ABSTRACT

JENNINGS, DIANNE BROOKS. The Role of Mannitol and Mannitol Dehydrogenase in Plant-Pathogen Interactions. (under the direction of John David Williamson and David Mason Pharr.)

Reactive oxygen species (ROS) are known to act as both signaling molecules and direct antimicrobial agents in plant defense against pathogens. Many plant pathogens have the ability to synthesize mannitol, a potent ROS quencher, and there is growing evidence that at least some phytopathogenic fungi use mannitol to suppress ROS mediated defenses. Here we show that mannitol production and secretion in the phytopathogenic fungus, Alternaria alternata is induced in the presence of host plant extracts. Additionally we demonstrate that the catabolic enzyme mannitol dehydrogenase (MTD) is induced in a non-mannitol-producing plant in response to both fungal elicitor and specific inducers of plant defense responses. This indicates a mechanism whereby the plant can counteract fungal suppression of ROS-mediated defenses by catabolizing mannitol of fungal origin. To further clarify this interaction, tobacco plants were transformed with celery Mtd cDNA under a constitutive promoter, resulting in production of an enzymatically active MTD protein. This constitutive MTD expression conferred enhanced resistance to A. alternata, and this resistance did not correlate with expression of PR1a, a protein often used as an indicator of systemic acquired resistance. Constitutive Mtd expression did not enhance tolerance to two non-mannitol secreting pathogens, the fungal pathogen Cercospora nicotianae and the bacterial pathogen Pseudomonas syringae pv. tabaci. These results are consistent with the hypothesis that MTD plays a role in plant resistance to mannitol secreting fungal pathogens by catabolizing mannitol of fungal origin.
The Role of Mannitol and Mannitol Dehydrogenase in Plant-Pathogen Interactions

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1-25</td>
</tr>
<tr>
<td>Chapter 1: Roles for Mannitol and Mannitol Dehydrogenase in Active Oxygen-Mediated Plant Defense</td>
<td>26-47</td>
</tr>
<tr>
<td>Abstract</td>
<td>27</td>
</tr>
<tr>
<td>Introduction</td>
<td>28-30</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>30-32</td>
</tr>
<tr>
<td>Results</td>
<td>32-35</td>
</tr>
<tr>
<td>Discussion</td>
<td>35-37</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>38</td>
</tr>
<tr>
<td>References Cited</td>
<td>38-40</td>
</tr>
<tr>
<td>Figure Legends</td>
<td>41-42</td>
</tr>
<tr>
<td>Chapter 2: Constitutive Expression of Celery Mannitol Dehydrogenase in Tobacco Provides Enhanced Tolerance to the Mannitol-Secreting Fungal Pathogen Alternaria alternata</td>
<td>48-81</td>
</tr>
<tr>
<td>Abstract</td>
<td>49</td>
</tr>
<tr>
<td>Introduction</td>
<td>50-53</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>53-58</td>
</tr>
<tr>
<td>Celery Mtd encodes an enzyme with mannitol dehydrogenase activity in tobacco</td>
<td>58</td>
</tr>
<tr>
<td>Transgenic plants show enhanced resistance to Alternaria alternata</td>
<td>59-60</td>
</tr>
<tr>
<td>Constitutive expression of MTD does not enhance resistance to non-mannitol secreting plant pathogens</td>
<td>60-63</td>
</tr>
<tr>
<td>Discussion</td>
<td>63-68</td>
</tr>
<tr>
<td>References Cited</td>
<td>69-72</td>
</tr>
<tr>
<td>Figure Legends</td>
<td>73-75</td>
</tr>
<tr>
<td>Appendix</td>
<td>82-88</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

### Introduction

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mannitol metabolism and catabolism in celery</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Reactions involved in the mannitol cycle in imperfect fungi</td>
<td>8</td>
</tr>
</tbody>
</table>

### Chapter 1: Roles for Mannitol and Mannitol Dehydrogenase in Active Oxygen-Mediated Plant Defense

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fungal induction of an endogenous mannitol dehydrogenase (MTD) in tobacco</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>Induction of mannitol dehydrogenase (MTD) in tobacco leaf discs and NT-1 suspension cells</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>MTD activity and protein levels in response to INA dose and treatment time</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>Immunotitration of MTD activity in extracts from INA-induced tobacco leaf discs</td>
<td>46</td>
</tr>
</tbody>
</table>

### Chapter 2: Constitutive Expression of Celery Mannitol Dehydrogenase in Tobacco Provides Enhanced Tolerance to the Mannitol-Secreting Fungal Pathogen *Alternaria alternata*

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Celery <em>Mtd</em> encodes an active enzyme in transgenic tobacco</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>Effect of constitutive MTD expression on resistance to the mannitol-secreting fungus <em>Alternaria alternata</em></td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>Induction of MTD in three tobacco cultivars</td>
<td>78</td>
</tr>
<tr>
<td>4</td>
<td>Infection of <em>Mtd</em> transformed and untransformed tobacco plants with the fungal pathogen <em>Cercospora nicotinae</em></td>
<td>79</td>
</tr>
<tr>
<td>5</td>
<td>Effect of constitutive MTD expression on resistance to the non-mannitol-secreting fungus <em>Cercospora nicotinae</em></td>
<td>80</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

Chapter 1: Roles for Mannitol and Mannitol Dehydrogenase in Active Oxygen-Mediated Plant Defense

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mannitol accumulation in fungal cultures grown with and without host leaf extract</td>
<td>47</td>
</tr>
</tbody>
</table>
INTRODUCTION

Although sucrose is the most commonly studied form of photosynthetically fixed carbon, it has been estimated that up to 30% of all primary production in plants goes through polyols (Bieleski, 1982). Of the 13 different polyols that have been isolated from higher plants, mannitol, a six-carbon non-cyclic sugar alcohol, appears to be the most widespread, being found in over 70 families (Lewis and Smith, 1967). In plants mannitol is made in addition to sucrose and is a phloem-translocated photoassimilate. Mannitol has been shown to have many roles in the plants in which it is made including serving as a carbon storage compound (Lewis, 1984), as a store of reducing power (Loescher, 1987; Stacy, 1974; Stoop and Pharr, 1992), as a compatible solute (Brown and Simpson, 1972, Yancey et al, 1982) and in osmoregulation (Hellebust, 1976). It has also been shown to be an oxygen radical quencher both \textit{in vitro} (Smirnoff and Cumbes, 1989) and \textit{in vivo} (Shen et al., 1997a, 1997b). In the dark, mannitol is the main translocated carbohydrate when the sucrose pool is exhausted (Davis and Loescher, 1990). Mannitol may also be involved in the utilization of photochemical energy. Synthesis of mannitol utilizes NADPH generated in the cytosol during the conversion of triose phosphate, a photosynthesis product that is transported from the chloroplast, to 3-phosphoglyceric acid. The 3-phosphoglyceric acid is then transported into the chloroplast (Rumpho et al., 1983; Loescher, 1987).

\textbf{Mannitol metabolism and catabolism}

Mannitol metabolism and catabolism have been extensively studied in celery (\textit{Apium graveolens} L.) where mannitol is a major photoassimilate, comprising up to
50% of the newly fixed carbon in mature leaves. Mannitol is translocated in the phloem of celery and utilized and/or stored in sink tissues (Keller and Matile, 1989, Loescher et al., 1992, Stoop and Pharr, 1993). The mannitol synthetic pathway has been well described in celery and is known to be comprised of three enzymatic steps (Fig. 1); (a) isomerization of fructose-6-phosphate (Fru-6-P) to mannose-6-phosphate (Man-6-P) by phosphomannose isomerase (PMI), (b) reduction of Man-6-P to mannitol-1-phosphate by NADPH-dependent mannose-6-phosphate reductase (M6PR), and (c) dephosphorylation of mannitol-1-phosphate to mannitol by mannitol-1-phosphate phosphatase (Everard et al. 1997). This pathway also appears to be present in other mannitol-synthesizing vascular plants, such as parsley and snapdragon (Loescher et al., 1992; Harloff and Wegmann, 1993; Simier et al., 1994).

![Fig.1. Mannitol metabolism and catabolism in celery. Enzymes involved in pathways are a) PMI; phosphomannose isomerase, b) M6PR: mannose-6-phosphate reductase, c) mannitol-1-phosphate phosphatase, d) MTD: mannitol 1-oxidoreductase/ mannitol dehydrogenase, e) HK: hexokinase.]
M6PR is a key enzyme in the mannitol biosynthetic pathway (Rumpho et al., 1983). M6PR is active in mature photosynthetic tissues but its activity is very low or not detectable in sink tissues, e.g. young roots, young petioles, young leaves [except under salt stress (Everard et al., 1994)], or mature petioles (Stoop and Pharr, 1994). Increasing salt treatments, up to 300mM NaCl applied to the root environment, increased mannitol accumulation in leaf tissues and correlated with increased M6PR activity (Everard et al., 1994). While increases in M6PR protein in mature tissues were not apparent, M6PR activity increased 2-fold in mature tissues and 6-fold in young leaves. Previous work by Everard et al. (1993) showed a close relationship between cellular distribution of M6PR and Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the enzyme catalyzing the initial step in photosynthetic carbon reduction. Both enzymes are present in the mesophyll and vascular companion cells and absent in the epidermis and non-photosynthetic vascular tissues. Data indicate that the final steps for mannitol synthesis in celery occur outside the chloroplast, in the cytoplasm (Rumpho et al., 1983). This is in keeping with the enzyme’s substrate specificities, M6P being derived from the same cytoplasmic hexose-P pool as that utilized for sucrose biosynthesis, and a pH optimum of 7.5 (Loescher et al, 1992). The presence of M6PR at primary sites of carboxylation, as well as its absence from non-photosynthetic tissues, may indicate that the primary role of M6PR is the assimilation of carbon being exported from the chloroplasts. In light of this role it is not surprising that M6PR activity changes during celery leaf development, increasing in parallel with photosynthetic capacity and export of mannitol from source leaves (Davis et al, 1988, Davis and Loescher, 1990).
In celery mannitol dehydrogenase (MTD), mannitol: mannose 1-oxidoreductase constitutes the first step in the enzymatic catabolism of mannitol (Stoop et al., 1995). Mannitol use is spatially separated from its synthesis (Fig. 1). MTD activity is not detectable in mature leaves but is present at high levels in sink tissues of whole plants (roots, immature leaves) and in cell suspension cultures grown on mannitol (Stoop et al., 1996b). The MTD from celery is unique in that it is the first NAD-dependent enzyme described that oxidizes alditols to aldoses at the carbon-1 position (Stoop et al., 1996a). All previously reported NAD(P) dependent polyol dehydrogenases were identified as 2-oxidoreductases, including mannitol dehydrogenases found in fungi and bacteria. In these organisms mannitol or mannitol phosphate are converted to fructose or fructose phosphate, respectively, often using NADP as an oxidant (Stoop et al., 1996a).

MTD in celery has been shown to have a complex regulation pattern and, in fact, is thought to be a key regulator of mannitol pool size in mannitol producing plants. When celery plants are exposed to high salinity, mannitol accumulation increases, while mannitol use is drastically reduced. As previously mentioned, increased accumulation of mannitol occurs because M6PR activity increases in both fully expanded mature leaves and in young tissue (Everard et al., 1994), while at the same time MTD activity in sink tissue decreases (Stoop and Pharr, 1994). Corresponding to decreased MTD activity there is a decrease in the amount of MTD protein in sink tissues (Pharr et al., 1995). Subsequent work (Williamson et al., 1995) showed that changes in MTD activity due to NaCl stress were paralleled by changes in the amount of Mtd transcript. Together these data indicate that decreases in MTD activity under
salt stress are due to reduction in the accumulation of the protein and its transcript. This is important in light of the fact that mannitol plays a role in osmoregulation and is further supported by the fact transgenic tobacco plants expressing the bacterial gene mtlD, which encodes a mannitol biosynthetic/catabolizing enzyme, exhibit increased tolerance to salt stress (Tarczynski et al., 1993). Similarly, mannitol levels in the marine alga *Platymonas subcordiformis* also increase or decline in response to changes in external salinity and osmotic conditions (Richter and Kirst, 1987).

