ABSTRACT

PATEL, TAKSHAY KISHORBHAI. Role of Mannitol Dehydrogenase in Fungal Resistance in Tomato (*Botrytis cinerea* and *Alternaria solani*). (Under the direction of Dr. Dilip R. Panthee and Dr. John D. Williamson).

The oxidative burst is a critical early event in plant-pathogen interactions that leads to a localized, programmed cell death (PCD) called the hypersensitive response (HR). The HR and associated PCD retard infection by biotrophic pathogens, but in fact, enhance infection by necrotrophic pathogens like *Botrytis cinerea* and *Alternaria solani*. In addition to signaling the HR, reactive oxygen species (ROS) produced during the oxidative burst are antimicrobial. In spite of this, pathogens like *B. cinerea* and *A. solani* survive. We hypothesize this is due in part to the pathogen's capacity to secrete the antioxidant mannitol during infection. Previous work supports the notion that overexpression of the catabolic enzyme mannitol dehydrogenase (MTD) can decrease a plants susceptibility to mannitol secreting pathogens. To extend the above hypothesis, and test the general utility of this approach in an important horticultural crop, we overexpressed a celeryMTD in tomato (cv. 'Moneymaker' and 'NC1 grape'). In these studies, we observed a significant increase in resistance to *B. cinerea* and *A. solani* in transgenic 'Moneymaker' and 'NC1 grape'tomatoes, respectively, expressing high amounts of MTD. Although *Mtd*gene homologs are naturally present in non-mannitol producing plants such as tobacco, tomato and Arabidopsis, expression of these endogenous MTD homologs is usually low. This, together with results presented here, suggests that in addition to transgenic strategies, selection of breeding lines with naturally high MTD expression could provide a useful approach to conventional breeding for resistance against *B. cinerea* and *A. solani*.

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Role of Mannitol dehydrogenase in Fungal Resistance in Tomato (*Botrytis cinerea* and *Alternaria solani*)

by
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DEDICATION

I dedicate my work to Bhagwan Krishna, whose words encouraged the best in me. Also I dedicate it to my parents Kishor and Leela for their constant support. Finally I dedicate it to my Love Sakshi, for patiently being with me during this work.

BIOGRAPHY

Takshay Patel, son of Kishor and Leela Patel, born on November 7, 1988 in Gujarat, India. Takshay is loving brother of Rutika, and eldest of the two children. Takshay was raised in Nashik, Maharashtra, where his father runs a real estate business and mother is caring housewife. Takshay graduated in 2009 from Pune University with a Bachelor of Science in Biotechnology. He further completed his Bachelor of Science in Plant Biotechnology at Pune University in 2010. In 2012 Takshay started his MS program in Horticultural Science.

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I took my first steps towards understanding plants and I am grateful to all the people who made these steps stronger.

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CHAPTER 1
REVIEW OF LITERATURE

Title
Mannitol in plants and fungi, and its role in plant-pathogen interactions

ABSTRACT

The occurrence of mannitol in organisms as diverse as plants and fungi clearly demonstrates the importance of this compound, yet our understanding of such fundamental processes as mannitol transport in plants and the interaction of mannitol metabolism in plants and fungal pathogens is still developing. In spite of great inroads in understanding the importance of mannitol and its metabolic roles during salt, osmotic and oxidative stresses in both in plant and fungi, our current understanding of mannitol's specific functions in reactive oxygen chemistry is still in its infancy. Finally, among the exciting areas of research addressed here, characterizing the dynamic interface between fungal mannitol synthesis and secretion and plant mannitol catabolism and protein transport during infection is a novel example of the complex interactions that can develop during the coevolution of plants and their pathogens.

INTRODUCTION

MANNITOL IN PLANTS

Polyols or sugar alcohols are structurally the reduced forms of aldose or ketose sugars where a hydroxyl group has been chemically reduced to a carboxyl group. Mannitol is one such polyol that is structurally related to the aldohexose mannose and is perhaps one of the

most abundant soluble carbohydrates in the biological world. Mannitol is found in algae, fungi, lichen and higher plants (Lewis and Smith, 1967). In fact, mannitol is one of the most common polyols in higher plants being found in more than 100 plant species. It is a major translocated carbohydrate in plants such as celery, carrot, olive, coffee, and parsley (Lewis and Smith, 1967). Mannitol is always found in combination with at least one other major carbohydrate. In celery (*Apium graveolens),* for instance*,* fixed carbon is partitioned between sucrose and mannitol, with up to 50%of the plants photoassimilate accumulating as mannitol (Stoop et al., 1996, Rumpho and Edwards, 1983).

