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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 antimicrobial defense mechanism (1). Plants produce an analogous, localized oxidative burst (2), wherein massive amounts of antimicrobial 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 antimicrobial activity, H_2O_2 also triggers the hypersensitive response, 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, in which localized infection of a plant confers enhanced systemic resistance to subsequent attack by the same or unrelated pathogens (5, 6). Systemic acquired resistance 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 systemic acquired resistance 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 also is emerging that pathogens suppress ROS-mediated defenses by nonenzymatic quenching of ROS. Mannitol has long been recognized as a potent ROS quencher *in vitro* (13) and has widely been used as a laboratory reagent to scavenge hydroxyl radicals (HO·) generated by the phagocyte respiratory burst or by cell-free oxidant systems (14). *In vivo*, increased mannitol production protects *Saccharomyces cerevisiae* from oxidative injury (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% nucleotide and >90% amino acid) to the *Eli3* pathogen-induced transcripts from parsley and from *Arabidopsis* (19, 20). The dramatic induction of MTD expression in celery-cell suspensions on treatment with SA provided further evidence that MTD and hence mannitol may 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.

Here 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 may 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. Kentucky 326; K326) was obtained from M. Daub (North Carolina State University, Raleigh, N.C.) 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 (North Carolina State University, Raleigh, N.C.) and grown in Murashige and Skoog medium (21) supplemented with 0.5 μg/ml 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were shaken at 100 rpm under constant light (150 $\mu E/m^2 \cdot sec^{-1}$) at 22°C and transferred into fresh medium every 7 days.

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Abbreviations: gfw, gram fresh weight; INA, 2,6-dichloroisonicotinic acid; MTD, mannitol dehydrogenase (mannitol:mannose 1-oxidoreductase, EC no. pending); PR, pathogenesis-related; ROS, reactive oxygen species; SA, salicylic acid.

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Protein Extraction and Enzyme Assays. Proteins were extracted and assayed as described (22), except the extraction buffer contained 0.1 mM phenylmethylsulfonyl fluoride but not Triton X-100 and extracts were not desalted before they were assayed. 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 1-oxidoreductase plant enzyme MTD (EC no. requested), catalyzes the NAD^+-dependent oxidation of mannitol to mannose. In contrast, fungal and bacterial mannitol dehydrogenases (e.g., MTLK and MTLD; EC nos. 1.1.1.67 and 1.1.1.17, respectively) are normally 2-oxidoreductases and catalyze the conversion of mannitol or mannitol phosphate to fructose or fructose phosphate, respectively, often by 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 using SDS/PAGE, blotted onto nitrocellulose, and probed with a polyclonal anti-MTD serum diluted 1:6,000 (18). Serum crossreacting proteins were visualized by using an alkaline phosphatase-linked secondary antibody (Promega). RNA extraction and blot analyses using a ^{32}P-labeled, full-length celery *Mtd* cDNA as a probe were as described (19), except final washes were performed in 0.1× SSC 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, North Carolina State University, Raleigh, NC; ref. 25). All treatments were for 48 hr unless otherwise indicated. Disks were harvested and frozen in liquid N_2 and stored at −80°C. MTD activity was measured as described above. NT-1 tobacco cell suspensions were amended with either 1:50 (vol/vol) fungal elicitor (a gift from R. Dixon, Samuel Roberts Noble Foundation; ref. 26) or an equal volume of sterile distilled water and incubated for 12 hr.

Immunotitration. Protein extracts (50 μl) from leaf discs treated with 3 mM INA for 48 hr were incubated for 12 hr 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·gfw^{-1}.

Fungal Culture Growth and Treatment. Mycelial inocula were prepared as described (27) from *A. alternata* and *Cercospora 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 (*N. tabacum*, cv. Kentucky 326) as described (27) and sterilized by filtration through a 0.2-μm nitrocellulose filter. Fungal mycelia (1 mg/ml, fresh weight) was inoculated into 125 ml of 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 ($^1O_2$) in *C. nicotianae*. Fungal tissue was recovered by centrifugation (6,000 × *g*), 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 of frozen mycelia as described (22), except initial extraction volumes were reduced by 30%. Media and internal soluble sugars were analyzed as described (22) by using a Waters HPLC system equipped with a guard column (C_{18} Corasil, Bio-Rad) and in-line cation and anion guards (Micro-Guard, Bio-Rad). Carbohydrates were separated isocratically on either Sierra Separations (Sparks, NV) carbohydrate, Ca^{2+} (flow rate of 0.5 ml/min water at 75°C) or fast carbohydrate, Pb^{2+} columns (flow rate of 0.8 ml/min water 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 may play a protective role in plant defense, we had transformed tobacco 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 plants 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 crossreacting protein corresponding in size to celery MTD (data not shown). Subsequent analyses revealed that significant MTD 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 does not produce mannitol, 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

