
<td>1.39</td>
<td></td>
<td>4.85</td>
<td>1.36</td>
<td>51.80</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>33.69</td>
<td>1.23</td>
<td></td>
<td>3.21</td>
<td>1.23</td>
<td>45.45</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>34.37</td>
<td>1.23</td>
<td></td>
<td>4.04</td>
<td>1.23</td>
<td>48.90</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.93</td>
<td>0.03</td>
<td></td>
<td>1.06</td>
<td>0.04</td>
<td>1.70</td>
</tr>
<tr>
<td>Mesocotyl Top</td>
<td>Cont</td>
<td>26.46</td>
<td>1.85</td>
<td>55.41</td>
<td>6.25</td>
<td>1.29</td>
<td>35.20</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>21.77</td>
<td>1.60</td>
<td>51.31</td>
<td>6.61</td>
<td>1.24</td>
<td>37.19</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>34.16</td>
<td>2.23</td>
<td>72.05</td>
<td>6.14</td>
<td>1.41</td>
<td>26.13</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>34.25</td>
<td>1.93</td>
<td>67.65</td>
<td>5.36</td>
<td>1.38</td>
<td>32.96</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.97</td>
<td>0.06</td>
<td>3.23</td>
<td>0.34</td>
<td>0.03</td>
<td>2.36</td>
</tr>
<tr>
<td>Mesocotyl Bottom</td>
<td>Cont</td>
<td>28.82</td>
<td>2.80</td>
<td>64.34</td>
<td>5.37</td>
<td>1.13</td>
<td>41.77</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>25.84</td>
<td>2.42</td>
<td>56.99</td>
<td>5.65</td>
<td>1.06</td>
<td>43.29</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>40.43</td>
<td>4.67</td>
<td>81.85</td>
<td>6.75</td>
<td>1.18</td>
<td>51.10</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>41.53</td>
<td>3.78</td>
<td>84.74</td>
<td>6.67</td>
<td>1.20</td>
<td>52.26</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.70</td>
<td>0.12</td>
<td>3.51</td>
<td>0.67</td>
<td>0.05</td>
<td>2.12</td>
</tr>
<tr>
<td>Root</td>
<td>Cont</td>
<td>23.69</td>
<td>6.47</td>
<td></td>
<td>2.93</td>
<td>1.37</td>
<td>54.44</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>23.52</td>
<td>5.49</td>
<td></td>
<td>4.63</td>
<td>1.32</td>
<td>46.84</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>23.26</td>
<td>6.58</td>
<td></td>
<td>4.33</td>
<td>1.38</td>
<td>50.08</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>24.77</td>
<td>6.03</td>
<td></td>
<td>4.55</td>
<td>1.54</td>
<td>45.69</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.81</td>
<td>0.20</td>
<td></td>
<td>0.94</td>
<td>0.05</td>
<td>2.89</td>
</tr>
</tbody>
</table>
decline in total CAT activity in the Ch treatment appeared to result from a decline in both CAT1 and CAT3, perhaps moreso from CAT1. This decline was consistent among replicate gels.

**Peroxidase Isozymes**

Total POX activity (Table II) was generally the lowest in the coleoptile+leaf, higher in the mesocotyl, and highest in the root. Chilling caused a significant reduction of POX activity in both the mesocotyl and the root. Significant induction of total POX activity by acclimation occurred only in the mesocotyl, in the bottom more so than in the top. When acclimated seedlings were subsequently chilled, POX activity was lower than when seedlings were acclimated without chilling. Examination of the POX isozyme profiles by IEF revealed a number of changes that occurred in response to acclimation and chilling (Fig. 4). All major isozymes observed using \( p \)-phenylenediamine as the substrate were also responsive to guaiacol, indicating that these results should be comparable to the total activities in Table II. In the coleoptile+leaf, POX activity was very low but there were two isozymes, focusing at pH 8.0 and 8.4 that were higher in both the Ac and AcCh treatments. In the mesocotyl top, we saw induction of nearly all of the major bands in the Ac and AcCh treatments; bands at pH 5.1, 6.7, 7.6, 8.0, 8.4, 8.8, and 9.0 were most notable. These bands were also induced by acclimation from a higher Cont level in the bottom of the mesocotyl (when compared to mesocotyl top) except those at pH 6.7, 7.6, and 8.0. No notable induction of any POX isozyme was observed from the constitutively high levels present in the root. Even though the band at pH 8.4
Figure 4. Separation of guaiacol peroxidase (POX) isozymes on a 5% IEF gel, pH 3-10, from seedlings exposed to acclimation and chilling. Cell-free extracts from coleoptile+leaf, mesocotyl top, mesocotyl bottom, and root of Cont (1), Ch (2), Ac (3), and AcCh (4) treatments were loaded at a 75 μg total protein level and stained for POX activity. PIs were determined using an IEF marker kit, pI range 3.5-9.3, and stained according to manufacturer's instructions (Sigma, catalog # I-3018). Precipitation at the applicator is indicated as "appl."
<table>
<thead>
<tr>
<th>Coleoptile+leaf</th>
<th>Mesocotyl Top</th>
<th>Mesocotyl Bottom</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
appears to be a doublet in Ac and AcCh treatments in the mesocotyl bottom and in the root, we have questioned whether these are separate isozymes (see Discussion). Until we obtain evidence to the contrary, we have considered the doublet as one isozyme and discuss its intensity in terms of both bands. The band at pH 6.7 was found to be a mitochondrial peroxidase (T.K. Prasad, M.D. Anderson, C.R. Stewart, manuscript submitted) and is likely responsible for the induction of POX by acclimation in mitochondrial preparations (Prasad et al., 1994b). Several bands appeared to decline somewhat in response to chilling. In the mesocotyl bottom, bands focusing at pH 7.6 and 8.8 were most notably reduced. One band, focusing at pH 7.0 was elevated in only the AcCh treatment in coleoptile+leaf and in the mesocotyl top and in both Ch and AcCh treatments in the bottom of the mesocotyl, indicating that it may be an isozyme that was induced, not by acclimation, but by chilling.

There was considerable POX activity present at the cathode, peroxidases with pl's higher than the pH range of the gel. In order to determine whether there were any effects of treatment on these isozymes, gels were underfocused, in an effort to observe the fast-migrating bands before they reached the cathode. These results are shown in Fig. 5. We were able to visualize at least three additional bands. In the 1.5 h gel section, the uppermost arrow indicates an isozyme that was both induced by acclimation and reduced by chilling and the lower arrow indicates an isozyme that was unaffected by treatment. In the 1.33 h section, the arrow indicates a band that was strongly induced by acclimation.