Mannitol metabolism has been most extensively studied in celery, where it is found in source, sink and phloem tissues, in amounts ranging from a trace in root tips to 30 mg/g fresh weight in mature petioles (Stoop and Pharr, 1992). Although mannitol is found throughout the plant, its anabolic (synthesis) and catabolic (utilization/ breakdown) pathways are compartmentalized and highly regulated in response to tissue age, metabolic state, as well as biotic and abiotic stresses. For instance, the ability to synthesize mannitol increases with leaf age, but only young leaves and other sink tissues catabolize mannitol (Davis and Loescher, 1990; Davis et al., 1988;Stoop et al., 1996). In mature source leaves mannitol synthesis occurs primarily in the cytosol of mesophyll cells where photosynthetically fixed carbon in the form of fructose-6-P (Fru-6-P) is partitioned between sucrose and mannitol (Figure 1). During this process Fru-6-P is converted sequentially to mannose-6-P (Man-6-P), mannitol-1-P (Mtl-1-P) and mannitol (Mtl) by the enzymes phosphomannose isomerase (PMI), Man-6-P reductase (M6PR) and Mtl-1-P phosphatase (M1PP), respectively (Rumpho et al., 1983, Stoop et al., 1996). In sink tissues mannitol catabolism is initiated by the conversion of

mannitol directly to mannose by the 1-oxidoreductase mannitol dehydrogenase (MTD). Mannose is then converted to Man-6-P and finally to Fru-6-P by the enzymes hexokinase (HK) and PMI, respectively (Stoop et al., 1996).

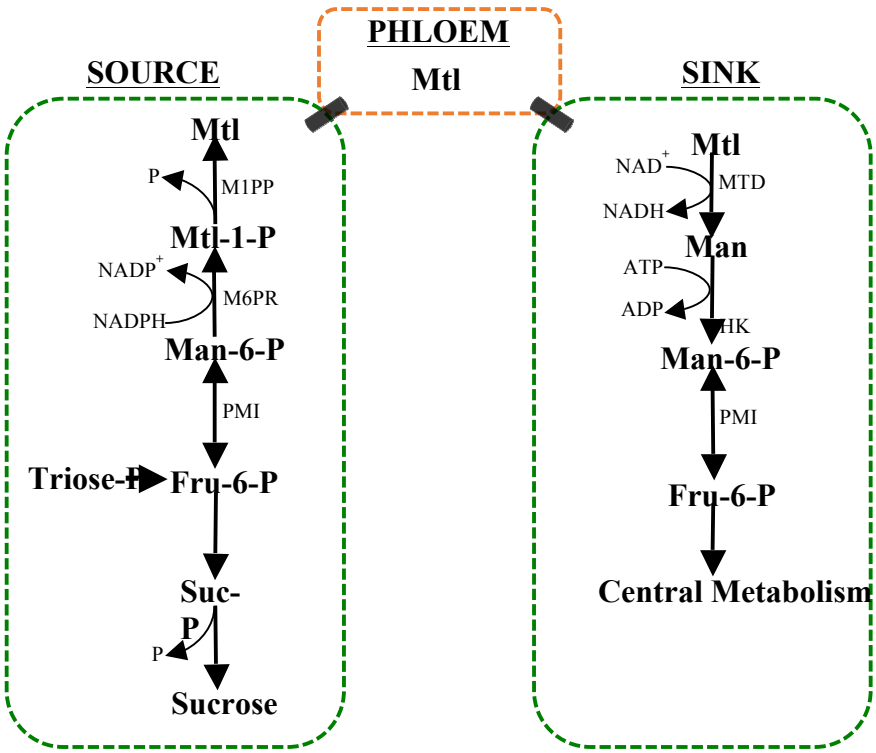

Figure 1: Mannitol metabolism in plants.Fru-6-P: fructose 6-phosphate; Man-6-P: mannose 6-phosphate; Mtl-1-P: mannitol 1-phosphate; Mtl: mannitol; Man: mannose; Suc-P: Sucrose Phosphate; PMI: Phosphomannose isomerase; M6PR: Mannose-6-phosphate reductase; M1PP: Mannitol-1-phosphate phosphatase; MTD: Mannitol dehydrogenase; HK: Hexokinase. ▬ :Mannitoltransports- *AgMat1, AgMat2, AgMat3, OeMat.*