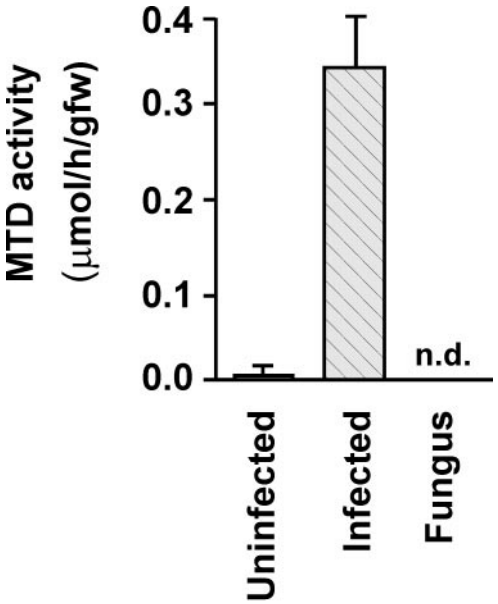

FIG. 1. Fungal infection elicits MTD activity in tobacco. Tissue from uninfected and infected tobacco and an isolated culture of the infecting fungus were collected, frozen in liquid nitrogen, and stored at −80°C. MTD activity was measured in extracts as the rate of mannitol-dependent conversion of NAD^+ to NADH as described in *Materials and Methods*. Error bars represent the SE of three independent observations. n.d., not detected.

MTD activity is a PR response, we incubated tissues and cells of untransformed tobacco with several known inducers of PR proteins. First, leaf discs of tobacco (*N. tabacum*, cv. Kentucky 326, K326) were treated with 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 activity together with a parallel increase in anti-MTD sera crossreacting protein and *Mtd* RNA (Fig. 2*A*). 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. 2*B*). In additional analyses using INA-treated K326 leaf disks, increases in MTD enzyme activity were observed to be rapid and linear with time (Fig. 3*A*), and both MTD activity and anti-MTD serum crossreacting protein(s) increased in a linear fashion with respect to INA concentration (Fig. 3*B*).

The Anti-MTD Crossreacting Protein Is MTD. To establish a functional link between the observed increase in MTD activity and the parallel induction of the anti-MTD sera crossreacting 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 show a decrease in activity, indicating that one or both of the observed anti-MTD sera crossreacting 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 may be expected to elicit changes in both fungal mannitol production and secretion. Two fungal pathogens of tobacco, *C. nicotianae* and *A. 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 or absence of aqueous tobacco-leaf extract for 7 days, after which the amounts of mannitol both in the fungal mycelia (internal) and in the culture filtrate (secreted) were determined by using 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- to 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 (3.7 mg 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 also is found in numerous species of vascular plants (17). Mannitol metabolism in plants primarily has been studied in celery