In addition to its response to salt stress, MTD’s expression is also strongly regulated by sugars. While mannitol itself does not induce expression of MTD, sugars may suppress MTD expression (Pharr et al., 1995). When celery cell cultures were grown in the presence of mannose or sucrose plus mannitol they expressed less MTD than cell cultures grown with mannitol alone (Stoop and Pharr, 1993). In addition, glucose grown cells that have low MTD activity during active growth, underwent a marked increase in MTD activity, with corresponding increases in the levels of protein and transcripts, upon glucose starvation (Prata et al., 1997). In the presence of mannoheptulose, a competitive inhibitor of hexokinase, derepression of MTD activity also occurred. Addition of the sugar analog 2-deoxyglucose, which is phosphorylated by HK but not further metabolized, resulted in repression of MTD activity in cell cultures grown in the presence of mannitol (Prata et al., 1997). This indicates that hexokinase and sugar phosphorylation may be involved in signaling mannitol repression. In plants the regulation of gene expression by sugars may act to control carbohydrate distribution amongst tissues and organs (Koch, 1996). In the case of mannitol, the repression of MTD by sugars may serve to create a pool of mannitol.
when the plants are not under stress. This mannitol reserve can then be used when environmental conditions/developmental states are not as favorable for sugar production or when additional energy/carbon is required.

**Mannitol pathways in other plants**

Mannitol metabolism has also been found in the *Orobanchaceae*, a group of holoparasitic flowering plants (Harloff and Wegmann, 1993). All enzyme activities necessary for mannitol synthesis (mannitol-6-phosphate reductase, mannitol-6-phosphate isomerase, and mannitol-1-phosphatase) occur in several tissues, e.g. the subterranean storage tuber, the emerging young spikes and the older flowering spikes. Fructose reductase (mannitol dehydrogenase) was found in the flowering spikes (Harloff and Wegmann, 1993), and appears to be NADH dependent, however, the 'end' product appears to be fructose rather than mannose, indicating that it is a 2-oxidoreductase.

Mannitol is the end product of photosynthesis, the main soluble carbohydrate in leaves and the carbohydrate that is translocated in the phloem of *Thesium*, a root hemiparasite (Fer et al., 1993). The physiological roles of mannitol in *Thesium* are similar to those proposed for celery, including storage of carbon and reducing power, osmoregulation and service as a compatible solute. Smith et al. (1969) proposed two additional functions for mannitol in parasitic plants; (1) it may be involved in osmotic adjustment, facilitating water movement from the host to the parasite, (2) as parasitic plants are able to convert other carbohydrates, such as sucrose, derived from the host plant, into mannitol, it could be considered a trapping mechanism for host
photosynthetic. *Thesium* and celery, however, differ in some aspects of mannitol synthesis and function. In *Thesium*, mannitol plays a continuous osmoregulatory role because leaves continue to exhibit high transpiration rates in the dark (Fer et al., 1994). Furthermore, mannitol synthesis occurs in young leaves as well as mature leaves of *Thesium*. In addition, MTD activity in young *Thesium* leaves was detected using either NAD$^+$ or NADP$^+$ as cofactors (Simier et al., 1998). Whether this activity was due to lack of specificity of a single enzyme or the presence of two different enzymes is not clear.

**Mannitol production in fungi**

Until now our main focus has been on plant production and utilization of mannitol. Mannitol, however, is also present in most imperfect fungi, some ascomycetes (Hult et al., 1980) and a few basidiomycetes (Speth and Niederpruem, 1976). In imperfect fungi the mannitol cycle (Fig. 2) is made up of the following enzymes: mannitol 1-phosphate dehydrogenase, mannitol 1-phosphatase, mannitol dehydrogenase, and hexokinase. With the exception of mannitol 1-phosphate dehydrogenase, all the above enzymes have also been found in the Ascomycetes *Gibberella, Ceratocystis* and *Neurospora* (Hult et al., 1980). While originally considered only as a pathway for NADPH regeneration and NADH oxidation, the mannitol cycle is now thought to play a central role in metabolism of fungi (Hult et al., 1980).
Mannitol dehydrogenases in fungi differ from MTD in plants in that they are usually NADP-dependent 2-oxidoreductases, where the product is fructose rather than mannose (fig. 2). Several fungi that infect humans and animals have been shown to produce mannitol in culture as well as in infected hosts (Wong et al., 1989, Wong et al., 1990). In addition, Joosten et al. (1990) found that mannitol levels in the apoplast of tomato plants increased when the plants were attacked by the fungus *Cladosporium fulvum*, corresponding with increased mannitol production by the fungus.

Mutants of the animal pathogenic fungus *Cryptococcus neoformans* that under-produce and hence under-accumulate mannitol are more susceptible to osmotic and heat stress, and are hypersusceptible to oxidative killing (Chaturvedi et al., 1996a, 1996b). In addition these mutants were hypovirulent in mice, suggesting that mannitol functions in *C. neoformans* as an intracellular osmolyte and antioxidant, and that its
presence is required for wild-type virulence (Chaturvedi et al., 1997). It is hypothesized that polyols produced by fungi “quench” oxidants generated by the phagocytic oxidative burst, and therefore, the ability to synthesize and accumulate these polyols is necessary for the fungi to be able to suppress or resist this mammalian host defense (Chaturvedi et al., 1997).

As previously mentioned, mannitol has been shown to be an efficient antioxidant, 20mM mannitol “quenching” approximately 60% of the hydroxyl-radicals generated in in vitro assays (Smirnoff and Cumbes, 1989). Recent work has demonstrated that mannitol is also an effective antioxidant in vivo. Transgenic tobacco plants containing the bacterial enzyme mannitol-1-phosphate dehydrogenase (mtd) targeted to the chloroplast accumulated up to 100mM mannitol in these organelles (Shen et al., 1997a). Mtd containing tobacco plants were more resistant to oxidative damage induced by exposure to methyl viologen, which causes a 2-fold increase in the production of hydroxyl radicals. Further work by Shen et al. (1997b), suggests that mannitol in mtd transformed tobacco plants may also function to shield thiol-regulated enzymes, like phosphoribulokinase, that are susceptible to inactivation by hydroxyl radicals. However, it should be noted that mannitol is not produced in the chloroplast of mannitol containing plants, but rather is thought to be synthesized in the cytoplasm (Rumpho et al., 1983). Moore et al. (1997), however, found mannitol about equally distributed between the cytosol and chloroplast of the mannitol producing plants, snapdragon and parsley. This seems to indicate that mannitol can enter the chloroplast of these species. Regardless of its exact localization in the cell, the
antioxidant function of mannitol may in fact play a significant role in plant-pathogen interactions.

**Plant Defense Responses**

Plants are constantly being challenged by pathogens, yet not all plant-pathogen interactions result in the appearance of disease. There are many possible reasons why a pathogen fails to infect a host. For example, the plant may possess constitutive non-specific characters that prevent pathogen infection (e.g. waxy cuticle, preformed toxins) or alternatively, the plant may be able to recognize the pathogen and then turn on specific defense responses. Regardless of how the plant evades the pathogen the end result is an incompatible interaction, in which the pathogen fails to infect and cause disease. In contrast, during a compatible interaction the pathogen is able to overcome both native and induced defenses and cause an infection.

Incompatible interactions are often characterized by localized cell death at the site of infection, a phenomenon called the hypersensitive response (HR). This can occur within a few hours of pathogen attack and is thought to serve to limit the spread of the pathogen. There is, however, a great deal of variation in the degree of the HR, ranging from death of a single cell to spreading necrotic areas accompanying limited pathogen colonization (Holub et al., 1994). Although cell death itself may limit the spread of some pathogens (non-necrotrophic ones) by removing a source of potential nutrients, during decompartmentalization of cells preexisting toxic compounds may also be released from cell structures and contribute to pathogen death. HR-type resistance is the most rapidly detectable symptom of pathogenesis in many systems, and usually
involves the interaction of gene products of both the plant and the pathogen (Ellingboe, 1982).

After formation of a necrotic lesion, either as part of the HR or as a symptom of disease, the systemic acquired resistance (SAR) pathway is activated (Ryals et al., 1996). SAR is a defense response, invoked by one pathogen that leads to host resistance to subsequent infection by a wide range of pathogens. Inherent to SAR are changes in salicylic acid (SA) and hydrogen peroxide levels, as well as expression of specific pathogenesis-related (PR) proteins.

### Role of Salicylic Acid in Defense Responses

A key compound in plant defense responses is salicylic acid (SA). Pathogen attack results in an increase in cellular SA concentration by de novo synthesis or release of SA from inactive conjugates. However, marked increases are not always correlated with the induction of defense responses. Factors such as SA compartmentation, intracellular redox state and presence of other defense inducing compounds may also be involved (Conrath et al., 1995). One mechanism of SA action could be to bind to and inhibit catalase, thereby elevating $\text{H}_2\text{O}_2$ levels (Chen et al., 1993). $\text{H}_2\text{O}_2$ and/or other reactive oxygen species derived from it may then activate plant defense-related genes. On the other hand, SA accumulation in response to pathogen infection may be regulated by $\text{H}_2\text{O}_2$ (Sharma et al., 1996). A previous study indicated that $\text{H}_2\text{O}_2$ could induce accumulation of free benzoic acid (BA), a precursor of SA, as well as SA itself, in tobacco (Leon et al., 1995). When tobacco plants were infiltrated with $\text{H}_2\text{O}_2$ there was a rapid accumulation of benzoic acid 2-hydroxylase
(BA2H) which catalyzes the formation of SA from BA. However, the exact amount contributed by H$_2$O$_2$-mediated SA formation in pathogen-induced SA accumulation is still unclear.

The role of SA in plant signaling has been investigated using a series of mutants and transformants that affect the SA signaling pathway. The first of these were *Arabidopsis* transformants in which a bacterial salicylate hydroxylase gene (*nahG*) had been introduced (Gaffney et al., 1993). This gene from *Pseudomonas putida* encodes an enzyme that replaces the carboxyl group from SA with a hydroxyl group. This converts SA to catechol in a very specific reaction that utilizes NADH as a cofactor (White-Stevens and Kamin, 1972). The result is a plant that is unable to accumulate SA, and is thus unable to induce systemic acquired resistance (Gaffney et al., 1993). In addition to its role in SAR induction, SA appears to be required for the expression of pathogen specific resistance as *nahG* plants support substantial growth of normally incompatible races of some pathogens (Delany et al., 1994). Interestingly NahG plants still retain their ability to respond to the SA-analog 2,6-dichloroisonicotinic acid (INA), indicating that INA acts through the same pathway(s) and at, or downstream of, the site of SA action (Delany et al., 1994: Vernooij et al., 1995). Tobacco plants expressing *nahG* also exhibit an inability to accumulate SA and an enhanced susceptibility to normally non-specific viral, fungal and bacterial pathogens (Ryals et al., 1995).
The Oxidative Burst and Reactive Oxygen Species

Upon pathogen attack plants initiate an oxidative burst whereby active oxygen species are produced. The production of reactive oxygen species (ROS) plays a central role in pathogen defense both in plants and animals. In animals, ROS production by phagocytic leucocytes (macrophages) is a well-characterized anti-microbial defense mechanism (Rotrosen and Gallin, 1987). Plants produce an analogous, localized oxidative burst, wherein massive amounts of anti-microbial ROS ($\cdot O_2^-$) are generated by a pathogen induced NADPH oxidase localized on the plasma membrane (Apostol et al., 1989). The $\cdot O_2^-$ produced is rapidly dismutated either non-enzymatically or by superoxide dismutase (SOD) catalysis to hydrogen peroxide ($H_2O_2$) (Sutherland, 1991; Levine et al., 1994, Mehdy, 1994; Nurnberger et al., 1994). ROS, in particular $H_2O_2$, may function to directly inhibit microbial growth, induce programmed cell death, or serve as a substrate in lignification, which strengthens the walls of uninfected cells by cross-linking. More globally, and perhaps more importantly, $H_2O_2$ acts as a signal to induce the expression of an array of PR genes (Dong, 1995).