Mannitol synthesis occurs primarily in source tissues (mature leaves) whereas its utilization or catalysis occurs in sink tissues (young leaf and root) requiring metabolite

transport. This translocation of mannitol from source to sink is assumed to occur through the phloem, as mannitol amounts as high as 730 mM(Nadwodnik and Lohaus, 2008) are found in celery phloem. As might be expected, the ability of a leaf to synthesize and transport mannitol is coordinated, as both the activities increase during the developmental transition from sink to source in leaves (Stoop et al., 1996).

Although mannitol metabolism is relatively well characterized, the mechanisms of mannitol transport including loading and unloading in phloem, as well as mechanisms of movement through mesophyll cells to the vascular bundle in source tissue and vice versa in sinks are still being investigated. Initial hypotheses from celery studies suggested that mannitol is actively loaded into phloem (Davis et al., 1988). This hypothesis is consistent with the high mannitol concentration observed in phloem sap as compared to concentrations in apoplast and cytosol of celery (Nadwodnik and Lohaus, 2008).

The recent identification of mannitol transporters AgMaT1, AgMaT2 and AgMaT3 in celery (Noiraud et al., 2001; Juchaux-Cachau et al., 2007; Landouar-Arsivaud et al., 2011) and OeMaT1 in olive (Conde et.al, 2007) has provided a clearer, although still incomplete, picture of mannitol transport in phloem. Expression of both AgMaT1 and AgMaT2 in yeast resulted in mannitol transport at acidic pH. Further, transport was inhibited by the proton ionophore carbonylcyanide m-chlorophenylhydrazone, suggesting active mannitol/H^+ cotransport. Finally, as shown in Table 1, localization of AgMaT1and AgMaT2 by RNA *in situ* localization and protein immunolocalization supports their role in phloem loading. AgMaT2 RNA accumulation, for instance, was low in young leaf tissue but high in mature leaves, consistent with a role in mannitol export (loading) from source leaf. More

Table 1: Expression and localization of mannitol transporters in various cells/tissues.

Cell/Tissue/ Organ	AgMaT1[a] (RNA) ML standard 100%	AgMaT2[b]		
		RNA	Protein	
			Anti –AgMaT2 Ab	AgMat2 Protein in EM
YL Petiole	ND			
CZ	ND	Present	Present	ND
PC	ND	Present	Absent	ND
XY	ND	Absent	Absent	ND
YL Veins	**40%**			Plasma membrane of Sieve elements, companion cells and Phloem Parenchyma cells
CZ	ND	Present	Present, less than ML	
PC	ND	Absent	Absent	
XY	ND	Absent	Absent	
ML Petiole	**40% PH 15% storage PC**			Plasma membrane of Sieve elements, companion cells and Phloem Parenchyma cells
CZ	ND	Present	Present	
PC	ND	Absent	Absent	
XY	ND	Absent	Absent	
ML Veins	**100%**			Plasma membrane of Sieve elements, companion cells and Phloem Parenchyma cells
CZ	ND	Present	Present More than YL	
PC	ND	Absent	Absent	
XY	ND	Absent	Absent	
Roots	**15%**	Stele phloem zone		
		Low level in cortical parenchyma		
Floral stalk		Present in CZ	Phloem cells	

ML- mature leaf, YL- young leaf, CZ- conducting zone, PC- parenchyma cells, XY- xylem, PH- phloem, ND- not determined, EM- electron microscope, Ab- antibody. AgMat1- Only data on RNA in tissues is available.[a]Noiraud et al. (2001);[b] Juchaux-Cachau et al. (2007).

specifically, immunoelectron microscopy showed that AgMaT2 protein was localized in the plasma membranes of sieve element (SE), companion cells (CC) and phloem parenchyma cells (PPC) (Juchaux-Cachau et al., 2007).

