INTRODUCTION

Reactive oxygen species (ROS) produced by both fungal and plant NADPH oxidases (Nox’s) are important components of the plant-pathogen interaction. Characterization of Nox in Alternaria alternata (Morita et al. 2013; Yang and Chung 2012) showed that AaNox expression is critical for virulence and necrotic lesion formation. Plants also produce ROS as a key component of multiple defense responses against these same pathogens (Levine et al. 1994). As a result, necrotrophic pathogens like Alternaria spp. require very effective mechanisms to survive the antimicrobial effects of ROS produced both by themselves and the host plant (Dulermo et al. 2010; Véllez et al. 2008). Although induction of global stress responses via expression of transcription factors such as YAP1 (Lin et al. 2009; Yang et al. 2009) are reported to help Alternaria spp. survive oxidative stress, few single fungal compounds have been shown to provide effective ROS resistance. One of these few, however, is the host-induced, antioxidant/compatible solute mannitol (Chaturvedi et al. 1997; Smirnoff and Cumbes 1989), which is secreted by fungal pathogens during plant infection (Jennings et al. 1998). In fact, A. alternata mutants defective in mannitol production and secretion have been shown to be less virulent than wildtype strains on tobacco (Véllez et al. 2008). Consistent with this proposed role for extracellular fungal mannitol, tobacco plants expressing a mannitol transporter gene (AgMaT2) (Juchaux-Cachau et al. 2007) were found to be more resistant to Alternaria longipes. This is presumably due to the plant’s newly acquired ability to remove fungal mannitol from the site of infection by cellular uptake.

On the other side of this interaction, all plants assessed to date make and/or secrete the mannitol catabolic enzyme mannitol dehydrogenase (MTD) in response to pathogen infection (Blackburn et al. 2010; Cheng et al. 2009; Jennings et al. 1998; Jennings et al. 2002; Williamson et al. 1995), presumably to convert mannitol to the non-quenching sugar mannose. If this hypothesis is so, then increased levels of MTD would be expected to provide increased resistance to mannitol-secreting fungal pathogens. This is, in fact, consistent with our previous demonstration that transgenic tobacco overexpressing MTD was more resistant to A. alternata (Jennings et al. 2002). More recently, we showed that the necrotrophic pathogen Botrytis cinerea also secretes mannitol, and that overexpression of MTD in geranium (Williamson et al. 2013) and the greenhouse tomato ‘Moneymaker’ (Patel et al. 2015) confers significant resistance to B. cinerea. Here, we assessed the effect of MTD overexpression in Solanum lycopersicum ‘NC1 Grape’, an elite tomato breeding line (Gardner and Panthee 2010), on resistance to the early blight fungus, Alternaria solani.
OVEREXPRESSION OF MTD IN THE TOMATO BREEDING LINE ‘NC1 GRAPE’

*Solanum lycopersicum* ‘NC1 Grape’ was transformed using *Agrobacterium tumefaciens* strain GV3101 containing the plasmid pT2N214N8 (Williamson et al. 2013) as described in Patel et al. (2015). Resulting kanamycin-resistant regenerants were screened for the presence of the 35S-Mtd transgene by PCR, using forward primers GAACCTCGCTAAAGACTGG and AAACCTCCTCGGATTCCATT (35S-FWD1 and 35S-FWD2, respectively) and reverse primers GAACCTCGCTAAAGACTGG and AAACCTCCTCGGATTCCATT (nosT-REV1 and nosT-REV2, respectively). A total of fifteen kanamycin-resistant regenerants were screened, of which nine plants had the Mtd transgene.

To assess expression of these transgenes, proteins were first extracted from 0.2 gm tissue from young, fully expanded leaves (5 to 6 cm) of primary transformants (T₀’s) as well as nontransformed (wildtype, WT) and vector-transformed controls (kanamycin resistant but lacking the Mtd transgene) by grinding directly in Bio-Rad (Richmond, CA) SDS sample buffer (1:2 w/v). Protein concentrations were determined by the method of Bradford (1976). Proteins (20 µg) were then separated by SDS-PAGE (Laemmli 1970), blotted onto nitrocellulose, and probed with an anti-MTD serum (1:2,000) (Stoop et al. 1995). Serum cross-reacting proteins were visualized using an alkaline phosphatase (AP)-linked secondary antibody (1:2,400) (Promega Corp., Madison, WI). Of the nine transformants displaying a wildtype phenotype (i.e., displaying no transformation/tissue culture-induced developmental abnormalities), only one expressed high levels of MTD protein (NCG12, Fig. 1); three expressed levels intermediate between wildtype and NCG12 (e.g., NCG19, Fig. 1); and the remaining five expressed MTD at approximately the same level as wildtype (data not shown).

TESTING RESISTANCE TO EARLY BLIGHT

Spores of the fungal pathogen *Alternaria solani* (a field strain isolated from tomato by Dr. Tika Adhikari, Department of Plant Pathology, NC State University, and subsequently identified using species-specific primers, microsatellite analysis, and pathogenicity assays) were stored at −20°C in 15% glycerol. To initiate sporulation, 50 µl of this spore stock was transferred onto potato dextrose agar (PDA) (Benton, Dickinson Co., Sparks, MD) pH 5 and maintained at 25°C with an 8/16-h light/dark photoperiod in a controlled humidity chamber (Percival Scientific, Inc., IA). Significant sporulation was observed 14 to 18 days after plating. Sporulating cultures were overlaid with 10 ml sterile 0.01% aqueous Tween 80 and the resulting spore suspensions collected. Spore suspensions were filtered through two layers of sterile cheesecloth, quantified using a hemocytometer and diluted to desired concentrations.