Since peroxidase involvement in lignification is well established (Gaspar et
Figure 5. Underfocusing peroxidase isozymes so that those with pI > 9.3 could be visualized. Cell-free extracts from mesocotyl bottom of Cont (1), Ch (2), Ac (3), and AcCh (4) treatments were loaded at a 75 µg total protein level and run at 20 W constant power for various lengths of time. Isozymes were most visible in the gel sections run for 1.33 h and 1.5 h. Three additional isozymes not observed in Fig. 4 are indicated by arrows. The lowest band on the 1.5 h gel section had reached the cathode and was better represented on the 1.33 h section.
al., 1991), it was of interest to determine which of these POX isozymes was cell wall localized. The POX isozyme profiles in cell wall and protoplast fractions are shown in Fig. 6. Bands at pH 4.7 and 8.0 were clearly cell wall peroxidases. The band at pH 8.4 appeared most abundantly in the cell wall but was also prominent in the protoplast fraction. It may be a cell wall peroxidase that was loosely associated with the cell wall so that a significant portion was extracted into the protoplast fraction. The band appearing at ~pH 7.5 in the cell wall fraction did not have the same appearance as the other bands, but was reminiscent of protein precipitation on the gel. At a lower loading level, this band was not present. Thus, it was likely not a true isozyme. All other POX isozymes were localized in the protoplast.

Lignin Content

The fact that of the three POX isozymes localized in the cell wall, two were induced by acclimation (the band at pH 8.0 was induced in the mesocotyl top and the band at pH 8.4 was induced in both the top and bottom of the mesocotyl) suggested that lignification may be a process affected by acclimation. Lignin contents are reported in Table II and indeed, lignin content was significantly higher in both the top and bottom of the mesocotyl in the Ac and AcCh treatments when compared to the Cont and Ch treatments.

Superoxide Dismutase and Ascorbate Peroxidase Isozymes

We have previously reported, without showing data, that total SOD activity was unaffected by acclimation and/or chilling in mesocotyls of dark-grown maize.
Figure 6. Separation of guaiacol peroxidase isozymes in cell wall (CW) and protoplasmic (P) fractions from the bottom of the mesocotyl of Ac and AcCh seedlings. Lanes 1 and 2 are total protein extracts (75 μg total protein) from the mesocotyl bottom and root of the AcCh treatment respectively. Lanes 3 and 4 are cell wall (15 μg total protein) and protoplasmic (15 μg total protein) fractions from Ac and lanes 5 and 6 are cell wall and protoplasmic fractions from AcCh, all taken from the bottom of the mesocotyl. Those bands that were induced by acclimation in both the top and bottom of the mesocotyl (Fig. 4) are marked as **. Bands induced in the top but not the bottom of the mesocotyl are marked as *. The band induced by chilling is marked as #. Precipitation at the applicator is indicated as "appl." The band at ~pH 7.5 in the cell wall fractions did not have the same appearance as other bands and was considered an artifact.
<table>
<thead>
<tr>
<th>Total Protein</th>
<th>Ac</th>
<th>AcCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB R CW P CW P pi</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pl

4.7
5.1**
5.5
Appl

6.7*
7.0#

7.6*
8.0*

8.4**
8.8**
9.0**
Cathode
seedlings (Prasad et al., 1994a). Total SOD activities are given in Table II and, although there appeared to be a small induction of SOD by acclimation in the bottom of the mesocotyl, there were no significant differences in SOD activity among treatments in any of the tissues tested. This was verified by examining the SOD isozymes on nondenaturing polyacrylamide gels (data not shown). We observed four SOD isozymes but none of them were strikingly affected by acclimation or chilling. APX activity is also reported in Table II and like SOD, there were no meaningful differences among treatments in any of the tissues tested. Nondenaturing polyacrylamide gels, stained for APX, revealed four APX isozymes, two of which were prominent and two were very faint. None of the bands were affected by treatment. With the lack of response of SOD and APX isozymes to acclimation or chilling, no effort was made to further characterize these enzymes.

Glutathione Reductase Isozymes

Only minor changes in total GR activity (Table II) were observed in response to acclimation and chilling. GR activity was increased slightly in the Ac and AcCh treatments in the bottom of the mesocotyl. No other significant differences among treatments were observed in the other tissues tested. However, examination of GR isozyme profiles (Fig. 7A), revealed three isozymes that were greatly affected by acclimation in the mesocotyl. Activity in the three bands were dependent on the presence of GSSG during the staining reaction, verifying their identities as GR. In Cont seedlings, the top band was most prominent in the coleoptile+leaf and the mesocotyl and the bottom two bands were most
Figure 7A. Separation of glutathione reductase isozymes from seedlings exposed to acclimation and chilling. The same cell-free extracts used for catalase (Fig. 3) were loaded at a 100 μg total protein level. The three bands observed did not stain in the absence of GSSG, verifying their identity as glutathione reductases. B. Separation of glutathione reductase isozymes from plastid (P), mitochondria (M), and cytosol (C) fractions (all loaded at 100 μg total protein) from the mesocotyl of Ac seedlings. Plastid and mitochondrial samples contained 1% (v/v) Nonidet P-40 to disrupt membranes. A total protein extract (T, 75 μg total protein) containing all three bands in high quantities was run for comparison (ABACCh root was used, see Fig. 10).
prominent in the roots. No significant changes in the GR profile were observed
in the coleoptile+leaf or the roots in response to acclimation and chilling. In the
mesocotyl (both top and bottom), the upper band was greatly diminished in the
Ac and AcCh treatments while the lower two bands were greatly increased. The
net effect was the absence of a significant change in total GR activity (Table II).

Examination of GR isozyme profiles in plastidic, mitochondrial, and cytosolic
fractions (Fig. 7B) revealed that all three bands observed in the cell-free extracts
were cytosolic. Apparently, the plastidic and mitochondrial isozymes were too
dilute to be observed in the cell-free extracts. Plastid and mitochondrial isolates
each had one GR band that migrated differently than any of the cytosolic bands.
A rapidly migrating band also appeared in the plastids which was found to stain
at equal intensity in the absence of GSSG and was thus not GR.