In contrast to AgMaT1 and 2, the third celery mannitol transporter identified by Lemoine's group (AgMaT3) is apparently salt induced and is found in storage parenchyma and phloem of petioles (Landouar-Arsivaud et al., 2011). OeMaT1, currently the only known olive mannitol transporter, is homologous to AgMat2 and is likewise thought to cotransport mannitol/H^+ at acidic pH (Conde et.al, 2007). In suspension culture cells of olive, OeMaT1 transports mannitol under normal growth conditions, and is also further induced by salt.

Finally, radioactive tracer studies together with mannitol concentration gradient analyses suggest that passive mannitol transport also occurs in plants, and that mannitol is transported symplastically in source leaf ground tissues (mesophyll) (Reidel et al., 2009). Putting the pieces together then, mannitol appears to be translocated both symplastically (passive from mesophyll to phloem tissues) and apoplastically (active phloem loading) depending on the cell and tissue type. After synthesis in the mesophyll cells of source tissues, mannitol moves symplastically to the phloem parenchyma cells where it is then actively loaded in phloem. Mannitol is then translocated to sink tissues and unloaded.

Mannitol is also translocated intracellularly between the cytosol and the vacuole or chloroplast. Subcellular compartmentalization and transport of mannitol has been studied in celery, parsley and snapdragon (Keller and Matile, 1989; Moore etal., 1997; Greutert et al., 1998; Nadwodnik and Lohaus, 2008). Keller and Matile's (1989) initial assessment of mannitol localization in temporary sink tissues such as storage parenchyma cells of celery petioles showed that 81% of cellular mannitol was in vacuoles. Later uptake studies using ^{14}C-mannitol by Gruetert et al. (1998) led to the conclusionthat mannitol is transported across the vacuole tonoplast by facilitated diffusion. In mesophyll cells of celery leaves the

mannitol content of vacuole, chloroplast and cytosol is about 72, 16 and 12 percent respectively (Nadwodnik and Lohaus, 2008), while in mesophyll cells of snapdragon and parsley mannitol is distributed about equally among cytosol, chloroplast and vacuole with total amounts varying according to leaf age (Moore etal., 1997). Gruetert et al. (1998) proposed a model of facilitated diffusion of mannitol into vacuoles, with aquaporins acting as non-specific transporters. To date specific vacuolar mannitol transporters have notbeen reported.

Roles for mannitol metabolism in mannitol producing plants

Efficient carbon use

One of the first characterized roles of mannitol in plants was in carbon metabolism. Plants that make mannitol have higher photosynthetic rates as measured by rates of carbon fixation. For example, celery fixes carbon up to 60 mg CO_2 /dm^2/hour in leaves (Fox et al., 1986). This is not only an unusually high carbon fixation rate for a C3 plant, but is comparable to rates in a number of C4 plants. A current hypothesis (Pharr et al., 1995a) is that this higher carbon fixation might be due to mannitol acting as a carbon and energy sink in addition to or in parallel with sucrose, ultimately increasing the total carbon demand in the cytosol.

In addition to mannitol synthesis providing an additional carbon sink, mannitol catabolism is also more energetically efficient. Both mannitol and sucrose are converted to Fru-6-P for entry into central metabolism, after which they share the same chemical reactions. As discussed earlier, the conversion of mannitol to Fru-6-P yields one NADH and

uses one ATP (for a net gain of 2 ATP), whereas sucrose conversion uses two ATPs (Figure 2). In addition, sucrose, a disaccharide, yields 2 Fru-6-P molecules, while the equivalent complete oxidization of two molecules of mannitol yields4 more ATPs than complete catabolism of sucrose. Therefore, catabolism of mannitol along with sucrose would be an advantage in actively growing sink tissues where demand for both energy and carbon is high (Stoop et al.1996; Pharr etal., 1995). To test this hypothesized mannitol catabolic efficiency Pharr et al. (1995) compared dry weight gains of celery cells using sucrose or mannitol as the sole carbon source. In these *in vivo* experiments, they found that 2.93mg of mannitol was required for a milligram of dry weight increase, while 3.72mg of sucrose was required for the same gain, suggesting that mannitol utilization was 27% more efficient than sucrose for growth.