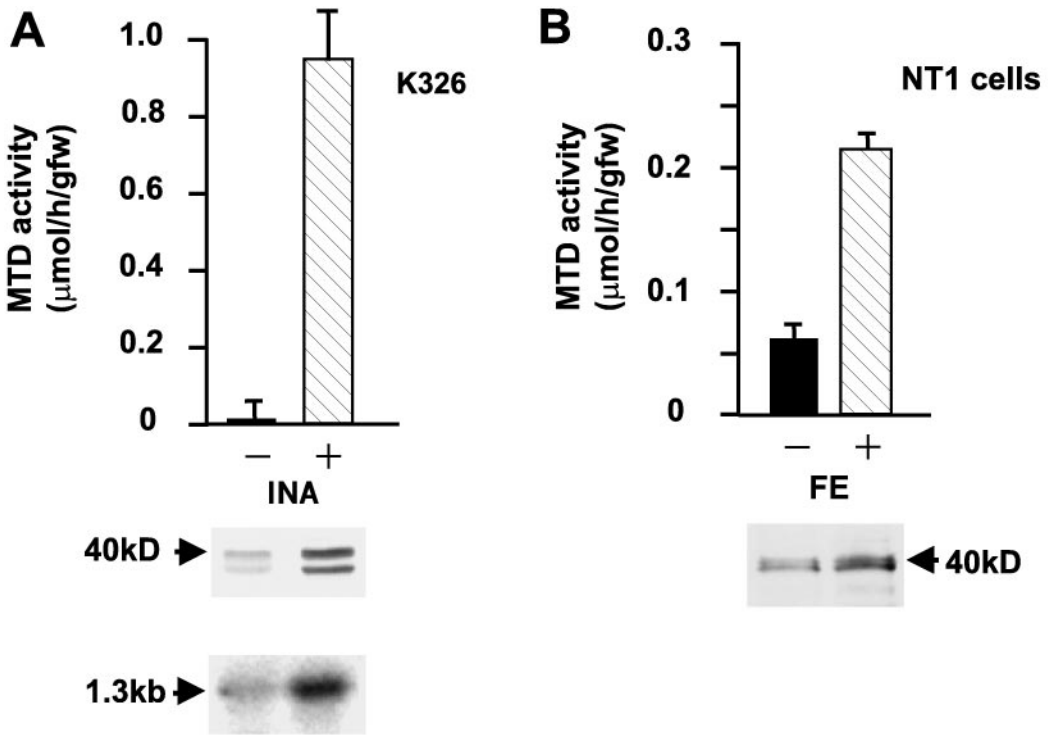

FIG. 2. Induction of MTD in tobacco by treatment with INA or fungal elicitor. (*A*) MTD activity (*Top*), protein (*Middle*), and RNA (*Bottom*) in extracts from tobacco leaf discs, cultivar K326. Discs were floated on sterile distilled water either in the absence (−) or presence (+) of 1 mM INA for 48 hr before extraction. MTD activity was measured in extracts as above. Data represent mean ± SE of three independent experiments. Equal protein (20 μg) 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 described in *Materials and Methods*. Total RNA was also extracted from these tissue samples, and relative amounts of *Mtd* transcript were determined by blot analysis by using a ^{32}P-labeled, full-length celery *Mtd* cDNA (19) as a probe under conditions of moderate stringency (washed in 0.1× SSC 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 12 hr. Cells were collected, and extracts were assayed for MTD activity (*Upper*) and protein (*Lower*) as described above. Data represent the means ± SE of three independent experiments.