Ascorbate and Glutathione Levels

Since $H_2O_2$ levels in Ch coleoptile+leaf were elevated but were maintained
at low levels in the AcCh treatment, some antioxidant mechanism must be
induced by acclimation in these tissues. However, none of the antioxidant
enzymes examined in this study (CAT, POX, SOD, APX, or GR) were affected
by acclimation in the coleoptile+leaf. Thus, the levels of ascorbate and
 glutathione were measured (Table III) to determine if there were changes in the
 pools of these antioxidants that could account for the low $H_2O_2$ levels in the
 AcCh coleoptile+leaf. In both coleoptile+leaf and in mesocotyl, we generally saw
 a decline in AsA, no significant change in DHA (although Cont DHA appears
 slightly higher in the coleoptile+leaf, it was not statistically higher), and a decline
Table III. Ascorbate and glutathione levels in various tissues of maize seedlings exposed to acclimation and chilling

Ascorbate (AsA) and dehydroascorbate (DHA) levels are expressed as μmol / g dry weight. Reduced (GSH) and oxidized (GSSG) glutathione are expressed as mg / g dry weight. Treatments were replicated three times. See Table I for treatment descriptions.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>AsA</th>
<th>DHA</th>
<th>Total</th>
<th>GSH</th>
<th>GSSG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptile+Leaf</td>
<td>Cont</td>
<td>21.91</td>
<td>7.09</td>
<td>29.00</td>
<td>2.49</td>
<td>1.07</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>14.97</td>
<td>4.49</td>
<td>19.46</td>
<td>2.19</td>
<td>1.54</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>21.09</td>
<td>4.24</td>
<td>25.33</td>
<td>2.70</td>
<td>1.70</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>19.06</td>
<td>3.98</td>
<td>23.03</td>
<td>2.53</td>
<td>1.98</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.77</td>
<td>0.69</td>
<td>0.91</td>
<td>0.08</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>Mesocotyl</td>
<td>Cont</td>
<td>12.80</td>
<td>4.75</td>
<td>17.56</td>
<td>2.72</td>
<td>1.57</td>
<td>4.29</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>9.70</td>
<td>4.98</td>
<td>14.68</td>
<td>2.43</td>
<td>1.94</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>14.00</td>
<td>5.08</td>
<td>19.08</td>
<td>2.70</td>
<td>1.95</td>
<td>4.65</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>12.05</td>
<td>3.74</td>
<td>15.79</td>
<td>2.69</td>
<td>2.22</td>
<td>4.92</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.56</td>
<td>0.60</td>
<td>0.91</td>
<td>0.07</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Root</td>
<td>Cont</td>
<td>8.79</td>
<td>4.02</td>
<td>12.93</td>
<td>1.94</td>
<td>0.88</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>Ch</td>
<td>6.35</td>
<td>3.38</td>
<td>9.73</td>
<td>1.46</td>
<td>1.23</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>Ac</td>
<td>8.93</td>
<td>3.01</td>
<td>12.10</td>
<td>1.84</td>
<td>1.01</td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td>AcCh</td>
<td>6.26</td>
<td>3.09</td>
<td>9.35</td>
<td>1.56</td>
<td>1.23</td>
<td>2.79</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.44</td>
<td>0.33</td>
<td>0.82</td>
<td>0.08</td>
<td>0.05</td>
<td>0.12</td>
</tr>
</tbody>
</table>

In the total ascorbate pool in response to chilling. Neither AsA nor DHA were significantly different in the Ac and AcCh treatments when compared to Cont. In the root, AsA was significantly lower in both Ch and AcCh treatments than Cont but DHA was unaffected by treatment. Like AsA, GSH was lower in both coleoptile+leaf and mesocotyl in the Ch treatment compared to Cont and GSSG was correspondingly higher resulting in no change in the total glutathione pool. However, in the Ac and AcCh treatments, GSH remained near Cont levels while GSSG was near Ch levels, resulting in an increase in the total glutathione pool. This effect was more pronounced in the coleoptile+leaf than in the mesocotyl. In
the root, there was no change in the total glutathione pool among treatments and there was lower GSH and correspondingly higher GSSG in the Ch and AcCh treatments than in Cont.

**Effects of ABA treatment**

We have previously reported that, like acclimation, exogenous ABA treatment both improved chilling tolerance and induced CAT activity (Anderson et al., 1994). Since acclimation induced changes in CAT, POX, GR, and lignin; we also examined these parameters in ABA-treated seedlings. Compared to Cont3d and ABA@4, total CAT activity (Table IV) was significantly lower in the Cont4d, ABA, and ABACh treatments in the coleoptile+leaf. In both the mesocotyl and root, total CAT activity was induced in the ABA, and ABACh treatments. CAT was also slightly lower in all tissues in the ABA@4 treatment but these were not significantly different than Cont3d. Fig. 8 shows the CAT isozyme profiles in response to these treatments. The decline in CAT activity in the Cont4d, ABA, and ABACh treatments of the coleoptile+leaf resulted from a loss of CAT1 activity, presumably a developmental effect. The dramatic increase in electrophoretic mobility in both CAT1 and CAT3 was reminiscent of the mobility shifts shown in Fig. 2, suggesting the possibility of developmentally regulated oxidation of CAT *in vivo*. The induction of CAT in mesocotyls of the ABA and ABACh treatments appeared to be due primarily to an induction of CAT1. There may have also been an increase in CAT3 but the differences in CAT3 activity in the ABA and ABACh treatments when compared to Cont4d was slight. In the roots, CAT1 was strongly induced by ABA.
Table IV. Lignin content and CAT, POX, and GR activities in maize seedlings exposed to various ABA and chilling treatments

Seedlings were grown in germination paper at 27°C in darkness and exposed to various ABA and chilling treatments. Cont3d = 3 d old seedlings; Cont4d = 4 d old seedlings; ABA = 3 d old seedlings exposed to 1 mM ABA for 1 d at 27°C in darkness; ABACh = ABA-treated seedlings subsequently exposed to a chilling treatment of 4°C for 4 d in darkness; ABA@4 = 3 d old seedlings chilled at 4°C for 2 d, treated with 1 mM ABA for 1 d at 4°C, then chilled an additional day. Treatments were replicated three times. Lignin content and enzyme units are expressed as in Table II.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatment</th>
<th>CAT</th>
<th>POX</th>
<th>Lignin</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptile+leaf</td>
<td>Cont3d</td>
<td>39.00</td>
<td>1.35</td>
<td>50.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont4d</td>
<td>26.83</td>
<td>1.18</td>
<td>47.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA</td>
<td>27.45</td>
<td>1.21</td>
<td>51.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABACh</td>
<td>28.84</td>
<td>1.18</td>
<td>42.72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA@4</td>
<td>32.61</td>
<td>1.23</td>
<td>46.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.80</td>
<td>0.06</td>
<td>3.52</td>
<td></td>
</tr>
<tr>
<td>Mesocotyl</td>
<td>Cont3d</td>
<td>31.04</td>
<td>1.77</td>
<td>66.07</td>
<td>37.85</td>
</tr>
<tr>
<td></td>
<td>Cont4d</td>
<td>34.58</td>
<td>2.15</td>
<td>76.35</td>
<td>35.41</td>
</tr>
<tr>
<td></td>
<td>ABA</td>
<td>46.03</td>
<td>2.18</td>
<td>78.03</td>
<td>44.54</td>
</tr>
<tr>
<td></td>
<td>ABACh</td>
<td>43.25</td>
<td>1.85</td>
<td>73.57</td>
<td>39.87</td>
</tr>
<tr>
<td></td>
<td>ABA@4</td>
<td>25.30</td>
<td>1.49</td>
<td>55.91</td>
<td>32.45</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.67</td>
<td>0.05</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>Cont3d</td>
<td>34.77</td>
<td>5.11</td>
<td>63.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont4d</td>
<td>31.09</td>
<td>5.95</td>
<td>47.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA</td>
<td>41.63</td>
<td>5.19</td>
<td>81.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABACh</td>
<td>43.30</td>
<td>4.84</td>
<td>90.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA@4</td>
<td>26.37</td>
<td>4.48</td>
<td>57.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.74</td>
<td>0.19</td>
<td>3.70</td>
<td></td>
</tr>
</tbody>
</table>