In plants $H_2O_2$ is destroyed predominately by ascorbate peroxidases (APX) and catalases (Asada, 1997; Willekens et al., 1997). However, the oxidative burst is thought to produce such large quantities of $H_2O_2$ that these antioxidant mechanisms are temporarily overwhelmed (Lamb and Dixon, 1997; Wojtaszek, 1997). APX as well as the antioxidant enzymes, superoxide dismutase, glutathione reductase and monodehydroascorbate reductase are found in substantial amounts in the apoplast of healthy barley leaves and increase in response to pathogen inoculation (Vanacker et al., 1998). Plants that were susceptible had higher levels of antioxidant enzyme
activity, suggesting that increased apoplastic antioxidant defenses are a feature of the establishment of infection in a susceptible host (Vanacker et al., 1998). H₂O₂ can easily diffuse through membranes and serve as a signal to surrounding cells to induce defense responses (Halterman and Martin, 1997), such as PR protein production and SA accumulation (Leon et al., 1995).

**Plant Pathogenesis-Related Proteins**

Integral to the defense response is a subset of host-encoded polypeptides, which are synthesized in response to infection or related stresses, and are thus classified as pathogenesis related (PR) proteins (Mouradov et al., 1994). PR proteins appear to occur in two forms: basic PR proteins that are targeted to the vacuole and acidic PR proteins that carry out their function after being secreted outside the cell (Halterman and Martin, 1997). In addition to this classification, PR proteins have been divided into at least five different families, most of which have both basic and acidic forms. The first of these is the PR-1 group. Although the biological function of these proteins is as yet unknown, constitutive expression of several of these proteins in transgenic plants provides measurable protection against fungal pathogens (Lawton et al., 1993). Several PR-1 proteins also have activity *in vitro* against *Phytophthora infestans* (Niderman et al., 1993). The next two families/groups, PR-2 and PR-3 proteins have been identified as β-1,3-glucanases and chitinases, respectively. Both have been shown to have direct antifungal activity, presumably by degrading fungal cells walls (Boller et al., 1983; Mauch et al., 1988). PR-4 proteins, like PR-1s are of unknown function. They have some similarity to the C-terminal sequence of hevein, a
protein from latex of *Hevea brasiliensis* (Stintzi et al., 1993). They are all extracellular and while serologically related to each other, they are not related to other PRs or PR-groups (Stintzi et al., 1993). Finally there are the PR-5 proteins, also termed the thaumatin-like proteins because of their sequence similarity to a sweet tasting protein, thaumatin, from the african shrub *Thaumatococcus danielli* (Stintzi et al., 1993). Several members of the PR-5 family have been found to have differential antifungal activities with specificity for different fungal species (Vigers et al., 1992). *In vitro* assays have shown that they inhibit hyphal growth and mediate hyphal and spore lysis (Roberts and Selitrennikoff, 1990, Vigers et al., 1991, 1992, Woloshuk et al., 1991). While most of the PR proteins can be considered to have direct antimicrobial activity, some might also be indirectly antimicrobial, releasing elicitor-active oligosaccharides (PRs-2 and PRs-3) or, in the cases of peroxidases, by catalyzing cross-linking of molecules in the cell wall (Stintzi et al., 1993). In addition to the five families mentioned above there are other less well-characterized PR proteins. Recently a potentially new class of PR proteins has been described; the enzyme mannitol dehydrogenase has been shown to be induced by SA in celery cells and INA in tobacco plants (Williamson et al., 1995, Jennings et al., 1998).

### Mannitol Dehydrogenase as a PR Protein

Characterization of a cDNA encoding celery MTD revealed an unexpectedly striking nucleotide homology (>70%) to elicitor induced (Eli3) genes from parsley and their homologs in *Arabidopsis* (Williamson et al., 1995). Moreover, salicylic acid treatment evoked a dramatic increase in *Mtd* expression in celery cells (Williamson et
al., 1995) suggesting that MTD and hence mannitol play roles in plant defense. Recently MTD has been found to be present in a non-mannitol producing plant, tobacco, where expression is increased by fungal infection and 2,6-dicloroisonicotinic acid (INA) (Jennings et al., 1998). Initially it seemed surprising to find enzymatically active MTD in tobacco, however, the apparent link to fungal infection suggested a potential role for MTD in non-mannitol producing plants. As plants use ROS in plant defense, successful pathogens presumably have evolved mechanisms to avoid or suppress these defenses. For example, it has been hypothesized that phytopathogenic bacteria secrete catalase (an enzyme that detoxifies \( \text{H}_2\text{O}_2 \) by converting it to water) to suppress ROS-mediated plant defenses (Klotz and Hutcheson, 1992; Katsuwan and Anderson, 1990). Conversely, plants transformed to express glucose oxidase, an enzyme that generates \( \text{H}_2\text{O}_2 \), become more resistant to pathogens (Wu et al., 1995). As previously mentioned, the active oxygen quenching sugar alcohol mannitol is produced by all ascomycete fungi examined to date (Bielski, 1982; Jennings and Burke, 1990). It is hypothesized that mannitol producing phytopathogenic fungi might use mannitol to suppress, at least partially, the ROS-mediated plant defenses. In turn, plants counter this fungal stratagem by inducing MTD to catabolize the pathogen generated mannitol, and thus potentiate the oxidative burst and the associated \( \text{H}_2\text{O}_2 \)-mediated induction of the SAR response.

The first step in elucidating this potential interaction was to determine if MTD was indeed a PR protein and is discussed in detail in the first chapter. This was accomplished through a series of induction experiments in tobacco involving the use of known PR protein inducers (INA, SA, benzothiadiazole (BTH) and fungal elicitors).
Induction of MTD activity by the aforementioned compounds paralleled increases in MTD protein and RNA levels. In addition, mannitol production and secretion, in one of two fungal phytopathogens, were induced by a simple, aqueous tobacco (host) leaf extract.

The second chapter deals with direct testing of the efficacy of MTD in plant-pathogen interactions. Tobacco plants transformed to constitutively express a chimeric celery Mtd gene were used to determine if constitutive expression of MTD provided increased resistance to two fungal and one bacterial pathogen. Western blot analyses were performed to ascertain if resistance specifically correlated with the transgenic expression of MTD protein.
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CHAPTER 1

Roles for mannitol and mannitol dehydrogenase in active oxygen mediated plant defense

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ABSTRACT Reactive oxygen species (ROS) are both signal molecules and direct participants in plant defense against pathogens. Many fungi synthesize mannitol, a potent quencher of ROS, and there is growing evidence that at least some phytopathogenic fungi use mannitol to suppress ROS mediated plant defenses. Here we show induction of mannitol production and secretion in the phytopathogenic fungus *Alternaria alternata* in the presence of host plant extracts. Conversely, we show that the catabolic enzyme mannitol dehydrogenase is induced in a non-mannitol producing plant in response to both fungal infection and specific inducers of plant defense responses. This provides a mechanism whereby the plant can counteract fungal suppression of ROS mediated defenses by catabolizing mannitol of fungal origin.
Compelling evidence has arisen over the last decade demonstrating that reactive oxygen species (ROS) play a central role in pathogen defense in both animals and plants. In animals, ROS production by phagocytic leukocytes (macrophages/neutrophils) is a well-characterized anti-microbial defense mechanism (1). Plants produce an analogous, localized oxidative burst (2) wherein massive amounts of anti-microbial ROS (e.g. superoxide, \( \cdot O_2^- \); and hydrogen peroxide, \( H_2O_2 \)) are generated by a pathogen induced NADPH oxidase localized on the plant plasma membrane (3). In addition to its direct anti-microbial activity, \( H_2O_2 \) also triggers the hypersensitive response (HR), in which plant programmed, localized cell death at the site of infection limits pathogen spread (4). \( H_2O_2 \) also plays a central role in signaling a unique phenomenon known as systemic acquired resistance (SAR), in which localized infection of a plant confers enhanced systemic resistance to subsequent attack, by the same or unrelated pathogens (5,6). SAR is correlated with the systemic induction of a large number of defense related proteins collectively labeled pathogenesis-related (PR) proteins. In addition to \( H_2O_2 \), the endogenous signal molecule salicylic acid (SA) is implicated in PR protein induction and has been used extensively as an exogenous stimulator of the SAR response (7,8).

A successful pathogen must be able to overcome or suppress this complex array of ROS-mediated host defenses. In fact, microbial suppression of ROS-mediated defenses by secretion of ROS scavenging enzymes such as superoxide dismutase and catalase, which convert ROS into less reactive species, has been extensively documented in both plant and animal pathogens (9-12). Evidence is also emerging that pathogens suppress ROS-mediated defenses by non-enzymatic quenching of ROS. Mannitol has long been recognized as a potent ROS quencher \textit{in vitro} (13), and has been widely used as a laboratory reagent to scavenge hydroxyl radicals (OH·) generated by the phagocyte respiratory burst or by cell free oxidant systems (14). \textit{In vivo},
increased mannitol production protects *Saccharomyces cerevisiae* from oxidative killing (15). Furthermore, it was recently shown that the human fungal pathogen, *Cryptococcus neoformans* (syn. *Filobasidiella neoformans*), produces mannitol to quench neutrophil-generated ROS and thereby suppress this animal disease defense (16).

In addition to microbes, over 100 species of vascular plants synthesize mannitol (17). Our recent research has focused on the role(s) of mannitol metabolism in plants, in particular celery, where mannitol serves as an alternate metabolic reserve, as well as an osmoprotectant. In celery, the enzyme mannitol dehydrogenase (MTD), a 1-oxidoreductase, catalyzes the direct conversion of mannitol to mannose, and is a key regulator of mannitol pool size (18). Characterization of a cDNA encoding MTD revealed a striking sequence similarity (>70% nt and >90% aa) to the *Eli3* pathogen induced transcripts from parsley and *Arabidopsis* (19,20). The dramatic induction of MTD expression in celery cell suspensions upon treatment with SA provided further evidence that MTD and hence mannitol might play a role in plant-pathogen interactions. We originally hypothesized that, given its antioxidant properties, the large pools of mannitol in celery and parsley (up to 50 and 20%, respectively, of their soluble carbohydrate) would seriously handicap ROS-mediated plant resistance responses. However, removal of mannitol via the pathogen-induced production of MTD would allow these defense responses to proceed.

Herein we report the discovery of the pathogen-induced expression of MTD in the non-mannitol-producing plant tobacco, as well as the plant-induced production and secretion of mannitol in the tobacco pathogen *Alternaria alternata*. Together these data suggest that, like their animal counterparts, plant pathogenic fungi produce the ROS quenching sugar alcohol mannitol as a means of suppressing ROS-mediated plant defense mechanisms. However, unlike
animals, the pathogen induced expression of plant MTD might serve to counter this fungal suppressive mechanism by catabolizing mannitol of fungal origin.

**MATERIALS AND METHODS**

**Plant Materials and Growth.** Tobacco (*Nicotiana tabacum* L. cv. K326) was obtained from M. Daub and grown in a growth chamber at 22°C with a 14:10 hr light:dark cycle. NT-1 tobacco cell suspension cultures were obtained from S. Spiker and grown in Murashige and Skoog-medium (21) supplemented with 0.5 µg·ml⁻¹ 2,4-dichlorophenoxyacetic Acid (2,4D). Cultures were shaken at 100 x rpm under constant light (150 µE·m⁻²·sec⁻¹) at 22°C, and transferred into fresh medium every 7 days.