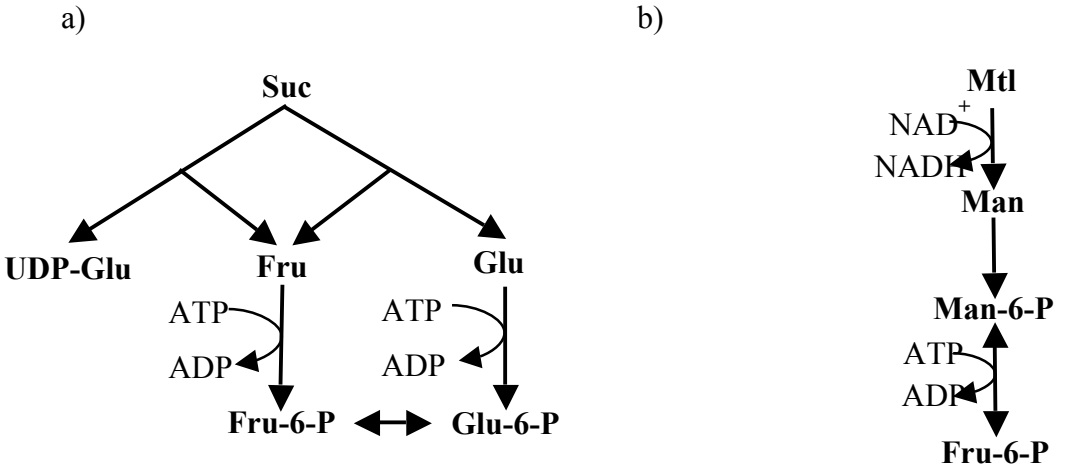

Figure 2: Sucrose vs. Mannitol metabolism. A) Sucrose utilizes 2 ATP to yield 2 Fructose 6-P molecules. B) Two mannitol molecules utilize 2 ATP and give 2 NADH to yield 2 Fructose 6-P molecules. Suc: Sucrose; Glu: Glucose; Fru: Fructose; Fru-6-P: Fructose 6-phosphate; Glu-6-P: Glucose 6 –phosphate; Mtl: mannitol; Man: mannose; Man-6-P: mannose 6-phosphate.

Salt and drought stress

In addition to its impact on metabolic efficiency, mannitol has well documented roles in mediating tolerance to several abiotic stresses. These include salinity and drought, of which salt stress is the best studied. The initial observation that mannitol-synthesizing plants like celery were tolerant to salt concentrations as high as 30% that of seawater (Pharr et al., 1995a) led to extensive assessment of mannitol's potential role in salt tolerance.

Salt stress induces a complex set of physiological responses in plants. One of the first effects of both salinity and drought on plant cells is hyperosmotic stress or a decrease in the water content of cells, initially in the roots (Parida and Das, 2005; Mahajan and Tuteja, 2005). Celery, when grown under salt stress, shows a number of typical responses including reduced fresh weight, leaf growth rate, leaf length, leaf size, and petiole area (Stoop et al., 1996; Everard et al., 1994; Pharr and Stoop, 1994; Landouar-Arsivaud et al., 2011). Everard et al. (1994) and Landouar-Arsivaud et al. (2011) also observed a decrease in leaf emergence and senescence of older leaves. The first response to salinity is an outward movement of water from the roots due to osmosis. This leads to the synthesis of ABA in the roots, transport of ABA to the shoots and ultimately initiates closure of stomata to decrease transpiration (Parida and Das, 2005). Stomatal closure then leads to decreased CO_2 exchange, a subsequent decrease in carbon fixation and thus slower growth in a typical plant that does not produce mannitol. Although there was a fresh weight decrease in salt stressed celery due to outward movement of water, no significant decrease in dry weight was recorded even at 300 mM salt (Everard et al., 1994). This observation implies that the total photo-assimilation in celery was unaffected by salinity. In celery, this is associated with an increase in the

relative concentration of mannitol versus sucrose (Everard et al., 1994; Pharr and Stoop, 1994; Landouar-Arsivaud et al., 2011). Everard et al. (1994) also observed that increase in carbon partitioning to mannitol was due to an increase in the total activity of mannitol biosynthetic enzyme mannose-6-phosphate reductase (M6PR). This increase in M6PR activity is consistent with the observation that, while there is normally little to no M6PR activity in sink tissue (young leaves), mannitol synthesis in sink tissues increased 7-fold under salt stress (Everard et al., 1994). Under these same conditions, however, there was little significant increase in M6PR activity observed in mature (source) leaves.