No changes in total POX activity in response to ABA (Table IV) were observed in coleoptile+leaf. In mesocotyls, there was a small increase in the Cont4d and ABA treatments relative to Cont3d. In both mesocotyl and root, there was a slight reduction in POX activity when the seedlings were chilled.
Figure 8. Separation of catalase isozymes from seedlings exposed to various ABA and chilling treatments. Cell-free extracts from coleoptile+leaf, mesocotyl, and root of Cont3d (5), Cont4d (6), ABA (7), ABACh (8), and ABA@4 (9) treatments (see Table IV for treatment descriptions) were loaded at a 30 μg total protein level. In order to better observe treatment differences for CAT1, root samples were also loaded at 15 μg total protein. The position of a faint CAT1 band in the Cont4d, ABA, and ABACh treatments of the coleoptile+leaf is indicated by the arrow.
(ABA@4 < Cont3d, ABACCh < ABA), as was seen in the acclimation experiments. POX isozyme profiles (Fig. 9) corroborated these data. In the mesocotyl, bands at pH 5.1 and 8.4 were induced by ABA and ABACCh relative to Cont3d, but not compared to Cont4d. Thus, it appeared that ABA had no effect on POX isozymes and any changes observed among treatments were developmental effects. We also observed a difference in the overall isozyme profile when compared to the acclimation treatments (Fig. 4). The band at pH 8.8 was much more intense in the roots and a band at pH 7.4 appeared that was indistinguishable in the roots of the acclimation treatments. The source of these effects is unknown. To accompany POX, lignin contents are also given in Table IV and a similar interpretation is suggested. While lignin contents were higher in ABA and ABACCh mesocotyls with respect to Cont3d, they were not higher than in Cont4d. Thus, these differences in lignin content were likely due to developmental, and not ABA effects.

Total GR activities (Table IV) were not affected by any of the ABA treatments in either the coleoptile+leaf or the mesocotyl. In the roots, GR was significantly induced in the ABA and ABACCh treatments. The data in Fig. 10, show that ABA did not induce the same response in GR isozymes as acclimation, i.e. there was no decrease in the top band or increases in the bottom bands. The cause of the increase in total GR in the roots in response to ABA was an induction of the top band. Interestingly, we observed a decreased mobility in the top and middle bands and an increased mobility in the bottom band in the Cont4d, ABA, and ABACCh coleoptile+leaf. The source of these changes is unknown but as we observed with CAT, it appeared to be a developmental phenomenon.
Figure 9. Separation of guaiacol peroxidase isozymes on a 5% IEF gel, pH 3-10, from seedlings exposed to various ABA and chilling treatments. Cell-free extracts from coleoptile+leaf, mesocotyl, and root of Cont3d (5), Cont4d (6), ABA (7), ABAC (8), and ABA@4 (9) treatments were loaded at a 75 μg total protein level. PIs are indicated to the right of the figure.
<table>
<thead>
<tr>
<th></th>
<th>Coleoptile+leaf</th>
<th>Mesocotyl</th>
<th>Root</th>
<th>pi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 6 7 8 9</td>
<td>5 6 7 8 9</td>
<td>5 6 7 8 9</td>
<td>7 8 9</td>
</tr>
<tr>
<td>4.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathode</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. Separation of glutathione reductase isozymes from seedlings exposed to various ABA and chilling treatments. The same cell-free extracts used for catalase (Fig. 8) were loaded at a 75 μg total protein level.
Discussion

In this study, we have observed numerous effects on antioxidant enzymes in response to acclimation. While there was no effect on SOD or APX by acclimation, CAT3, at least nine POX isozymes, and three cytosolic GR isozymes all responded to acclimation. These responses were observed primarily in the mesocotyl and few changes were observed in the coleoptile+leaf or the root. It is understandable that if the mesocotyl is the most sensitive to chilling damage, that acclimation-induced chilling tolerance mechanisms would be focused on this organ. We also noted that the mature cells of the mesocotyl appeared to be more susceptible to chilling damage than the cells in the apical region. Total CAT and POX activity (Fig. 2) were induced by acclimation more strongly in the mature cells, supporting the correlation of chilling sensitivity of the tissue with induction of antioxidant enzymes during acclimation. These responses were confirmed by examination of isozyme profiles. While there were dramatic changes in CAT3, POX, and GR in the top of the mesocotyl in response to acclimation (Figs. 3, 4, 7A), the changes were more pronounced in the bottom of the mesocotyl.

The Role of Catalase during Acclimation and Chilling

We observed (Fig. 3) that CAT3 activity was induced by acclimation. This corroborates the induction of CAT3 previously reported (Prasad et al., 1994a,b) in which CAT3 activity was determined spectrophotometrically in cell-free extracts in the presence of 3-amino-1,2,4-triazole (differentially inhibits CAT1 over CAT3) or in mitochondrial preparations. We have previously suggested that
in nonphotosynthetic tissue such as the mesocotyl of maize, or in dark-grown seedlings, the mitochondria are a primary source of active oxygen during chilling (Prasad et al., 1994b). Thus, induction of CAT3 in the mitochondria is likely important in protecting mitochondrial components from oxidative damage. Mitochondria have also been implicated as being a major source of extramitochondrial H₂O₂ (Puntarulo et al., 1991) in nonphotosynthetic tissue and thus, CAT3 may serve as a first line of defense against oxidative damage throughout the cell.