**Protein Extraction and Enzyme Assays.** Proteins were extracted and assayed as described (22), except the extraction buffer contained 0.1mM PMSF, but no Triton X-100, and extracts were not desalted prior to assay. MTD activity was determined by measuring the rate of mannitol-dependent conversion of NAD⁺ to NADH. To confirm the identity of the tobacco MTD as a 1-oxidoreductase, the ability of extracts to catalyze the NADH dependent reduction of mannose (i.e. the “reverse reaction”, ref. 23) was also assayed in representative samples. For simplicity, only forward reactions are depicted. The plant enzyme MTD (EC requested), being a 1-oxidoreductase, catalyzes the NAD⁺-dependent oxidation of mannitol to mannose. In contrast, fungal and bacterial mannitol dehydrogenases (e.g. MTLK and MTLD; EC's 1.1.1.67 and 1.1.1.17, respectively) are normally 2-oxidoreductases, and catalyze the conversion of mannitol or mannitol-P to fructose or fructose-P, often using NADP⁺ as an oxidant (17). As such microbial mannitol dehydrogenases would not be detected by our assays. Proteins were quantified by the method of Bradford (24) before analysis.
**Blot Analyses.** Protein extracts (20 μg per lane) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with a polyclonal anti-MTD serum diluted 1:6000 (18). Serum cross-reacting proteins were visualized using an alkaline phosphatase-linked secondary antibody (Promega). RNA extraction and blot analyses, using a 32P-labeled, full length celery Mtd cDNA as a probe, were as described (19), except final washes were performed in 0.1XSSC at 50°C.

**Induction of MTD.** Tobacco leaf discs, 9 mm in diameter, were floated on sterile distilled water with or without the indicated concentration of 2,6-dichloroisonicotinic acid (INA, synthesized by Novartis and a gift from J. Burton, ref. 25). All treatments were for 48hr unless otherwise indicated. Disks were harvested and frozen in liquid N₂ and stored at -80°C. MTD enzyme activity was measured as described above. NT-1 tobacco cell suspensions were amended with either 1:50 (v/v) fungal elicitor (a gift from R. Dixon, Samuel Roberts Nobel Foundation, ref. 26) or an equal volume of sterile distilled water and incubated for 12hr.

**Immunotitration.** Protein extracts (50 μl) from leaf discs treated with 3mM INA for 48hr was incubated for 12hr at 0°C with increasing volumes of either preimmune or anti-MTD serum (18). Proteins were quantified by the method of Bradford (24), and total protein in samples was equalized by the addition of BSA. MTD activity was measured as described above and is expressed as a percentage of the enzyme activity in the absence of either preimmune or anti-MTD serum. Activity of untreated control extract was 2.03 μmol/hr⁻¹·gfw⁻¹.

**Fungal Culture Growth and Treatment.** Mycelial inocula were prepared as described (27) from A. alternata and C. nicotianae cultures (gifts from M. Daub) grown in shake culture at 25°C on a 12:12 hr light:dark cycle for 7 days in malt extract medium (28). Plant extracts were prepared from 10-cm-long primary leaves of greenhouse-grown tobacco (Nicotiana tabacum, cv. K326) as described (27), and sterilized by filtration through a 0.2 μM nitrocellulose filter. Fungal
mycelia (1 mg·ml⁻¹fresh weight) was inoculated into 125 ml malt medium containing either 10% (vol/vol) aqueous tobacco leaf extract or an equal volume of sterile distilled water. Cultures were grown in continuous darkness to prevent the synthesis of the photosensitizing toxin cercosporin and subsequent generation of the ROS singlet oxygen (¹O₂) in C. nicotianae. Fungal tissue was recovered by centrifugation (6000xg), washed with sterile distilled water to remove residual medium and stored at –80°C. Supernatants (culture filtrate) from each culture were also collected and stored at –80°C.

Sugar Analyses. Internal soluble sugars were extracted from 200 mg frozen mycelia as described (22), except initial extraction volumes were reduced by 30%. Media and internal soluble sugars were analyzed as described (22), using a Waters HPLC system equipped with a guard column (C-18 Corasil, Bio-Rad, Hercules, CA) and in-line cation and anion guards (Micro-Guard, Bio-Rad, Hercules, CA). Carbohydrates were separated isocratically on either a Sierra Separation (Sparks, NV) Carbohydrate, Ca²⁺ (flow rate of 0.5 ml water min⁻¹ at 75°C) or a Fast Carbohydrate, Pb²⁺ column (flow rate of 0.8 ml water min⁻¹ at 85°C) with essentially identical results. Carbohydrate identity and quantity were determined by comparison to standards using a differential refractometer (model 410, Waters) coupled to a computing integrator (model SP4200, Spectra-Physics).

RESULTS

Fungi Induce an Endogenous MTD Activity in Tobacco. To test the hypothesis that MTD might play a protective role in plant defense, we had transformed tobacco, a non-mannitol containing plant, with a constitutively expressed MTD construct. Tobacco, unlike celery, does not contain endogenous pools of mannitol (29), and so was assumed to lack endogenous
mannitol catabolic activity (i.e. MTD). During screening of these transgenic tobacco we noted that, although untransformed control plants grown in sterile culture lacked detectable MTD activity, protein blot analyses detected traces of an anti-MTD sera cross-reacting protein corresponding in size to celery MTD (data not shown). Subsequent analyses revealed that significant MTD enzyme activity could be detected in extracts from untransformed control plants that had trace fungal contamination in the medium (Fig. 1). In contrast, MTD activity was not detected in extracts from uncontaminated plants. To confirm that the observed activity was not a fungal NAD$^+$-dependent mannitol dehydrogenase, an extract from fungal mycelia isolated from infected cultures was assayed and no detectable activity was observed (Fig. 1). These results suggested that, although tobacco is a non-mannitol producing plant, it does have an endogenous MTD that is pathogen-induced.

Expression of Tobacco MTD is Up-regulated by Inducers of Plant Defenses. To assess the hypothesis that induction of MTD activity is a pathogenesis related (PR) response we incubated tissues and cells of untransformed tobacco with several known inducers of PR proteins. First, leaf discs of tobacco (*Nicotiana tabacum*, cv. Kentucky 326, K326) were treated with 2,4-dichloroisonicotinic acid (INA), a synthetic analog of SA that induces the same set of PR-proteins in tobacco but is less phytotoxic (25). INA treatment elicited a >10-fold increase in MTD enzyme activity together with a parallel increase in anti-MTD sera cross-reacting protein and *Mtd* RNA (Fig. 2A). To verify that this response was not specific for INA, but represented a general response to various inducers of plant defense, we evaluated the effects of the SA and fungal elicitor on NT-1 tobacco suspension cells. MTD activity and protein were, in fact, comparably induced in NT-1 tobacco suspension cells by both SA (not shown) and fungal elicitor (Fig. 2B). In additional analyses using INA-treated K326 leaf disks, increases in MTD
enzyme activity were observed to be rapid and linear with time (Fig. 3A), and both MTD activity and anti-MTD serum cross-reacting protein(s) increased in a linear fashion with respect to INA concentration (Fig. 3B).

**The Anti-MTD Cross-Reacting Protein is MTD.** To establish a functional link between the observed increase in MTD activity and the parallel induction of the anti-MTD sera cross-reacting protein(s), extracts from INA-treated leaf discs were assessed by immunotitration. Extracts incubated with increasing amounts of anti-MTD sera showed a dose-dependent decrease in MTD activity (Fig. 4). In contrast, extracts incubated with equivalent amounts of preimmune sera did not, indicating that one or both of the observed anti-MTD sera cross-reacting proteins was responsible for the observed activity.

**Host Plant Extract Induces Increased Fungal Mannitol Production and Secretion.** If phytopathogenic fungi employ mannitol to quench plant generated ROS then host plant extracts might be expected to elicit changes in both fungal mannitol production and secretion. Two fungal pathogens of tobacco, *Cercospora nicotianae* and *Alternaria alternata*, each having potentially different modes of attack with respect to ROS, were used to assess possible effects of host plant extracts on fungal mannitol production and secretion. Each was cultured in the presence and absence of aqueous tobacco leaf extract for 7 days, after which the amounts of mannitol in both the fungal mycelia (internal) and in the culture filtrate (secreted) were determined by HPLC. Fungal growth was essentially unaffected by plant extract (data not shown). However, *A. alternata*, a known mannitol producer (30), responded to the presence of plant extract by accumulating both substantially higher levels of total mannitol, and a 3-5 fold increase in secreted mannitol (Table 1). In contrast, *C. nicotianae* did not secrete detectable amounts of mannitol in either the presence or absence of plant extract, nor did internal mannitol
accumulation respond significantly to plant extracts. Mannitol was not detected in either uninoculated growth medium or in tobacco leaf extracts. In addition, the total amount of mannitol precursors (e.g. fructose) present in these leaf extracts (3.7mg in a culture volume of 125 ml), even if completely and preferentially converted to mannitol, was grossly insufficient to account for the observed results.

**DISCUSSION**

The sugar alcohol mannitol is not only a commonly occurring carbohydrate in bacteria, yeast, fungi, and lichens, but is also found in numerous species of vascular plants (17). Mannitol metabolism in plants has been primarily studied in celery (*Apium graveolens*), where mannitol can comprise up to 50% of the soluble carbohydrate (17). The plant enzyme mannitol dehydrogenase (MTD), a 1-oxidoreductase, catalyzes the conversion of mannitol to mannose, thus acting as a key regulator of mannitol pool size in celery (18). Initially it seemed surprising to find an enzymatically active mannitol-catabolizing enzyme in tobacco, a plant that does not contain mannitol. In fact, not only are tobacco and celery MTD biochemically similar (both are 1-oxidoreductases), but they also appear to be structurally quite similar. Anti-sera raised against purified celery MTD not only cross-reacts with an appropriately sized, INA/SA-induced protein in tobacco, but also effectively immunotitrates INA-induced tobacco MTD activity. This similarity apparently extends to the nucleotide level, because a celery *Mtd* cDNA hybridizes at moderate stringency with an appropriately sized INA-induced tobacco RNA.

The observed correlation between MTD expression in tobacco and fungal infection, however, suggested a potential role for MTD in a non-mannitol producing plant. Recent research has revealed a strong link between production of ROS and the appearance of the
hypersensitive response (4,31). Additional studies suggest that active oxygen, most notably hydrogen peroxide, acts not only as an antimicrobial agent (2), but also as an extracellular signal that mediates numerous plant defense responses (6, 8). If plants use ROS in defense against pathogens, successful pathogens presumably have evolved mechanisms to avoid or suppress these defenses. For example, phytopathogenic bacteria secrete catalase (an enzyme that detoxifies H$_2$O$_2$ by converting it to water). It has been hypothesized that this secreted catalase is used to suppress ROS-mediated plant defenses (9,10). Conversely, plants transformed to express glucose oxidase, an enzyme that generates H$_2$O$_2$, become more resistant to pathogens (32).

Combined with the observation that most ascomycete fungi examined to date produce mannitol (30), the presence of a pathogen induced MTD in tobacco suggests a pervasive role for MTD in pathogen resistance in plants. While mannitol in fungi may serve primarily as an osmolyte or metabolic reserve, it seems increasingly likely that, like human fungal pathogens, some phytopathogenic fungi use mannitol to suppress ROS-mediated plant defenses. This is supported by the observation that production of mannitol is necessary for pathogenicity of the tomato pathogen *Cladosporum fulvum* (33). Moreover, Lauter (34) recently reported that tomato has a gene with high homology (70% nt identity) to celery *Mtd*. The pathogen response of this gene was not examined, but as tomato lacks mannitol it seems likely that tomato MTD plays a role similar to that proposed for tobacco MTD.

If mannitol-mediated quenching is a common mechanism by which fungi evade ROS-mediated plant defenses, then suitable host plant extracts might be expected to induce mannitol production in fungal pathogens. The observed increase in production and secretion of mannitol by the tobacco pathogen *A. alternata* in response to leaf extract is consistent with mannitol’s proposed role as a ROS quencher during the infection process. In contrast, production and
secretion of mannitol by \textit{C. nicotianae} was not significantly affected by the presence of host plant extract. This is consistent with the fact that the photosensitizing toxin cercosporin, a producer of the ROS singlet oxygen (\(1^1\text{O}_2\)), is required for \textit{C. nicotianae} pathogenicity (35).