Coincident with this increase in mannitol synthesis, plants like celery also decrease the usage of mannitol in sink tissues by decreasing production of the catabolic enzyme MTD (Stoop and Pharr, 1994). Specific down-regulation of *Mtd* RNA accumulation and *Mtd* gene expression in the presence of salt were demonstrated in cultured celery and olive cells (Williamson et al., 1995; Zamski et al., 2001,Conde et al., 2007). As a result, in celery plants under salt and drought stress, mannitol content in tissues such as leaves and roots should increase significantly (Stoop and Pharr, 1994). Finally, in addition to decreased *Mtd* expression, olive cells and celery grown under salt or osmotic stress increased expression of a mannitol transporter OeMaT1and AgMaT3 respectively (Conde et al., 2007; Landouar-Arsivaud et al., 2011). Together these observations suggest that total mannitol content and localization in celery and olive is determined by balancing mannitol synthesis, mannitol use, and mannitol transport in different tissues with response to salt and drought stress.

The resulting increase in mannitol is hypothesized to afford increased salt and drought stress tolerance. This is consistent with the observation that both celery and olive

cells grown in culture with mannitol as the sole carbon source are more resistant to growth inhibition by NaCl than cells grown in media with sucrose (Stoop and Pharr, 1996; Conde et al., 2007). The effect of drought/osmotic stress induced by polyethylene glycol (PEG) was likewise reported to be less severe in mannitol-grown olive cells than in cells growing in sucrose (Conde et al., 2007).

To more directly assess the potential role(s) of mannitol in salinity and drought tolerance, a bacterial mannitol biosynthetic gene (*mtlD*) has been expressed in various plants that do not produce mannitol including tobacco (Tarczynski et al., 1993; Karakas et al., 1997), wheat (Abebe et al., 2003), potato (Rahnama et al., 2011) and *Arabidopsis* (Thomas et al., 1995). In all cases *mtlD* expression resulted in increased salt and drought tolerance in plants ectopically producing mannitol compared to their wild type (WT) counterparts. With transgenic *Arabidopsis,* germination in a saline environment was also enhanced when compared to germination of WT plants (Thomas et al., 1995).

How does mannitol protect against salt and osmotic stress?

Mannitol as an osmolyte/osmoprotectant

Although mannitol clearly does protect against salt and drought stresses, the precise mechanism remains unknown, which is possibly due to salt and drought affecting a plant in a variety of ways. Among these effects are the aforementioned "simple" changes in water and osmotic potential. Perhaps because of its use in cell culture to adjust osmotic potential (Edwin et al., 2008) mannitol has been hypothesized to be a simple osmolyte *in vivo*. This is also consistent with the high concentrations of mannitol found in some salt tolerant plants

like celery and olive. However, mannitol concentrations and water and osmotic potentials measured in mannitol-producing, salt-tolerant transgenic callus and transgenic plants under both normal and salt stress conditions found that the amount of mannitol present *in vivo* was not sufficient for mannitol to function purely as an osmolyte (Rahnama et al., 2011; Karakas et al., 1997). These findings suggest that,rather than acting strictly as an osmolyte,mannitol can act as an osmoprotectant. This hypothesis is supported by the observation that celery cells grown in media with mannitol as the sole carbon source had higher growth rates under salt stress than cells using sucrose as the sole carbon source, even though the measured internal osmolality of the cells was identical (Pharr et al., 1995b).