While acclimation did not appear to significantly induce CAT1 (Fig. 3) we observed that CAT1 was susceptible to inactivation by chilling. A number of studies have been conducted to show that catalase activity declines in response to chilling in combination with high light (MacRea and Ferguson, 1985; Volk and Feierabend, 1989; Feierabend et al., 1992; Mishra et al., 1993). The proposed mechanism of catalase photoinactivation is through generation of active oxygen species from excess light energy absorbed by chlorophyll (Feierabend and Engel, 1986). Active oxygen species, possibly in the form of organic peroxides, inactivate catalase (this was peroxisomal catalase in rye, possibly functionally analogous to maize CAT2). In our system, chilling also caused inactivation of CAT1 in the dark, although the degree of inactivation was not as pronounced and a longer duration of exposure to chilling is required for notable inactivation. In vitro, CAT1 is susceptible to oxidative inactivation (Fig. 2). Although we cannot rule out differences in cat1 gene expression, we have shown that H₂O₂ levels are elevated in response to chilling and it is possible that one of the effects is oxidative inactivation of CAT1. It is noteworthy that CAT1 activity was
maintained in the AcCh treatment. Whether acclimation directly affects CAT1 or indirectly prevents its inactivation through maintenance of low H₂O₂ levels is unknown.

The Role of Peroxidase during Acclimation and Chilling

We observed the induction of at least nine major POX isozymes in response to acclimation. Thus, POX may be involved in a number of different processes that play some role in chilling tolerance. Conservatively, we observe a total of ten distinct bands in Fig. 4 and an additional three bands in Fig. 5. Numerous minor POX bands were also present although most were too faint to be confidently discussed in terms of treatment effects. Jackson and Ricardo (1994) warned against interpreting all bands as distinct isozymes because of possible in vitro modifications or other artifacts. Since the band at ~pH 6.4 was directly beneath the applicator, we are not confident that it is a properly focused isozyme. Further, we are not convinced that the doublet observed at pH 8.4 in Fig. 4 truly represents two separate isozymes, especially given the fact that it occurs as a doublet only in samples with very high activity (Ac and AcCh treatments in the bottom of the mesocotyl and in roots), and only one band appeared in mesocotyls of the ABA-treated samples (Fig. 9). At a lower loading level, only one band appeared. Likewise, the band at pH 5.1 was always observed as a doublet in the AcCh mesocotyl bottom (and also in Ac mesocotyl bottom in one replicate gel). It is possible that these are in vitro artifacts but they may also be true treatment effects where the treatment resulted in a post-translational modification of the isozyme. Savich (1990) presented evidence for
qualitative changes in basic POX isozymes in response to low temperature in maize and we may be seeing similar effects. Even if such is the case and they are not genetically distinct isozymes, they may be important aspects of the acclimation process.

At this point, we have made no effort to identify the substrates most reactive with individual isozymes and cannot suggest possible functions. In general, peroxidases involved in lignification and suberization have proven to be anionic while those involved in IAA catabolism and ethylene synthesis are thought to be cationic (Gaspar et al., 1991). We identified three prominent isozymes as being ionically bound to the cell wall and thus potentially involved in lignification or suberization (Fig. 6). However, the ones that were induced by acclimation were both cationic (pI 8.0 and 8.4). There are a number of examples where lignification has been correlated with cationic POX isozymes (Church and Galston, 1988; Chabanet et al., 1993; Smith et al., 1994), thus the notion that POX isozymes involved in lignification are anionic, may not hold for all species. In maize, Grison and Pilet (1985) reported 11 cytosolic POX isozymes, four ionically bound to the cell wall, and four covalently bound to the cell wall. Thus, their results are similar to ours, at least in terms of number of major isozymes detected (covalently bound isozymes would not be present in our samples).

Regardless of whether induction of the two cell wall peroxidases are directly responsible for the increased lignification, the fact that lignin content is elevated in acclimated seedlings (Table II) is important in itself. It is another means by which chilling tolerance may be enhanced by acclimation. Lignin is a complex polymer of phenolic alcohols that is a major structural component of cell walls.
and is especially prevalent in the vasculature. Its function is primarily to confer mechanical strength. In accordance with this function, acclimated mesocotyls would have an increased rigidity, allowing them to remain intact while unacclimated mesocotyls become desiccated and mechanically weak in response to chilling. Lignin deposition in cell walls is a process that occurs in mature tissues after cell expansion has ceased and increases in cell wall peroxidases are correlated with the increased lignification during the aging process (Siegel, 1993). In this respect, the increased POX activity and lignification induced by acclimation may be considered an accelerated aging process, possibly as a result of the transient increase in $H_2O_2$ that we have previously reported (Prasad et al., 1994a). In this way, acclimation produces a more mature mesocotyl that is more able to withstand a chilling stress.

The Role of Glutathione Reductase during Acclimation and Chilling

Glutathione reductase is best known in plants from its participation in the ascorbate-glutathione cycle (see review by Smith et al., 1989). In the light, excess electrons from the photosystems reduce molecular oxygen to form superoxide. Under unstressed conditions, superoxide is effectively catabolized by SOD and ascorbate to form $H_2O_2$. The scavenging of $H_2O_2$ is then accomplished by the action of ascorbate peroxidase which utilizes $H_2O_2$ to oxidize ascorbate to monodehydroascorbate radical, which disproportionates to dehydroascorbate (DHA) nonenzymatically. Monodehydroascorbate reductase regenerates ascorbate at the expense of NAD(P)H and dehydroascorbate reductase regenerates ascorbate utilizing GSH to form GSSG. GSH is
regenerated at the expense of NADPH by the action of GR, the rate-limiting step of the cycle (Jablonski and Anderson, 1981). While this cycle is known to be primarily responsible for H₂O₂ scavenging in chloroplasts, its importance in the cytosol and in nonphotosynthetic tissues is becoming apparent (Alscher, 1989). GR is also present in mitochondria (Edwards et al., 1990).

We observed one plastidic, one mitochondrial, and three cytosolic isozymes of GR in maize (Fig. 7B) although we don't discount the possibility of undetected isozymes that require 2-d PAGE (as in pea, Edwards et al., 1990). Only the cytosolic isozymes were observed in cell-free extracts (Fig 7A). It was interesting that the upper band was the prominent form in the coleoptile+leaf and mesocotyl and the two lower bands predominated in the root. Acclimation resulted in a shift in the intensity from the upper band to the lower two bands, but only in the mesocotyl. It is unknown whether this is due to differential expression of distinct GR genes, or a post-translational modification of one or more of the GR subunits. The appearance of the two lower bands in acclimated mesocotyls is suggestive of an involvement in chilling tolerance. Similar to our results, Edwards et al. (1994) observed a small increase in total GR activity but an altered isozyme profile in pea leaves exposed to low temperature. They also observed an increased affinity of GR for GSSG and suggested that the change in isozyme profiles might be responsible. Guy and Carter (1984) noted the disappearance of one and the appearance of two higher mobility isozymes in cold-hardened spinach. These two higher mobility isozymes had greater stability in the presence of freezing stress, and a higher affinity for GSSG and NADPH at 5°C (but with a lower affinity at 25°C). If such is also the case with the two lower
bands in our system, it would enhance the ability of acclimated seedlings to maintain GSH in the reduced form in the cytosol, contributing to the ability to tolerate chilling-induced oxidative stress.