Hence, secretion by \textit{Cercospora} of an ROS quencher, i.e. mannitol, would be counterproductive. Although mannitol oxidation appears to be the primary metabolic function of MTD, further analyses show additional \textit{in vitro} activities. Purified celery MTD, for example, catalyzes the reduction of aldopentose and aldohexose substrates with the same stereochemical configuration at C-2 as that of D-mannose (36). In addition, proteins produced by heterologous expression of the \textit{Mtd} homolog \textit{Eli}3 in \textit{E. coli} have a measurable ability to catalyze the reduction of several phenylpropanoid pathway intermediates such as cinnamyl-aldehyde (37,38). It is possible that these other reported activities catalyzed by MTD might also play a role in plant-pathogen interaction. Our data linking pathogen-induced expression of MTD in a non-mannitol-producing plant with host-induced mannitol biosynthesis in the fungal pathogen, however, strongly implicates a specific function for MTD in mannitol degradation.

The work presented here suggests that \textit{Mtd} represents a new class of non-specific pathogen resistance gene that plays a role in the complex process of fungal resistance in plants. Akin to a growing class of other PR-proteins, MTD is an enzyme with clearly defined roles in central metabolism, which when specifically activated during pathogen attack can perform a very different biological role. Moreover, fungi normally produce mannitol as an osmolyte and metabolic reserve. On induction by host signals, however, mannitol could be mobilized to act as a suppressor of plant defenses. Hence, both pathogens and host plants appear to recruit existing enzymes or metabolites to serve unique functions during host pathogen interactions.
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Fig. 1. Fungal infection elicits MTD activity in tobacco. Tissue from uninfected and infected tobacco and an isolated culture of the infecting fungus were collected, frozen in liquid nitrogen, and stored at -80°C. MTD activity was measured in extracts as the rate of mannitol-dependent conversion of NAD\(^+\) to NADH as described in Materials and Methods. Error bars represent the SE of three independent observations. n.d., not detected.

Fig. 2. Induction of MTD in tobacco by treatment with INA or fungal elicitor. (A) MTD activity (top), protein (middle) and RNA (bottom) in extracts from tobacco leaf discs, cultivar K326. Discs were floated on sterile distilled water either in the absence (-) or presence (+) of 1mM INA for 48hrs before extraction. MTD activity was measured in extracts as above. Data represent means ± SE of three independent experiments. Equal protein (20µg) from each sample was separated by SDS/PAGE and blotted onto nitrocellulose. Blots were incubated with anti-MTD serum for 1 hr and visualized as previously described in Materials and Methods. Total RNA was also extracted from these tissue samples, and relative amounts of Mtd transcript determined by blot analysis using a \(^{32}\)P-labeled, full-length celery Mtd cDNA (19) as a probe under conditions of moderate stringency (washed in 0.1XSSC at 50°C). (B) NT-1 tobacco cell suspensions were amended with either 1:50 (vol/vol) fungal elicitor (+, ref. 26) or an equal volume of sterile distilled water (-) and incubated for 12hr. Cells were collected, and extracts assayed for MTD activity (upper) and protein (lower) as described above. Data represent the means ± SE of three independent experiments.
Fig. 3. Changes in MTD activity and protein in response to INA treatment time and concentration. (A) MTD activity in extracts from K326 leaf discs treated with 3mM INA (●) or distilled water (■). Discs were randomly selected at 0, 6, 12 and 24 hr, and MTD activity in extracts was assayed as described above. Data points are means of two independent experiments. (B) MTD activity in extracts from K326 tobacco leaf discs treated with 0, 0.25, 0.5, 1 or 2 mM INA for 48 hr. Data points are means of two independent experiments. Equal protein (20µg) from each leaf disc extract was separated by SDS/PAGE and blotted onto nitrocellulose. Blots were incubated with anti-MTD serum for 1 hr and visualized as described above.

Fig.4. Immunotitration of MTD activity in extracts from INA-induced tobacco leaf discs. Extract(s) (50 µl) from leaf discs treated with 3mM INA for 48hr were incubated for 12hr at 0°C with increasing volumes of preimmune (■) or anti-MTD (●) serum. Data are means ± SE of data from two independent experiments.
Figure 4

MTD Activity (% unamended) vs. µl serum
CHAPTER 2

Constitutive expression of a celery mannitol dehydrogenase in tobacco provides enhanced tolerance to the mannitol-secreting fungal pathogen *Alternaria alternata*.

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ABSTRACT

Constitutive expression of a cDNA encoding the mannitol-catabolizing enzyme mannitol dehydrogenase (MTD) from celery in the non-mannitol producing plant tobacco resulted in production of an enzymatically active MTD protein. This constitutive expression of celery Mtd in tobacco conferred enhanced tolerance to the mannitol secreting plant pathogen, *Alternaria alternata*. Enhanced resistance did not correlate with expression of PR1a, a protein often used as an indicator of systemic acquired resistance. This confirmed that the enhanced resistance observed was due to the constitutive presence of *Mtd* rather than a coincident induction of the SAR response. In contrast, constitutive *Mtd* expression did not enhance tolerance to the non-mannitol secreting fungal plant-pathogen *Cercospora nicotianae* or the non-mannitol secreting bacterial plant-pathogen *Pseudomonas syringae pv. tabaci*. These results are consistent with the hypothesis that MTD plays a role in plant resistance to mannitol secreting fungal pathogens by catabolizing mannitol of fungal origin.
INTRODUCTION

In mannitol-producing plants such as celery and parsley, mannitol serves as a carbon and energy storage compound (Lewis, 1984, Loescher, 1987; Stacy, 1974; Stoop and Pharr, 1992), as a compatible solute (Brown and Simpson, 1972; Yancey et al., 1982) and in osmoregulation (Hellebust, 1976). In these plants mannitol accumulation is at least partly modulated by the regulated expression of the mannitol catabolic enzyme mannitol dehydrogenase (MTD) (Stoop and Pharr, 1992, 1993). MTD has also been postulated to be a pathogenesis related (PR) protein in celery due to its strong sequence similarity to the elicitor-induced ELI3 proteins from parsley and Arabidopsis, as well as its massive induction by salicylic acid (SA) in cultured celery cells (Williamson et al., 1995).

In addition to being a metabolite and osmoprotectant, mannitol has been shown to be a powerful antioxidant, both in vitro (Smirnoff and Cumbes, 1989) and in vivo (Shen et al., 1997). Reactive oxygen species (ROS) play a central role in plant responses to pathogen attack. Upon recognition of an infecting pathogen, a plasma membrane associated NAD(P)H oxidase is activated and superoxide (\( \cdot \text{O}_2^- \)) is produced (Doke, 1985). \( \cdot \text{O}_2^- \) is subsequently dismutated to \( \text{H}_2\text{O}_2 \), either spontaneously or via an apoplastic superoxide dismutase (Alvarez et al., 1998; Vanacker et al., 1998). \( \text{H}_2\text{O}_2 \) then diffuses into cells and, depending on the concentration, activates apoptosis (cell death) or a variety of other defense mechanisms (Levine et al., 1994; Draper, 1997).
ROS serve as direct antimicrobial compounds, as substrates for lignification, or more globally as signal molecules for induction of SA synthesis and resulting induction of PR-proteins. Due to the potent antioxidant capacity of mannitol, the large amounts of mannitol present in celery (up to 50% of the soluble translocated carbohydrate) might be expected to quench ROS, and thus cripple the whole cascade of ROS-mediated defenses. As neither parsley nor celery is exceptionally noted for disease susceptibility, our initial hypothesis was that removal of mannitol via the SA-mediated induction of MTD acted to potentiate ROS-mediated defenses in celery.

Unexpectedly, MTD has also been found in the non-mannitol producing plant tobacco, where its expression is induced by the SA analog 2,6-dichloroisonicotinic acid (INA) as well as by fungal elicitors (Jennings et al., 1998). In addition to the pathogen-induced MTD homolog in Arabidopsis (ELI3), an MTD homolog has also been found in tomato (Lauter, 1996, van der Hoeven et al. 1999). Both Arabidopsis and tomato, like tobacco, are non-mannitol producing plants. Thus, MTD is not confined solely to mannitol producing plants, and the lack of its normal substrate in non-mannitol plants suggests that it is unlikely to have the same role in these plants.

Recent findings suggest a possible role for pathogen-induced MTD in non-mannitol producing plants. Because reactive oxygen serves such a central role in plant responses to pathogen attack, successful pathogens must be able to evade or suppress ROS-mediated defenses. In the case of phytopathogenic bacteria, oxidative damage may be prevented by enzymes that detoxify ROS,
such as catalases and superoxide dismutases (Katsuwon and Anderson 1989; Klotz and Hutcheson 1992). In contrast, many fungi, including plant pathogenic ones, produce the potent antioxidant mannitol (Bielski, 1982). Mannitol was originally thought mainly to play a role in metabolic processes and NAD(P)H recycling in these fungi. However, mannitol production appears to be essential for pathogenicity in at least some plant-pathogenic fungi. For example, Joosten et al. (1990) reported that the tomato pathogen *Cladosporium fulvum* produces and secretes mannitol during the infection process. In addition, *Cl. fulvum* mutants that had lost the ability to make or secrete mannitol were no longer pathogenic. Similarly, mutants of the animal pathogenic fungus *Cryptococcus neoformans* that under-produce and hence under-accumulate mannitol are more susceptible to osmotic and heat stress, and are hypersusceptible to oxidative killing (Chaturvedi et al., 1996a, Chaturvedi et al., 1996b). Moreover, these mutants were hypovirulent in mice, suggesting that mannitol functions in *Cr. neoformans* as an intracellular osmolyte and antioxidant and that its presence is required for wild-type virulence (Chaturvedi et al., 1997).

If mannitol-mediated quenching is a mechanism by which pathogens suppress ROS-mediated host plant defenses, then host plant extracts might be expected to induce mannitol production and/or secretion in corresponding pathogens. Previous work in our lab showed that mannitol production and secretion were induced in the tobacco pathogen, *Alternaria alternata*, by a simple aqueous tobacco leaf extract (Jennings et al., 1998). In contrast, *Cercospora nicotianae*, also a fungal pathogen of tobacco, did not show increased mannitol
production or secretion in the presence of tobacco leaf extract. This is consistent with the fact that the production of the ROS singlet oxygen (¹O₂) by the photosensitizing toxin cercosporin is required for C. nicotianae pathogenicity (Daub et al., 1998). Hence, secretion of the ROS quencher mannitol by C. nicotianae would be counterproductive.

To more directly assess the possible role of MTD, and hence mannitol in plant defense against pathogens, tobacco plants transformed to constitutively express a celery Mtd cDNA were generated and assessed for potential changes in resistance to both mannitol and non-mannitol secreting pathogens.

MATERIALS AND METHODS

Materials

All chemicals used in these studies were of Reagent grade or better and were acquired from Sigma, except DTT, which was obtained from Boeringher Mannheim. All DNA restriction and modification enzymes were obtained from Promega (Madison, WI). ECL Western Blot reagents were obtained from Bio-Rad (Hercules, CA). INA (2,6-dichloroisonicotinic acid) was a kind gift from Novartis Crop Protection, Inc. (RTP, NC)

Construction and transformation of 35S-Mtd chimera.

A DNA fragment containing full-length celery Mtd cDNA was excised from clone p5-4 (Williamson et al., 1995) with the restriction enzymes Smal and SnaBI. This blunt-ended fragment was used to replace the GUS reporter gene in a Smal-EcoRI-cut pBI121 plant transformation vector (Clontech, Palo Alto,
CA). The resulting clones were screened for desired insert orientation, and sequenced to verify promoter-cDNA borders. A suitable chimeric plasmid (p9-41) was identified, isolated and transformed into *Agrobacterium tumefacens*, strain EHA105 (Hood et al., 1993) using a freeze-thaw procedure (An et al., 1988). This 35S promoter-Mtd cDNA transcriptional fusion was then introduced into *Nicotiana tabacum* cv. Burley 21 by *Agrobacterium*-mediated transformation as described by Daub et al. (1994). Kanamycin-resistant primary transformants (R₀’s) were screened, and transformants expressing MTD activity were allowed to self. The resulting R₁ seed was collected, surface sterilized and germinated on 1/2 MS (Murashige and Skoog, 1962) medium containing 100mg/L kanamycin, 200 mg/L carbenicillin and 0.7% TC agar (Sigma). R₂ plants were grown from seed of representative R₁ plants that showed high levels of constitutive MTD expression in leaves.