Seed from selfed, primary transformants (T₀’s) were germinated on medium containing ½MS salts, kanamycin (100 mg/liter), and agar (6 g/liter) at pH 5.6 to produce second-generation tomato seedlings and plants (T₁’s). Seedlings from one high (plant 12) and one intermediate (plant 19) MTD expresser, as well as from a nontransformed control plant, were selected for analysis. This population was still segregating for the Mtd transgene, so only plants/seedlings with the Mtd transgene grew on kanamycin selection. Seedlings (6 to 8 cm in height) were transferred to small plastic pots with soil, and covered with clear plastic covers at 25°C for one week to acclimate. After acclimation, seedlings (12 to 15 cm in height) were arranged in a random complete block design with 8 to 12 plants per experimental unit for 8 replications and inoculated by spraying with *A. solani* spores (10⁶ spores/ml). The spore concentration used was determined in preliminary experiments by inoculating wildtype seedlings with a range of spore concentrations (10⁴ to 10⁷ spores/ml). The lowest spore concentration producing complete infection on WT plants was used for inoculations (in this case, 1.8 × 10⁶ spores/ml). Symptom development was assessed as the percentage of infected leaf tissue area, with the entire seedling leaf area being 100%. Means from 8 to 12 seedlings for each line are shown. Differences in resistance among the three lines on day 7 were compared by ANOVA. Means with different letters are significantly different from each other on day 7 at P < 0.05.

![Figure 1](image)

**FIGURE 1**

Resistance to *Alternaria solani* in progeny of MTD over-expressing breeding lines of *Solanum lycopersicum* ‘NC1 Grape’. MTD protein from T₀ plants of one high (plant 12) and one intermediate (plant 19) MTD-expressing lines, and nontransformed (WT) plants were visually assessed by protein blot analyses. Segregating seedling populations from MTD transformed lines NCG12, NCG19, as well as WT controls were assessed for resistance. Seedlings were arranged, inoculated and scored for resistance as indicated above. (A) Blot analysis of MTD protein in Mtd-transformed and nontransformed (WT) controls. The Ponceau-stained blot is included to document protein loading and transfer. (B) Disease development was scored over a period of 7 days as a relative percentage of the total leaf tissue area, with total plant leaf area being 100%. Means from 8 to 12 seedlings for each line are shown. Differences in resistance among the three lines on day 7 were compared by ANOVA. Means with different letters are significantly different from each other on day 7 at P < 0.05.
seedlings from each of two selfed, independent MTD overexpressing lines (plants 12 and 19), as well as non-transformed (WT) seedling controls, were assessed for A. solani resistance. Progeny from the high MTD-expressing line NCG12 showed significantly less infection than seedlings from the other groups (P < 0.05) (Fig. 1), with infection rates less than 65% and new growth appearing on day 7 post-inoculation. Seedlings from the intermediate MTD-expressing line NCG19 had intermediate resistance, with infection rates less than 75%, while the WT controls had a terminal disease index of greater than 95% by the end of day 7.

CONCLUSIONS AND POTENTIAL APPLICATIONS

In this study, plants expressing high levels of MTD suppressed early blight symptoms caused by the necrotrophic pathogen A. solani. These results are consistent with the hypothesis that mannitol secretion by necrotrophic pathogens like A. solani is important for pathogen survival, presumably by protecting the pathogen from the effects of ROS during infection. We further hypothesize that the timely/immediate removal of pathogen-produced mannitol by the plant-produced MTD exposes fungal structures to the antimicrobial effects of ROS. While Mtd homologs are present in tomato (Lauter 1996), the amount of MTD expressed naturally is typically much lower than in MTD overexpressing plants (Fig. 1A, WT). Further, salicylic acid, INA (2,6-dichloroisonicotinic acid), and fungal elicitors induce MTD expression in other non-mannitol plants (Jennings et al. 1998; Kiedrowski et al. 1992; Williamson et al. 1995) although the induced amounts reported are still lower than in MTD overexpressing plants. If this were also true for MTD induction in tomato, levels might be too low to provide resistance to mannitol-secreting pathogens. The decrease in susceptibility to A. solani in seedlings expressing high constitutive amounts of MTD (NCG12) supports the hypothesis that the interaction of mannitol and MTD forms an important and dynamic interface during infection by mannitol-secreting fungal pathogens. Although we have previously reported that overexpression of an Mtd transgene provides resistance to Botrytis gray mold in a greenhouse tomato, this is the first report of MTD overexpression in an elite breeding variety (NC1 Grape) providing seed-transmissible, whole-plant resistance to A. solani, the causal agent of tomato early blight. In addition, although Mtd gene copy number was not determined for the primary transformants (T₀’s), it should be noted that populations assessed here are segregating, and thus presumably consist of a mix of 1× and 2× the base Mtd gene copy of those primary transformants. Finally, these results suggest that selection of breeding lines with high naturally occurring MTD expression could provide a useful approach for conventional breeding of resistance against early blight.

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LITERATURE CITED