**Effect of Acclimation and Chilling on the Steady State Pools of Ascorbate and Glutathione**

We have shown that CAT, SOD, GR, and APX were unaffected by acclimation in the coleoptile+leaf (Table II, Figs. 3, 7A). In addition, whether the small induction of two POX isozymes (focusing at pH 8.0 and 8.4, Fig. 4) in the coleoptile+leaf resulted in adequate antioxidant protection is questionable, especially given the fact that they were cell wall localized. The fact that these seedlings were etiolated may explain the lack of response of these enzymes to acclimation. It is possible that normally autotrophic cells are not conditioned to respond to active oxygen generation arising from sources other than chloroplasts. Yet the fact remains that H$_2$O$_2$ in the AcCh treatment was maintained at low levels. Examination of the ascorbate and glutathione pools in Table III may provide some insight. Among other functions, ascorbate and glutathione are key elements in the scavenging of H$_2$O$_2$ through their participation in the ascorbate-glutathione cycle (Rennenberg, 1982; Foyer et al., 1991). Increased active oxygen generation during chilling would initially cause a depletion in AsA, both through the nonenzymatic oxidation of AsA to DHA by superoxide (Foyer et al., 1991) and through APX. The lack of a corresponding increase in DHA observed in Table III might be the result of oxidation to tartaric acid and oxalic acid (Loewus, 1988) and the oxidation of GSH. The oxidation of
DHA would be a loss to the ascorbate pool, which we observed. The lack of sufficient GR activity or the lack of NADPH would result in a net conversion of GSH to GSSG, which we observed. When seedlings are acclimated, the glutathione pool increases. This may provide sufficient GSH to reduce DHA so that the AsA level can be maintained when acclimated seedlings are subsequently chilled. The lower level of DHA in the AcCh treatment (although not statistically lower) may be interpreted as the combined effects of DHA oxidation and DHA reduction to AsA. We suggest that the maintenance of GSH in acclimated seedlings near that of unchilled controls, apparently through increased synthesis of GSH, may contribute to the prevention of H₂O₂ accumulation in the coleoptile+leaf. The importance of maintaining reduced glutathione as a factor in low temperature tolerance has also been suggested by Alscher (1989). Our results are similar to those of Walker and McKersie (1993) where they observed an increased synthesis of total glutathione and a maintenance of the reduced form in chilling-resistant tomato while the susceptible tomato species exhibited only an oxidation of GSH with no increase in the total glutathione pool.

The response of ascorbate and glutathione in mesocotyls to acclimation and chilling was generally the same as that in the coleoptile+leaf, although the increase in the total glutathione pools in response to acclimation was not as extensive. Induction of other antioxidants in the mesocotyl may reduce the need for GSH synthesis. Like the coleoptile+leaf, there was no effect of acclimation or chilling on any of the antioxidant enzymes tested in the roots. However, H₂O₂ levels in the roots were not greatly increased by chilling, indicating either that the
constitutive levels of antioxidants in the roots are adequate to avoid oxidative damage or that the response of roots to chilling stress (in terms of H₂O₂ generation) is not as great. Nevertheless, there appears to be no effect of acclimation on antioxidants or on H₂O₂ levels. In the Ch treatment, we observed a loss of AsA and total ascorbate and a conversion of GSH to GSSG, similar to the Ch treatment in the coleoptile+leaf. However, in the root, there is no increase in the total glutathione pools in response to acclimation and a loss of AsA and total ascorbate was observed when acclimated seedlings were subsequently chilled. Thus, the ascorbate and glutathione pools in the root reflect the lack of a response to acclimation.

It is important to note that all of the enzymes examined in this study were assayed at room temperature. These assay conditions are appropriate when comparing activity levels among treatments but these activities cannot be used to interpret substrate pools. Jahnke et al. (1991) reported that, among other antioxidant enzymes, GR, monodehydroascorbate reductase, and DHA reductase were particularly susceptible to a low assay temperature (5°C). Thus, even though we report no effect of chilling on GR (Table II), it is likely that the GR activity during chilling is much lower in vivo, and a net oxidation of glutathione would be observed.

Effect of ABA on CAT, POX, and GR

We have previously reported that exogenous treatment of maize seedlings with ABA resulted in an improvement of chilling tolerance. Further, inhibition of ABA biosynthesis with fluridone prevented acclimation-induced chilling tolerance,
indicating that ABA was required (Anderson et al., 1994). We also showed that ABA, like acclimation, induced total CAT activity. However, we did not indicate that acclimation was mediated by ABA and did not rule out the possibility that ABA and acclimation induced chilling tolerance through separate mechanisms. In this study, we wished to determine whether the response of antioxidant isozymes to ABA was the same as to acclimation, in effort to establish a link between acclimation- and ABA-induced chilling tolerance.

Despite the fact that ABA-treated seedlings grew little during the 1 d treatment period, many developmental changes still occurred. When comparing ABA-treated seedlings with controls of the same age, none of the prominent POX isozymes were affected by ABA (Fig. 9). Thus, even though ABA has been shown to induce certain POX isozymes in other systems (e.g. Cottle and Kolattukudy, 1982), we conclude that ABA has no effect on POX (or lignification) in our system. Similarly, the activity of CAT3 in ABA-treated seedlings was not much higher than in the Cont4d treatment (Fig. 8). Williamson and Scandalios (1992) have reported that CAT1, but not CAT3, activity and transcripts were induced by ABA in maize scutellar tissue and Guan and Scandalios (1993) have identified two putative ABA response elements in the promoter region of the cat1 gene. We show that CAT1 activity is induced by ABA in both mesocotyls and roots. The response of CAT1 activity to ABA is obscured in the coleoptile+leaf because of a possible oxidative phenomenon that results in a loss of CAT1 activity and an increased electrophoretic mobility of both CAT1 and CAT3. These responses are apparently developmental, not ABA-induced, since it was also observed in the Cont4d treatment. GR electrophoretic mobility was also
altered in these treatments (Fig. 10). These changes in isozyme mobility might reflect differing roles of CAT1 and GR as seedlings develop.