**Fungal and bacterial inoculations**

The fungal tobacco pathogens, *A. alternata* and *C. nicotianae* were grown on V-8 juice agar (Jenns et al., 1989) for 7 days. Sterile distilled water was then added to plates and the resulting spore suspensions collected. Spores were quantified using a hemocytometer and resuspended to the desired concentration in sterile distilled water. For *A. alternata* inoculations, a detached leaf assay similar to that of Spurr (1973) was used to assess resistance. Three leaves were harvested from 12 week old transformed and untransformed B21 plants grown at the Southeastern Plant Environment Laboratory (Phytotron) with a 12:12 hr light:dark cycle at 150 μEm⁻²sec⁻¹ at 22°C. Two 2"x2" squares were cut from
each leaf and placed abaxial side up on wire mesh suspended above water in a lidded, plastic container. Twenty-10µl drops of the A. alternata spore suspension (5 x 10⁴ spores/ml) were distributed evenly on each leaf section and allowed to air dry. Incubation boxes were then closed and placed in a 20°C incubator with continuous light (100 µEm⁻²s⁻¹). Leaf sections were scored at 5, 7 and 10 days after inoculation for lesion formation. The number of necrotic lesions per 20 inoculations sites was noted for each leaf section in each of three incubation boxes and scores for tissues from the same plant were averaged. Data for 7th day observations are shown.

For C. nicotianae inoculations, two leaves each on 16-week old greenhouse-grown untransformed and transgenic B21 plants were sprayed on the abaxial side with a 5 x 10⁴ per ml spore suspension of C. nicotianae. Spore suspensions were prepared from 7-day-old cultures as described above. Leaves were allowed to dry after inoculation, and the plants were placed in plastic bags in a shaded area in the greenhouse. Plants were removed from bags after 4 days, placed on greenhouse benches and monitored for development of lesions, and lesion coalescence.

The bacterial pathogen, Pseudomonas syringae pv. tabaci was grown in King’s B (KB) media (King et al., 1954) for 16 hours at which time 500 µl was removed and inoculated into 50ml of fresh KB. Cultures were grown at 25°C overnight and bacteria harvested by centrifugation. Bacterial pellets were washed once in distilled water, repelleted by centrifugation and resuspended in water at the indicated concentrations. Tobacco plants (untransformed and
transformed) were inoculated with *P. syringae pv. tabaci* by infiltration of 10⁵, 10⁶, 10⁷ or 10⁸ c.f.u./ml suspensions in distilled water. One leaf per plant was inoculated with distilled water as a control. Infiltration was into the abaxial side of intact leaves of 12 week-old plants with a plastic syringe without a needle (Glazebrook and Ausubel, 1994). Two inoculation sites per bacterial concentration were made in each leaf, and two leaves were inoculated per plant. Symptoms, development of necrosis and tissue degeneration, were evaluated 5, 7, and 10 days after infiltration.

**RNA Blot Analyses.**

RNA extraction and blot analyses, using a ³²P-labeled, full length celery *Mtd* cDNA as a probe, were as described (Williamson et al., 1995, Jennings et al., 1998), but with final washes performed in 0.1XSSC at 65°C to ensure detection of only the homologous celery *Mtd* transcript. The tobacco *Mtd* homologue is only detected at low stringency (50°C wash temp.).

**Induction of PR Proteins**

Tobacco (*Nicotiana tabacum* L. cvs.; Burley 21 (B21), K 326 and Petit Havana (SR1)) was grown in a growth chamber at 22°C with a 14:10 hr light:dark cycle at 150 μEm⁻²sec⁻¹. For PR protein induction, 9mm leaf discs were removed from plants and floated on either 3mM INA (2,6-dichloroisonicotinic acid), or distilled water (control) for 48 hrs.

**Protein Extraction, MTD activity determination and Blot Analyses**

Prior to infection, representative tissue from each transformed and untransformed B21 plant was harvested and frozen in liquid nitrogen. Tissue
was ground directly in Bio-Rad (Richmond, CA) SDS sample buffer (1:6 w/v). Proteins were quantified by the method of Bradford (1976) prior to analysis. Protein extracts (20µg per lane, unless otherwise noted) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with a polyclonal anti-MTD serum (1:6000) (Stoop et al., 1995) or anti-PR1a serum (a kind gift of D. Klessig, Rutgers University). Serum cross-reacting proteins were visualized using the ECL western blotting analysis system (Amersham Life Science, Buckinghamshire, England). MTD activity was determined by measuring the mannitol-dependent conversion of NAD⁺ to NADH. Prior to assay for MTD activity protein extracts were passed through a Sephadex G25-50 column to desalt the extracts. Desalting serves to inactivate endogenous tobacco MTD but does not negatively affect celery MTD (unpublished data).

**Sugar Analyses.**

Internal soluble sugars were extracted from 200 mg frozen bacterial cultures, or from 9mm discs of excised tobacco leaf tissue surrounding bacterial infections sites, as described (Stoop and Pharr, 1993), except initial extraction volumes were reduced by 30%. Media and internal soluble sugars were analyzed as described (Stoop and Pharr, 1993), using a Waters HPLC system equipped with a guard column (C-18 Corasil, Bio-Rad, Hercules, CA) and in-line cation and anion guards (Micro-Guard, Bio-Rad, Hercules, CA). Carbohydrates were separated isocratically on a "Carbohydrate, Ca²⁺" column (Sierra Separation, Inc., Sparks, NV) at a flow rate of 0.5 mL water min⁻¹ at 75°C. Carbohydrate identity and quantity were determined by comparison to standards.
using a differential refractometer (model 410, Waters/Millipore, Milford, MA) coupled to a computing integrator (model SP4200, Spectra Physics, San Jose, CA).

RESULTS

Celery Mtd encodes an enzyme with mannitol dehydrogenase activity in tobacco

As a first step, tobacco plants transformed with a CaMV 35S-celery Mtd construct were assayed for MTD activity to confirm that the cloned Mtd cDNA could produce an active MTD in a heterologous system. Leaf tissue from primary transformants (R₀’s) and untransformed control plants was extracted, and desalted crude extracts analyzed for MTD activity (Fig 1A). Desalting inactivates the endogenous tobacco MTD previously described (Jennings et al., 1998), ensuring specific detection of the cDNA-encoded celery enzyme. Several primary transformants (e.g. 1E5) had significant MTD activity, while other transformants, like 1E10, as well as untransformed B21 plants had little or no detectable MTD activity. The low level of activity in untransformed B21 plants was most likely due to endogenous MTD that was not completely inactivated by desalting. Samples from representative transformed plants (kanamycin resistant) with varying levels of MTD activity, as well as untransformed control plants were further analyzed for MTD protein (not shown) and Mtd RNA (Fig 1B). RNA blot analyses were performed at high stringency to ensure specific detection of the celery cDNA-encoded transcript (see materials and methods). MTD activity in
transformants paralleled the accumulation of MTD protein (not shown) and Mtd RNA, supporting the notion that the 35S-Mtd transgene makes an active enzyme in tobacco. As expected, there was no detectable Mtd RNA and little or no activity in the untransformed control.

**Transgenic plants show enhanced resistance to *Alternaria alternata***

If, as hypothesized, MTD metabolizes pathogen-secreted mannitol, and thus protects ROS-mediated defense responses, then constitutive expression of MTD should provide enhanced protection against a pathogen that secretes mannitol. To test this hypothesis, detached leaves from both Mtd transformed and untransformed plants were scored for resistance to the mannitol secreting fungal pathogen *A. alternata*. Leaf sections from R₁ and R₂ plants as well as untransformed controls were removed, placed in an incubation chamber and inoculated with an *A. alternata* spore suspension. The number of necrotic lesions per 20 inoculation sites was determined seven days after inoculation.

Susceptible interactions were characterized by the rapid formation of necrotic lesions at the site of inoculation, followed by the formation of a yellow halo and eventual necrosis of the entire leaf section (Fig 2A). Resistant reactions varied from no obvious symptoms to slight yellowing around infection sites, and in some cases the formation of a green halo around the infection site. Leaf tissue from R₁ transgenic plants exhibited many fewer necrotic lesions than untransformed leaf tissue (Fig 2B). Analyses of tissue from the same untransformed and transformed tobacco plants, taken just prior to infection,
indicated that lesion formation on transgenic plants was inversely correlated with levels of constitutively expressed MTD protein, i.e. plants expressing higher amounts of MTD protein had fewer lesions (Fig 2C). R_2 transgenic tobacco displayed the same inverse correlation between lesion formation and MTD protein expression (data not shown). To ensure that resistance was due to the presence of MTD and not a pre-existing generalized defense response, tissue extracts were also assessed for the presence of the PR1a protein which is often used as a marker of an induced defense response. There was no correlation between the presence of the PR1a protein and the resistance of transformants to _A. alternata_. Thus resistance was in fact due to the constitutive expression of MTD and not a pre-existing defense response.

Pathogen-induced expression of MTD appears to be a common response in tobacco. We previously noted that MTD was induced in the tobacco cultivar K326 by the PR protein inducer INA (Jennings et al, 1998). Similarly, although there is some variability between lines, MTD is also INA-induced in at least two other tobacco lines, Burley 21 (B21, the line used in this study) and Petit Havana (SR1) (Fig 3A). We were able to distinguish endogenous tobacco MTD activity from that of constitutively expressed celery MTD by use of a Sephadex G25-50 desalting column. Passage of protein extracts through the column inactivates endogenous tobacco MTD while not affecting the activity of celery MTD (Jennings et al., 1998 and unpublished data). Induction of MTD protein by INA correlated with increased levels of MTD activity (Fig 3B) in all three lines (K326 shown). As previously demonstrated by a large number of labs, PR1a protein
expression is also induced by INA treatment (Fig 3B). However, PR1a protein expression did not correlate with reduced lesion formation in the A. alternata detached leaf assays of Mtd transformed tobacco (Fig 3C). The constitutive expression of MTD alone appears to be sufficient for conferring enhanced resistance to A. alternata.

**Constitutive expression of MTD does not enhance resistance to non-mannitol-secreting plant pathogens**

As previously noted, C. nicotianae, unlike A. alternata, does not secrete mannitol in the presence of tobacco leaf extract (Jennings et al., 1998). If C. nicotianae does not secrete mannitol during the infection process, one might predict that constitutive expression of MTD would not affect the ability of this pathogen to cause infection. To test this hypothesis, 16-week old Mtd-transformed and untransformed tobacco plants were sprayed with a spore suspension of C. nicotianae, and the development of disease symptoms (lesion formation, coalescence and necrosis) was monitored. There was no detectable difference in symptom onset and severity between untransformed and transformed tobacco plants inoculated with C. nicotianae (Fig 4). All inoculated leaves showed formation of necrotic lesions by 10 days after inoculation (Fig 5A). Necrotic areas increased in size and coalesced by 14 days, at which point leaf yellowing precluded further scoring. Analyses of tissues taken prior to inoculation showed the presence of the MTD protein in transformed plants (Fig 5B), however there was no correlation between the presence of MTD protein and
lesion formation. Both untransformed and transformed plants were susceptible to infection by *C. nicotianae*, indicating that constitutive expression of MTD in tobacco does not appear to enhance resistance to the non-mannitol secreting plant fungal pathogen *C. nicotianae*.

*Pseudomonas spp.* produces mannitol in response to osmotic stress (Kets et al., 1996). However, secretion of mannitol by *P. syringae* during the infection process for quenching of ROS has not been reported. If *P. syringae* employs mannitol to quench plant generated ROS then host plant extracts might be expected to elicit changes in mannitol production as is observed for *A. alternata* (Jennings et al., 1998, and Figure 6A and B). To assess any potential role of mannitol in *P. syringae* infections, *P. syringae pv. tabaci* was grown in the presence and absence of aqueous tobacco leaf extract, and the amounts of mannitol in the resulting bacterial pellets (internal mannitol) determined by HPLC. These analyses showed that there was no detectable mannitol production when *P. syringae pv. tabaci* cultures were treated with water (Fig. 6D) or host leaf (tobacco) extract (Fig. 6E).