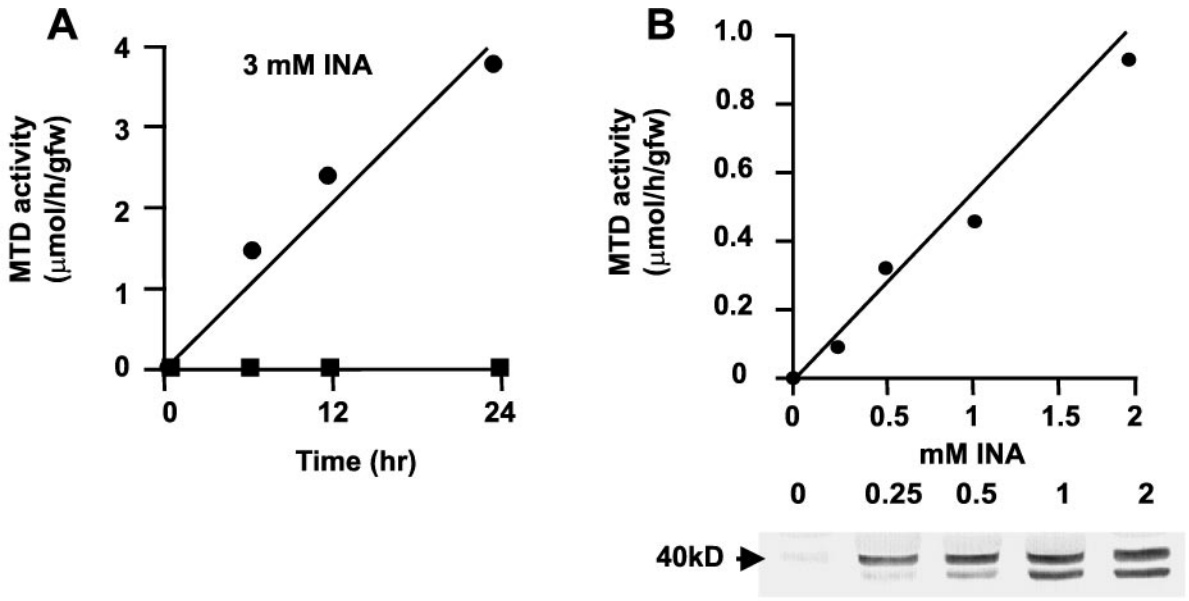

FIG. 3. Changes in MTD activity and protein in response to INA treatment time and concentration. (*A*) MTD activity in extracts from K326 leaf discs treated with 3 mM INA (●) or distilled water (■). Discs were randomly selected at 0, 6, 12, and 24 hr, and MTD activity in extracts was assayed as described 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 (20 μg) 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.

(*Apium graveolens*), where mannitol can comprise up to 50% of the soluble carbohydrate (17). MTD catalyzes the conversion of mannitol to mannose, thus acting as a key regulator of mannitol pool size in celery (18). Initially, it was 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. Antisera raised against purified celery MTD not only crossreacts 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.

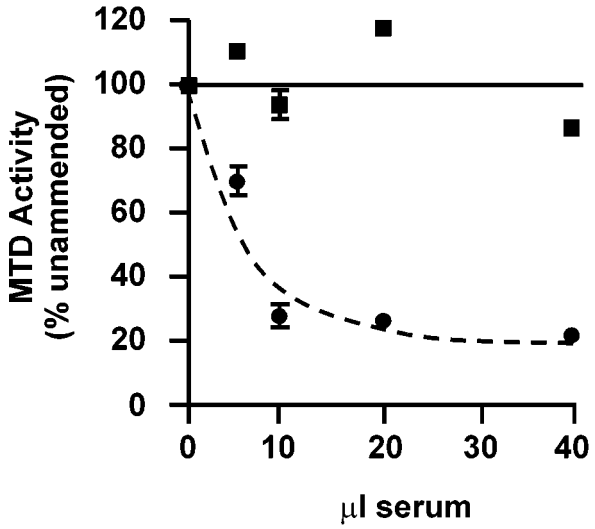

FIG. 4. Immunotitration of MTD activity in extracts from INA-induced tobacco-leaf discs. Leaf-disc extract(s) (50 μl) treated with 3 mM INA for 48 hr were incubated for 12 hr at 0°C with increasing volumes of preimmune (■) or anti-MTD (●) serum. Data are mean ± SE of data from two independent experiments.

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 ROS, most notably H_2O_2, 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 as a 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_2O_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_2O_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. Whereas mannitol in fungi may serve primarily as an osmolyte or as a 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

Table 1. Mannitol accumulation in fungal cultures grown with and without host-leaf extract

Mannitol production	Accumulation, mg/gfw mycelia			
	Alternaria alternata		*Cercospora nicotianae*	
	−L. E.	+L. E.	−L. E.	+L. E.
Internal	5.89 ± 0.08*	7.78 ± 1.1	3.39 ± 0.39	3.97 ± 1.17
Secreted	2.64 ± 0.31	9.45 ± 1.38	ND	ND

*Means of data from two independent experiments ±SE; ND, not detected; L. E., tobacco-leaf extract.

pathogenicity of the tomato pathogen *Cladosporum fulvum* (33). Moreover, Lauter (34) recently reported that tomato has a gene with high homology (70% nucleotide 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 may 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 an ROS quencher during the infection process. In contrast, production and secretion of mannitol by *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 ($^1O_2$), is required for *C. nicotianae* pathogenicity (35). Hence, secretion by *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 *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 *Mtd* homolog *Eli3* in *Escherichia coli* have a measurable ability to catalyze the reduction of several phenylpropanoid-pathway intermediates such as cinnamaldehyde (37, 38). It is possible that these other reported activities catalyzed by MTD may also play a role in plant–pathogen interactions. 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 implicate a specific function for MTD in mannitol degradation.

The work presented here suggests that *Mtd* represents an additional class of nonspecific 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 that 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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