We have determined that ABA had a significant influence on two antioxidant enzymes, CAT1 and the low mobility band of GR. Both were induced, somewhat in the mesocotyl, but to a much greater extent in the root, possibly as a result of a greater dose of ABA in the root. However, whether these effects contribute to ABA-induced chilling tolerance has yet to be determined. There were no similarities in isozyme patterns of CAT, POX, or GR between acclimation and ABA. While acclimation induced CAT3, numerous POX isozymes, and enhanced the two high mobility GR isozymes, ABA induced CAT1 and enhanced the low mobility GR isozyme. Clearly, the action of ABA differs from that of acclimation, at least in terms of antioxidant defenses.

Acknowledgments

The authors wish to thank L. Tabatabai for the use of equipment necessary for the separation of POX isozymes by IEF.
CHAPTER 4. SUMMARY AND CONCLUSIONS

We have identified a chilling acclimation phenomenon in dark-grown seedlings of chilling-sensitive maize inbred G50 (Pioneer). Growth and survival of seedlings was greatly reduced after exposure to a chilling stress of 4°C for 7 d in darkness. Damage symptoms were most evident in the mesocotyl. However, exposure of seedlings to an acclimation treatment of 14°C for 3 d in darkness before applying the chilling stress resulted in a dramatic improvement in the ability of seedlings to tolerate the chilling stress.

Examination of acclimated and unacclimated seedlings for differences in gene expression resulted in the identification of three transcripts that were up-regulated in response to acclimation. These clones were designated car333, car30, and car757 (chilling acclimation responsive) and represented 228, 231, and 696 base fragments from the 3’ end of each transcript respectively. Sequence comparisons with the GenBank/EMBL database revealed no homology of either car30 or car757 to any published sequence. However, car333 was 97.8% homologous with cat3, which encodes maize mitochondrial catalase. Characterization of the three transcripts in terms of location (coleoptile+leaf, mesocotyl, and root) and response to low temperature revealed that car30 was induced somewhat by chilling, but was much more strongly induced by acclimation in the coleoptile+leaf and root. In the mesocotyl, car30 was strongly induced from an undetectable level by acclimation, but not by chilling. Car757 was present in low levels in all organs but was induced by acclimation and especially by chilling, as if it was responding not to acclimation,
but to the severity of the low temperature. *Cat3* was induced somewhat by chilling, but much more strongly induced by acclimation in the coleoptile+leaf and especially in the mesocotyl. *Cat3* was undetectable in the root.

Measurement of catalase (CAT) activity in cell-free protein extracts from coleoptile+leaf, mesocotyl, and root of seedlings exposed to acclimation and chilling revealed that total CAT activity (consisting of both CAT1 and CAT3 activities) was induced by acclimation, but only in the mesocotyl. Neither acclimation nor chilling induced total CAT activity in the coleoptile+leaf or the root. Examination of CAT isozymes on zymograms revealed that the induction of CAT by acclimation in the mesocotyl was due entirely to induction of CAT3. CAT1 was unaffected by acclimation but was inactivated somewhat by chilling. This may be the result of the susceptibility of CAT1 to oxidative inactivation. CAT3 activity was not induced by chilling despite the fact that *cat3* transcripts were induced by chilling. Further, CAT3 activity in the coleoptile+leaf was unaffected by acclimation or chilling even though *cat3* transcripts were induced by these treatments. No effort was made to examine the post-transcriptional events that would account for these differences between *cat3* transcript levels and CAT3 activity. CAT3 was present in roots at very low levels, consistent with the undetectable levels of *cat3* transcript in the root.

The tissue-specific induction of *car30* and *car757* by low temperature may represent significant events in the acclimation process. However, since they remain unidentified, we cannot draw any conclusions about their role in chilling tolerance. On the other hand, the induction of *cat3* by acclimation implied that chilling tolerance might involve protection from chilling-induced oxidative stress.
In order to assess the oxidative status of seedlings exposed to acclimation and chilling, H$_2$O$_2$ levels were measured in coleoptile+leaf, mesocotyl, and root of seedlings exposed to the various low temperature treatments. Chilling without acclimation resulted in a 4-fold increase in H$_2$O$_2$ in both the coleoptile+leaf and the mesocotyl but H$_2$O$_2$ was held at much lower levels when seedlings were acclimated before applying the chilling stress. The prevention of H$_2$O$_2$ accumulation was consistent with the suggestion that elevation of antioxidants during acclimation protected seedlings from oxidative stress. H$_2$O$_2$ levels were elevated only slightly in roots exposed to acclimation and chilling, indicating either that the generation of H$_2$O$_2$ in roots was not greatly increased by chilling or that adequate antioxidants were constitutively present.

In addition to CAT, a number of other antioxidant enzymes were examined in response to acclimation and chilling in order to obtain a more complete picture of the basis for the acclimation-induced protection from oxidative stress. Neither superoxide dismutase (SOD) nor ascorbate peroxidase (APX) responded to acclimation or chilling in any of the organs tested. Although they certainly are part of the complete antioxidant system that protects seedlings from oxidative stress, they are apparently not responsible for the enhanced protection in acclimated seedlings.

Nine major guaiacol peroxidase (POX) isozymes were induced by acclimation in the mesocotyl. Two of these were localized in the cell wall and may be involved in the polymerization of lignin from monolignols. Lignin content was found to be elevated in mesocotyls in response to acclimation, possibly enhancing the mechanical strength of acclimated seedlings. The benefits of
increased lignification as a mechanism of chilling tolerance is apparent, especially since a loss of mechanical integrity is a characteristic of chilling damage. The role that the other seven POX isozymes play during acclimation is unknown. While the role of guaiacol peroxidases in scavenging H$_2$O$_2$ has not been established, it is possible that some of these isozymes function in that capacity. However, it is likely that some, if not all, of these peroxidases perform some function unrelated to H$_2$O$_2$ scavenging but might, nevertheless, be important in the acclimation process.