If, like *C. nicotianae*, *P. syringae* does not secrete mannitol during the infection process, one would predict that constitutive expression of MTD would not affect the ability of this pathogen to cause infection. To test this hypothesis, both *Mtd* transformed and untransformed plants were inoculated with a suspension of *P. syringae pv. tabaci* and disease development monitored. As with *C. nicotianae*, we were unable to detect any differences in symptoms between *Mtd* transformed and untransformed tobacco inoculated with *P. syringae*.
Additional Analyses of tissue in and around infection sites also did not detect the presence of any mannitol (Fig. 6F). Together these observations indicate that mannitol does not play a role in infection by *P. syringae pv. tabaci*.

**DISCUSSION**

Previous work from this lab suggested that *Mtd* is a non-specific pathogen resistance gene that plays a role in the complex process of fungal resistance in plants. We initially observed that celery *Mtd* has a striking sequence similarity (>90%) to the ELI3-pathogen response genes found in parsley and *Arabidopsis*. In addition, MTD was massively induced by the known PR protein inducer SA in celery suspension cells (Williamson et al., 1995). As noted previously, both parsley and celery produce large amounts of mannitol as a product of photosynthesis, and mannitol is a potent ROS quencher. As ROS are central to numerous aspects of plants defense responses, these levels of mannitol might be expected to severely cripple plant defenses. However, neither celery nor parsley is notably susceptible to disease. The induction of MTD by SA, together with the ability of mannitol to serve as a reactive oxygen quencher led us initially to hypothesize that MTD plays a role in plant-pathogen interactions simply by removing mannitol and thereby "potentiating" ROS-mediated PR responses.

Surprisingly, however, further studies revealed the presence of a pathogen-induced MTD in tobacco, a plant that does not contain mannitol. Although tobacco lacks mannitol, the normal substrate for MTD, several lines of
evidence suggested a role for MTD in protection against mannitol producing fungal pathogens (Jennings et al., 1998, Williamson et al., 1995). Mannitol has been found to be produced by most ascomycete, as well as some basidomycete fungi, but was thought mainly to function in the regeneration of NADPH (Hult et al., 1980). However, studies of human pathogenic fungi showed that mannitol functions in these pathogens not only as an intracellular osmolyte and energy store, but as an antioxidant that is essential for wild-type virulence (Chaturvedi et al., 1997). We have previously shown that the plant pathogenic fungus, *A. alternata*, produces and secretes massively elevated levels of mannitol in the presence of host plant (tobacco) extract (Jennings et al., 1998). In addition, the tomato pathogen *Cl. fulvum* apparently requires mannitol biosynthetic capacity to be pathogenic (Joosten et al., 1990). We propose that fungi, upon induction by host signals, produce/mobilize and secrete mannitol to act as a suppressor of ROS-mediated plant defenses. Analogous to the hypothesized degradation of H$_2$O$_2$ by secreted catalase in bacteria (Klotz and Hutcheson, 1992), we infer that, like their animal counterparts (Chaturvedi et al., 1996b), phytopathogenic fungi might suppress ROS-mediated plant defenses by secretion of the antioxidant mannitol.

If a pathogen secretes mannitol to quench the "oxidative burst" and associated defense responses, then plants with constitutive MTD expression should be more resistant to this pathogen as they can metabolize the active-oxygen quencher mannitol to the non-quenching sugar mannose. Conversely, constitutive expression of MTD would not be expected to protect against
pathogens, such as *C. nicotianae* or *P. syringae*, that appear not to use mannitol to suppress ROS-mediated plant defenses. To further study and perhaps help verify MTD's role in plant-pathogen interactions, a celery *Mtd* cDNA was expressed in tobacco. The research presented in this paper shows that this *Mtd* cDNA does, in fact, produce an active MTD when transformed into tobacco plants under the control of the CaMV 35S promoter. More importantly, we show that transgenic tobacco plants constitutively expressing celery *Mtd* do indeed display enhanced resistance to the mannitol-secreting plant pathogenic fungus *A. alternata*. Expression of PR1a, a protein often used as a molecular marker of SAR induction, was also monitored in transgenic and untransformed tissue prior to infection to rule out inadvertent SAR induction as a possible source of resistance. We found that resistance did not correlate with the presence of PR1a protein, and, therefore, was likely not due to generalized SAR induced PR protein expression at the time of inoculation. These results are consistent with the hypothesis that constitutively expressed *Mtd* functions in the interaction between tobacco and *A. alternata* to remove the active oxygen quencher mannitol produced by *A. alternata*, and thereby “protect” the oxidative burst and resulting ROS-mediated defense signaling processes.

We previously demonstrated that *C. nicotianae* does not secrete detectable amounts of mannitol in response to the presence of host plant (tobacco) leaf extract (Jennings et al., 1998). Herein we demonstrate that the constitutive expression of the mannitol-catabolizing enzyme MTD does not appear to enhance resistance of tobacco to *C. nicotianae*. This is consistent with
the work of Daub and colleagues demonstrating that C. nicotianae produces the photosensitizing toxin cercosporin, which in turn generates the ROS, singlet oxygen (Daub et al., 1998). As production of ROS by cercosporin is a necessary factor for pathogenicity of C. nicotianae, it would be counterproductive to also secrete an active oxygen quencher (i.e. mannitol).

Although there is evidence that Pseudomonas putida makes mannitol as an osmoprotectant (Kets et al. 1996), the work of Klotz and Hutcheson (1992) suggests that catalase, an enzyme that enzymatically converts H₂O₂ to H₂O and O₂, rather than mannitol appears to be involved in suppression of ROS-mediated host defenses. They showed that pathogenic strains of Pseudomonas syringae have elevated levels of periplasmic catalases compared to non-pathogenic strains. In fact, the specific activities of catalases in P. syringae increase in response to H₂O₂ (Klotz and Hutcheson, 1992). Here we show that P. syringae pv. tabaci does not produce/secrete mannitol either in planta or when grown in cultures in the presence of host plant (tobacco) extracts. Therefore, not surprisingly, the constitutive expression of Mtd in tobacco did not enhance resistance to this tobacco pathogen.

A wide variety of pathogenesis-related (PR) proteins play roles in plant defenses against fungal and bacterial pathogens (Bowles et al., 1990). Induction of PR proteins by salicylic acid (SA), or its analogs INA and benzothiadiazole (BTH) provides protection against an array of plant pathogens. However, neither BTH nor INA induce resistance to Alternaria either in tobacco (Friedrich et al., 1996) or in Arabidopsis (Thomma et al., 1998) respectively. Thus it may initially
seem surprising that the constitutive expression of a single BTH-induced protein such as MTD enhances resistance to *A. alternata*.

Unlike BTH induced plants, which don't develop equivalent MTD levels until 24-48hrs after BTH treatment, plants expressing *Mtd* constitutively have significant MTD activity at time the time of inoculation. In addition, although MTD is induced in tobacco as part of the normal defense response against pathogens, we have shown that different varieties clearly make different amounts (Fig. 3A). It is possible that both the timing or rapidity of MTD accumulation as well as the absolute quantity of MTD may be important in pathogen resistance.

The production of ROS during the very early stages of infection appears to be a key signal for the programmed cell death that occurs during the HR response. For instance, when plants have reduced ascorbate peroxidase and/or catalase activity, either through antisense expression of these genes or through the use of chemical inhibitors, they are hyperresponsive to pathogen attack (Mittler et al. 1999). Likewise, tobacco antisense transformants resulting in reduced catalase activity show enhanced expression of PR-proteins and increased accumulation of the antioxidant glutathione (Chamnongpol et al., 1996). Thus it seems that a successful resistance response requires a rapid balance in the amount of ROS and antioxidant enzyme activity. Therefore the ability of a pathogen to interfere with this balance, either through production of antioxidants or antioxidant enzymes, as well as a plant's ability to restore this balance, may be crucial for successful infection.

The data presented here strongly support the hypothesis that MTD
functions in interactions between mannitol secreting pathogens and plants. However, the exact arena and nature of the interactions between pathogen-produced mannitol and plant-produced MTD is still not defined. It may be that pathogen produced mannitol enters the plant cell and encounters MTD in the cytoplasm. Conversely, the normally cytoplasmic MTD might be secreted into the apoplastic space where it interacts with pathogen secreted mannitol. Interestingly, Vanacker et al. (1998) recently showed that normally cytoplasmic forms of the antioxidant enzymes superoxide dismutase, catalase, and ascorbate peroxidase were all found in the apoplast of infected but not uninfected barley leaves. As these enzymes are not normally found in the apoplast, and do not contain any reported apoplast targeting sequence, there must be a specific mechanism for secreting them only upon pathogen infection. Thus, while MTD is an enzyme with clearly defined roles in central metabolism in the cytoplasm, when specifically activated during pathogen attack it may not only play a very different physiological role, but may also be localized differently.
REFERENCES


Figure Legends:

Figure 1. Celery *Mtd* encodes an active enzyme in transgenic tobacco. Tobacco plants were transformed with a 3S promoter-celery *Mtd* cDNA construct. Leaf tissue from initial (R₀) kanamycin-resistant regenerants (1E-1, 1E2, etc.) as well as from untransformed tobacco (B21, UT) was screened for transgene expression. (A) MTD activity was measured in desalted extracts using the MTD enzyme assay described in materials and methods. Data represent means and S.E. of three (3) independent experiments. (B) Presence of the celery *Mtd* transcript was ascertained using high stringency RNA blot analyses. Total RNA (20µg) extracted from the above tissue samples was separated on formaldehyde-agarose gels, and blotted onto nitrocellulose. Relative amounts of *Mtd* transcript were determined by blot analysis using a [32P]-labeled, full-length celery *Mtd* cDNA (Williamson et al., 1995) as a probe under conditions of high stringency (washed in 0.1XSSC at 65°C). Transformant number 1E10, while kanamycin resistant, had little or no MTD activity (A). RNA blot analyses, however, revealed the presence of a ca. 300 nt RNA hybridizing with the *Mtd* probe, suggesting, among other possibilities, the expression of a truncated copy of the *Mtd* cDNA (<). Celery total RNA (celery)(5 µg) was run for comparison. Blots were subsequently reprobed with a rDNA (25S rRNA) probe to assess loading and transfer.

Figure 2. Effect of constitutive expression of celery MTD in tobacco on resistance to the mannitol secreting fungus *Alternaria alternata*. Leaf sections from *Mtd* transformed R₁ (1E3, s) and untransformed (UT) plants were inoculated with *A. alternata* and placed in incubation chambers. (A) Typical symptoms of *A. alternata* infections on leaf tissue from untransformed (B21) and transformed (1E15-?) plants. (B) Number of necrotic lesions per 20 inoculation sites scored 7 days after inoculation. Results shown are the means and S.E.s of results for 3 leaf sections from each plant. (C) Protein (20µg) extracted from tissue from each leaf prior to inoculation was separated by SDS-PAGE and blotted onto nitrocellulose. Blots were incubated with anti-MTD ,as well as an anti-PR1a sera,
and visualized as described in materials and methods. MTD and PR1a cross-reacting proteins are indicated (MTD, PR1a). Total Coomassie staining is shown to indicate relative gel loading; ribulose 1,5-bisphosphate carboxylase (RbC) is indicated.

Figure 3. Induction of MTD in three tobacco cultivars. (A) Leaf discs from tobacco cultivars, K 326, Burley 21 (B21), and Petit Havana (SR1) were treated with either 3mM INA (+) or water (-) for 48 hrs. Tissue samples were collected and undesalted extracts analyzed for MTD activity. (B) K326 leaf discs were treated with either 3mM INA or water for 48 hrs and then analyzed for MTD protein and activity as well as for PR1a protein expression. (C). Detached leaf tissue from R₁ tobacco plants transformed with celery Mtd Mtd (1E3-2,1E15-7, 1E15-4) as well as untransformed (B21) plants was infected with 20-10 µl drops of an Alternaria alternata spore suspension (5 x 10⁴ spores/ml). Tissue was scored for number of lesions formed per 20 inoculation sites at 7 days after inoculation. Figure shows representative tissue from the same plants taken prior to inoculation that was analyzed for MTD protein and PR1a protein presence.