Mannitol as a compatible solute/antioxidant

How mannitol functions as an osmoprotectant without being an osmolyte is the subject of at least two major hypotheses. One is that mannitol functions as a compatible solute. Organisms under stress can increase the cellular concentration of a number of compounds that are 'compatible' with normal metabolic and biochemical processes, even at high concentrations. Common compatible solutes include sugars, polyols, amino acids and amino acid derivatives. Most of these are also osmotically active compounds, and so can be easily assumed to be simple osmolyte. The term "compatible solute", however, is a physiological term that does not describe a protective mechanism (Bohnert and Shen, 1999). There are two proposed mechanisms by which a compatible solute protects cells and cellular structures under stress. Both of these hypotheses propose that the molecular structure of

compatible solutes promotes interaction with the hydration shell around proteins and other structures to protect and stabilize them under conditions of low osmotic potential.

Another widely accepted hypothesis is that mannitol protects against salt and drought stress by acting as an antioxidant. Decreased water potential can result in oxidative stress through formation of reactive oxygen species (ROS) in plant cells (Parida and Das, 2005). Salt-induced production of ROS in plants is also a potentially destructive effect of saline conditions (Bernstein et al., 2010; Leshem et al., 2007; Dat et al., 2000). The hypothesis that mannitol ameliorates these effects in plants is the result of several studies over the last two decades. For instance Smirnoff and Cumbes (1989) reported that up to 60% of the ·OH radicals generated in an *in vitro* assay were quenched by 33mM mannitol. Another *in vitro* study showed that in a system using isolated chloroplast extract, 125mM mannitol was required to provide complete protection of an indicator protein (phosphoribulokinase) from internally generated ·OH(Shen et.al 1997b). Further support for the antioxidant hypothesis was provided by *in vivo* experiments by Shen et.al (1997a) that showed that transgenic plants expressing a bacterial mannitol biosynthetic gene (*mtlD*) whose product was targeted to the chloroplast showed an increased resistance to the ROS generator methyl viologen in the light.

The above observations were interpreted as evidence that mannitol protects against the effects of ROS by acting as a specific ·OH "scavenger" and perhaps as a result also protects against salt and drought stress. This is also partly based on observation that mannitol has a higher reaction rate constant with ·OH in *in vitro* assays than other compatible solutes. For instance Buxton et al. (1988) determined that mannitol has a four-fold higher rate constant than proline for ·OH radicals, hence mannitol is a better quencher than proline.

However, measured levels of mannitol in transgenic plants seem, as with the osmoticum theory, are too low to produce the observed results as a result of ROS quenching. For mannitol to be an effective quencher at these concentrations, it would need to be the preferred target for reactive oxygen rather than just a quencher. Unfortunately, ·OH radicals are so reactive that they react with essentially any molecule inside the cell at a rate so high it exceeds the rate of diffusion. Thus, the relatively small differences in reaction constants determined for various carbon containing molecules do not give rise to specific ·OH radical quenching *in vivo*. Instead, as detailed by Czapski (1984), a ·OH radical produced inside a cell would react essentially instantaneously with any neighboring molecule. Thus for a molecule to be the primary ·OH quencher in a cell it must be present at such high concentrations that it is the first molecule encountered by ·OH. For mannitol, Czapski (1984) calculated that a concentration of 1.7 to 3.5M would be required for 90% protection of the cell. This, of course, assumes that the concentration of a quencher must be homogenous inside the cell to protect against ·OH. We hypothesize that this might, however, not be required, if a quencher is present at a high concentration in the close vicinity of key ·OH target molecules or at the site of ·OH production. In these situations the quencher molecules would react with and thus "quench" ·OH before it damaged critical cellular components. Thus, there are at least two possible mechanisms that require further research to distinguish: first, when a cell produces ·OH radicals they will react with protein, lipids and other structures unless a quencher is present at high enough concentration to effectively be the dominant organic molecule present either homogenously or localized around the target cell structures. However, as observed in transgenic plants expressing *mtlD* the overall

concentration of mannitol is not high enough to be an effective "quencher' if it were present homogenously inside the cell. Another logical scenario, however, might be that mannitol is concentrated primarily around key cellular structures or molecules. In this manner mannitol is effectively present at extremely high concentrations in these specific locations. Therefore, although the hypotheses that mannitol acts either as a compatible solute by forming a protective sphere of hydration around molecules or as an antioxidant might initially seem mutually exclusive, infact, both could be occurring simultaneously.