Glutathione reductase (GR) is the enzyme that catalyzes the rate-limiting step in the ascorbate-glutathione scavenging system in the plastids and cytosol. Three GR bands were observed in total protein extracts and all three were of cytosolic origin. The upper band dominated in the coleoptile+leaf and mesocotyl while the lower two bands dominated in the root. As with CAT and POX, the isozyme profile of GR was unaffected by acclimation and chilling in the coleoptile+leaf or root. However, a shift in the isozyme pattern was observed in the mesocotyl, where the activity of the top band declined and the activity of the lower two bands increased in response to acclimation. This shift occurred without a significant change in the total GR activity. Although no further studies have been conducted to characterize these bands, similar shifts in isozyme patterns have been reported in other systems (e.g. Guy and Carter, 1984; Edwards et al., 1994) and were associated with an increased affinity of GR for its substrates, NADPH and GSSG, at low temperature. Thus, this may be a case where the activity of the enzyme is enhanced, not by increasing enzyme abundance, but by increasing its effectiveness at low temperature.
We have observed changes in CAT, POX, and GR in response to acclimation, but only in the mesocotyl. None of the enzymes tested (including SOD and APX) responded to acclimation in the coleoptile+leaf despite the fact that acclimation protected this organ from chilling-induced oxidative stress. Measurement of ascorbate and glutathione pools may have provided some insight. The activity of GR has been reported as being greatly reduced at chilling temperatures (Jahnke et al., 1991). With no means to regenerate the reduced form of glutathione, the components of the ascorbate-glutathione cycle rapidly become oxidized and lose the capacity to scavenge H$_2$O$_2$. We observed that an increase in the total glutathione pool occurred in response to acclimation, resulting in a maintenance of the reduced forms of glutathione and consequently, ascorbate. Thus, we proposed that the synthesis of GSH during acclimation could substitute for the regeneration of GSH from GSSG, maintaining the H$_2$O$_2$ scavenging capacity of the ascorbate-glutathione cycle. This increased GSH synthesis was most pronounced in the coleoptile+leaf but was also present to a lesser extent in the mesocotyl. The enhancement of CAT3, POX, and GR in the mesocotyl during acclimation may reduce the need for GSH synthesis to maintain the H$_2$O$_2$ scavenging capacity of the cycle. In the root, ascorbate and GSH pools shifted to a more oxidized state in response to chilling, as in the coleoptile+leaf and mesocotyl. Thus, chilling resulted in some degree of oxidative stress although it may not have been severe enough to cause significant damage. Acclimation did not alter the shift of ascorbate and glutathione to their oxidized forms in the root.
Overall, we have observed that both the coleoptile+leaf and the mesocotyl were susceptible to chilling-induced oxidative stress but were protected by the changes induced by acclimation. In the coleoptile+leaf, the only source of antioxidant protection identified was an increase in GSH synthesis. Whether the maintenance of the H2O2 scavenging capacity of the ascorbate-glutathione cycle in the cytosol is solely responsible for the oxidative status of the coleoptile+leaf is unknown. More likely, there are other unidentified factors that contribute to the protection of the coleoptile+leaf from oxidative stress. In the mesocotyl, the organ that is most susceptible to chilling damage, acclimation alters numerous antioxidants which likely act in concert to prevent oxidative damage. We have suggested that the mitochondria are probably the primary source of active oxygen in dark-grown maize seedlings exposed to a chilling stress. Induction of CAT3 by acclimation may represent the first line of defense against mitochondria-generated H2O2. The combined effect of increased GSH synthesis and the appearance of GR isoforms that are potentially more active at low temperature would serve to maintain the H2O2 scavenging capacity of the ascorbate-glutathione cycle in the cytosol. These metabolic changes may provide the second line of defense against H2O2 diffusing from the mitochondria as well as H2O2 arising from other sources. It is also possible that some of the POX isozymes contribute as H2O2 scavengers as well. Induction of the two cell wall peroxidases by acclimation may be responsible for the observed increase in lignification. Acclimation likely induces numerous changes that combine to provide maize seedlings with a degree of chilling tolerance. We have identified two processes, protection from oxidative stress through enhancement of
antioxidants, and the increased mechanical strength afforded by a higher lignin content, as important aspects of the acclimation process.

In addition to acclimation, we have also examined the effects of ABA on chilling tolerance and the response of antioxidant enzymes. The primary goal of these studies was to determine whether the acclimation process was mediated by ABA. We demonstrated that exogenous application of ABA resulted in an improvement of chilling tolerance although it was not as effective as acclimation. We also observed that inhibition of ABA biosynthesis with fluridone abolished acclimation-induced chilling tolerance. This showed that the changes induced by acclimation were not sufficient to provide chilling tolerance in the absence of ABA. However, this did not necessarily mean that acclimation was mediated by ABA. It is possible that acclimation and ABA may be acting through independent mechanisms, each contributing to optimal chilling tolerance. The fact that levels of free ABA were not significantly affected by acclimation but responded instead to chilling was evidence that acclimation was not mediated by ABA.

After establishing that induction of antioxidants and protection from oxidative stress was an important component of acclimation-induced chilling tolerance, we investigated the effect of ABA on the isozyme profiles of CAT, POX, and GR. If ABA-induced chilling tolerance was due, in part, to enhanced antioxidant protection, it would at least show that ABA and acclimation utilized similar mechanisms. It was found that both CAT1 and CAT3, and number of POX isozymes, lignin content, and one GR isozyme were higher in ABA-treated mesocotyls with respect to 3 d old controls. However, only the activities of CAT1 and the GR isozyme were higher in ABA-treated mesocotyls than in 4 d old
controls. Even though ABA-treated seedlings were the same size as 3 d old controls, they were actually a day older. Thus, we concluded that the only effect of ABA, not related to chronological age, was the induction of CAT1 and the GR isozyme, and this occurred much more strongly in the root than in the mesocotyl. Thus, while acclimation induced CAT3, POX, and caused a shift in GR profiles in the mesocotyl, ABA induced CAT1 and GR, primarily in the root. Although we have not investigated the significance of these ABA-induced changes, the response of antioxidant enzymes to ABA is clearly different than that of acclimation.

The results of the ABA experiment demonstrated the importance of development in the profiles of these antioxidant isozymes. In retrospect, it is possible that many of the observed changes induced by acclimation might be developmental responses. Comparison of 3 d old controls with 4 d old controls in the mesocotyl revealed increases in CAT3, numerous POX isozymes, lignin, and the middle GR band with increasing age. If this trend were to continue, the antioxidant profiles might be similar to those of acclimated seedlings when they reach the same chronological age (6 d old). Acclimation may essentially provide a more mature seedling and at the same time restrict growth, resulting in a seedling more capable of tolerating a chilling stress. Note that this does not invalidate acclimation as a means of inducing chilling tolerance but instead provides more insight into the acclimation process. A more complete developmental study is needed to determine the relationship between acclimation and development, in terms of antioxidant isozyme profiles.
REFERENCES


Buescher RW (1975) Organic acid and sugar levels in tomato pericarp as influenced by storage at low temperature. Hortscience 10: 158-159


Mehler AH (1951) Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch Biochem Biophys 33: 65-77


Redinbaugh MG, Sabre M, Scandalios JG (1990b) Expression of the maize cat3 catalase gene is under the influence of a circadian rhythm. Proc Natl Acad Sci USA 87: 6853-6857


Stewart CR, Martin BA, Reding L, Cerwick S (1990b) Seedling growth, mitochondrial characteristics, and alternative respiratory capacity in corn genotypes differing in cold tolerance. Plant Physiol 92: 761-766