Figure 4. Infection of Mtd transformed and untransformed tobacco plants with the fungal pathogen Cercospora nicotianae. Two fully expanded leaves of sixteen week old tobacco plants were sprayed with a C. nicotianae spore suspension (5 x 10⁴ spores/ml). Development of disease (lesion formation and coalescence) was observed over a two week period. C. nicotianae lesions on (A) Untransformed tobacco plant, (B) 1E6 Mtd transformed plant, (C) 1E15 Mtd transformed plant, (D) 1E13 Mtd transformed plant, 10 days after inoculation

Figure 5. Mtd R₁ transformed (1E6-1, 1E13-1, etc.) and untransformed (B21) tobacco plants inoculated with Cercospora nicotianae. Numbers of lesions per leaf were counted at 7 days after inoculation. Two leaves per plant and three plants per transgenic line were used for each of two experiments and means are shown. Proteins were extracted from tissue prior to infection and analyzed by western blot for presence of MTD and PR1 proteins.
Figure 6. Mannitol accumulation in the presence and absence of plant extracts. HPLC analyses were used to examine soluble sugars in extracts from *Alternaria alternata* mycelia and *Pseudomonas syringae* pv. tabaci cells grown in the presence or absence of aqueous tobacco leaf extract or from leaf tissue from untransformed tobacco (B21) inoculated with *P. syringae* pv. tabaci. A solution containing 2mg/ml each sucrose (S), glucose (G), fructose (F), and mannitol (M) was run as a standard (A). As previously reported (Jennings et al., 1998), although mannitol was present in *A. alternata* in the absence of tobacco leaf extract (B), mannitol production/accumulation increased significantly upon treatment of *A. alternata* cultures with 10% (v/v) tobacco leaf extract (C). In contrast, cells from *P. syringae* p.v. tabaci cultures amended with either 10% water (D) or 10%(v/v) tobacco leaf extract (E), had no detectable internal mannitol. Arrows (↑) indicate position at which mannitol would elute from the column (retention time). Finally, 9mm of tissue centered on and including the *P. syringae* inoculation site was excised and soluble sugars extracted and analyzed by HPLC (F) to determine if *P. syringae* was secreting/producing mannitol *in planta.*
Figure 1

A

MTD Activity (µmol/h/gfw)

0.8
0.6
0.4
0.2
0

B

Mtd RNA

25S rRNA

Transformants (R₀)

B21 (UT) 1E-1 1E-2 1E-3 1E-4 1E-5 1E-10 celery

<
Figure 2
Figure 3

A

MTD activity (µmol/h/g fw)

cultivars

K326  B21  SR1

B

MTD Activity (µmol/h/g fw)

V1-  V1+

C

# Lesions

IE3-2  IE15-7  IE15-4  B21

MTD

PR1a
Figure 5

![Bar Chart](image)

- # of lesions/infected leaf
- MTD
- PR1a
- RbC

[Image Gallery]

80
ACRYLAMIDE GELS

5X SDS sample buffer

- 50 mM Tris-HCl (pH 6.8)
- 100 mM DTT
- 2% SDS
- 0.1% bromophenol blue
- 10% glycerol

Separating Gel (1 gel)

<table>
<thead>
<tr>
<th>Component</th>
<th>11% Gel</th>
<th>13% Gel</th>
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<tbody>
<tr>
<td>water</td>
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<td>3.01 ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>3.67 ml</td>
<td>4.33 ml</td>
</tr>
<tr>
<td>1.5 mM Tris (pH 8.8)</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 ul</td>
<td>100 ul</td>
</tr>
<tr>
<td>10% Am Persulfate</td>
<td>50 ul</td>
<td>50 ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 ul</td>
<td>5 ul</td>
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</tbody>
</table>

Stacking Gel (enough for 2 gels)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
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<tr>
<td>30% acrylamide mix</td>
<td>1.3 ml</td>
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<tr>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 ul</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>50 ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 ul</td>
</tr>
</tbody>
</table>

Tris-glycine electrophoresis buffer

- 25 mM Tris
- 250 mM Glycine (pH 8.3)
- 0.1% SDS

Gels were typically run for 150V hours.
MEDIA RECIPIES

Tobacco Plant Culture Media

4.4g MS salts
pH 5.7 with KOH
add dH₂O to 1 liter
9g TC agar
Autoclave
Cool to 55C in water bath
Add 10 ml/L antimycin
Add 10 ml/L ½ F

For transgenic selection add 100 µl Kanamycin (100mg/ml)

Malt Media for liquid fungal cultures

15 g Difco malt extract
3 g peptone
30 g glucose
bring volume to 1 liter with dH₂O

V8 media for Alternaria alternata spores

300 ml V-8 juice
4.5 g calcium carbonate (CaCO₃)
mix, place in 100 ml glass centrifuge tubes, centrifuge at 2000rpm
for 10 minutes. Decant off clear fluid (discard pellet). Add distilled
water to make 1 liter. For solid media add 15g agar.

NT-1 Suspension Cell Media

4g MS Media
500µl 2,4-D
10ml B-1/inositol stock (10g myo-inositol and 0.1g thiamin HCl/liter)
3ml Millers I (60g KH₂PO₄/liter)
30g sucrose
add distilled water to 1 liter. Autoclave before use.
Buffers

Plant Protein Extraction Buffer

50 mM MOPS  
5 mM MgCl₂  
5 mM dithiothreitol (DTT)  
1 mM EDTA  
.1 mM PMSF

Frozen plant tissue was ground 1:4 with extraction buffer in the presence of sea sand in a mortar and pestle. Extract was placed in a microcentrifuge tube and debris was pelleted. Supernatant was removed and frozen prior to assay.
Protocols

Production of Tobacco Leaf Extract for Fungal Induction Experiments

- 8g of leaf tissue from tobacco cultivar Kentucky 326 plants growing in the greenhouse was harvested in the morning
- Leaves were ground in a Waring blender with 100 ml of distilled water for 3-30 second pulses
- Resulting slurry was filtered through cheesecloth
- Extract was centrifuged repeatedly for 20-30 minutes at 10,000g, 4ºC until little or no material pelleted
- Resulting supernatant was filter sterilized with a .2µm syringe filter and stored at –80ºC until use.

Treatment of Fungal Cultures with Tobacco Leaf Extract

- *Cercospora nicotinae* and *Alternaria alternata* were inoculated into fresh malt extract media at 1mg/ml from previously frozen inoculum
- Fresh cultures were either left untreated, treated with 10%(v/v) sterile distilled water or 10%(v/v) tobacco leaf extract.
  - *Cercospora* cultures were wrapped in aluminum foil to prevent cercosporin production
- All cultures were placed on a shaker at 28ºC in light.
- Cultures were harvested 7 days after inoculation and mycelia spun down at 10,000g for 20 minutes.
- Representative samples of supernatants/culture media were frozen.
- Mycelial pellets were washed 2X with sterile distilled water, vacuum dried and frozen at –80ºC.
Extraction of Internal Soluble Sugars from Fungal Mycelia, Plant Tissue or Bacteria

- .2g of frozen mycelia/leaf tissue/bacteria was ground with 2 ml of 80%(v/v) aqueous ethanol.
- Slurry was placed in 15ml tube and mortar and pestle rinsed with an additional volume of 80% ethanol and combine in same tube.
- Tubes/extracts were incubated at 80ºC for 5 minutes then centrifuge at 4ºC for 5 minutes at 1,000g.
- Supernatants were moved to new tubes and pellets were re-extracted two additional times.
- All resulting supernatants were pooled and frozen at -80ºC overnight.
- Extracts were thawed and evaporated at 50ºC under vacuum then further dried in an oven for 1 hour at 70ºC.
- Resulting residue was dissolved in 1ml deionized distilled water and frozen at –80ºC until HPLC analysis.

Infection of Tobacco Plants with Pseudomonas syringae pv. syringae

- The bacterial pathogen, Pseudomonas syringae pv. tabaci was grown in King’s B (KB) media (King et al., 1954) for 16 hours at which time 500 µl was removed and inoculated into 50ml of fresh KB.
- Cultures were grown at 25ºC overnight and bacteria harvested by centrifugation.
- Bacterial pellets were washed once in distilled water, repelleted by centrifugation and resuspended in water at the indicated concentrations.
- Tobacco plants (untransformed and transformed) were inoculated with P syringae pv. tabaci by infiltration of 10⁹, 10⁸, 10⁷, or 10⁵ c.f.u./ml suspensions in distilled water. Infiltration was into the abaxial side of intact leaves of 12 week-old plants with a plastic syringe without a needle (Glazebrook and Ausubel, 1994). Two inoculation sites per bacterial concentration were made in each leaf, and two leaves were inoculated per plant.
- Symptoms, development of necrosis and tissue degeneration were evaluated 5, 7, and 10 days after infiltration.
Fungal inoculations of tobacco plants

- The fungal tobacco pathogens, *Alternaria alternata* and *Cercospora nicotinae* were grown on sporulation medium (V-8 agar REF) for 7 days.
- Sterile distilled water was added to plates and the resulting spore suspensions collected.
- Spores were quantitated using a hemocytometer and resuspended to the desired concentration in sterile distilled water.

- For *A. alternata* inoculations, a detached leaf assay similar to that of Spur (1973) was used to assess resistance.
  - Three leaves were harvested from 12 week old transformed and untransformed B21 plants grown under controlled conditions.
  - Two 2”x2” squares were cut from each leaf and placed abaxial side up on wire mesh in incubation boxes.
  - Twenty-10µl drops of the *A. alternata* spore suspension (5 x 10^4 spores/ml) were distributed evenly on each leaf section and allowed to air dry.
  - Incubation boxes were then closed and placed in a 20°C incubator with continuous light (??100µEm^-2s^-1).
  - Leaf sections were scored at 5, 7 and 10 days after inoculation for lesion formation. Data for 7 day observations are shown.

- For *Cercospora nicotinae* inoculations
  - two leaves each on 16-week old untransformed and transgenic tobacco plant were sprayed on the abaxial side with a 5 x 10^5 per ml spore suspension of *C. nicotinae*.
  - Spore suspensions were prepared from 5 days old cultures as described above. Leaves were allowed to dry after inoculation and then were placed in plastic bags in a shaded area.
  - Plants were removed from bags after 4 days, placed on greenhouse benches and monitored for development of lesions, and lesion coalescence.
**Construction and transformation of 35S-Mtd chimera.**

- A DNA fragment containing full-length celery Mtd cDNA was excised from clone p5-4 (Williamson et al., 1995) with the restriction enzymes SmaI and SnaBI.
- This blunt-ended fragment was used to replace the GUS reporter gene in a SmaI-EcoRI-cut pBI121 plant transformation vector (Clontech, Palo Alto, CA).
- The resulting clones were screened for desired insert orientation, and sequenced to verify promoter-cDNA borders.
- A suitable chimeric plasmid (p9-41) was identified, isolated and transformed into *Agrobacterium tumefacens*, strain EHA105 (Hood et al., 1993) by direct transformation (An et al., 1988).
- This 35S promoter-Mtd cDNA transcriptional fusion was then introduced into *Nicotiana tabacum* cv. Burley 21 by *Agrobacterium*-mediated transformation as described by Horsch et al. (1985) with later modifications by Daub et al. (1994).
- Kanamycin resistant primary transformants (R₀'s) were screened, and transformants expressing MTD activity were allowed to self.
- The resulting R₁ seed was collected, surface sterilized and germinated on ½ MS (Murashige and Skoog, 1962) containing 100mg/L kanamycin, 200mg/L Carbanicillin and 0.7% TC agar (Sigma).
- R₂ plants were grown from seed of representative R₁ plants that showed high levels of ectopic MTD expression.