MANNITOL IN FUNGI

In addition to being a significant photoassimilate in many plants, mannitol is also a common metabolite in microorganisms. Mannitol is especially prevalent in fungi and can be found in ascomycetes, basidiomycetes, fungi imperfecti and zygomycetes (Solomon et al., 2007).

Mannitol is common in fungi, but its role in metabolism and function(s) are not fully understood. Hult and Gatenbeck (1978) initially proposed that mannitol metabolism occurred as a cycle (Figure 3) in *Alternaria alternata*. The cycle involved the mannitol-1-phosphate dehydrogenase (M1PDH), mannitol-1-phosphatase (mannitol 1-P-phosphatase or MPP), mannitol dehydrogenase (MtDH) and hexokinase (HK), in which each catalyzed a step in a four-step cycle. In this model the enzymatic reaction catalyzed by M1PDH was proposed to be the major anabolic reaction while MtDH was the major catabolic reaction. The model was based on measurement of metabolic fluxes of intermediates and enzyme activities. The mannitol cycle was depicted as a branch from glycolysis starting with fructose-6-phosphate.

The proposed role of the mannitol cycle was the regeneration or production of NADPH along with the production of mannitol as a precursor to storage compounds (fats) and the toxin alternariol.

Although the model of Hult and Gatenbeck found wide acceptance, other investigations into mannitol metabolism at the time did not agree completely with the proposed cyclic nature of mannitol metabolism. Strandberg (1969) reported that when *Aspergillus candidus*was grown on media with mannitol as the sole carbon source, M1PDH activity was approximately six times that of MtDH. According to the proposed mannitol cycle the specific activity of MtDH should have been higher than M1PDH if mannitol was the sole carbon and energy source. Further, studies in *Sclerotinia sclerotiorum* showed that MPP has broad substrate specificity and is active when fructose-6-P, glucose-1-P, glucose-6-P, glucitol-6-P, fructose-1-P, fructose-1,6-P_2, and mannose-6-P are used as substrates, although the specificity is less than that for mannitol-1-P (Wang and Tourneau, 1972). Broad MPP substrate specificity suggests that reactions other than (or in addition to) those proposed for the mannitol cycle are possible. Interestingly, although both plants and fungi make and utilize mannitol, the pathways are quite different. For instance, in plant the catabolic enzyme MTD is a 1-oxidoreductase, while fungal MtDHs, are 2-oxidoreductase, producing mannose and fructose as products, respectively (Table 2).

Table 2: Mannitol metabolism enzymes in Fungi and Plants.

Enzyme	EC number	Substrate	Cofactor
Mannitol-1-phosphate dehydrogenase(M1PDH)[a,b]	1.1.1.17	Fru-6-P,Mtl-1-P	NAD^+
Mannitol-1-phosphate phosphatase(MPP)[b,c]	3.1.3.22	Mtl-1-P	
Mannitol-2-dehydrogenase(MtDH)[b]	1.1.1.138	Mtl, Fru	$NADP^+$
Mannitol dehydrogenase(MTD)[d]	1.1.1.255	Mtl	NAD^+
Phosphomannose isomerase(PMI)[c]	5.3.1.8	Man-6-P, Fru-6-P	
Mannose-6-phosphate reductase(M6PR)[e]	1.1.1.224	Man-6-P	NADPH

Fru-6-P: fructose 6-phosphate; Mtl-1-P: mannitol 1-phosphate; Mtl: mannitol; Fru: Fructose; Man-6-P: mannose 6-phosphate; Man: mannose. [a] Wang and Tourneau,(1972); [b] Hult and Gatenbeck (1978); [c] Rumpho et al.(1993); [d] Stoop and Pharr (1992); [e] Loescher et al. (1992).

Although much remains unknown, newly developed technologies have shed light on several major questions. For instance, gene expression (mRNA) and protein analyses found that MtDH and M1PDH are expressed and accumulate in different parts of *Aspergillus niger* (Aguilar-Osorio et al., 2010). MtDH protein and RNA were found only in spores, while M1PDHexpression was detected only in vegetative mycelia. The authors interpreted this result as a support for the absence of the mannitol cycle in *A. niger.* Even more conclusive evidence for the absence of a classical mannitol cycle has been provided by the elegant use of targeted gene disruption (knockout) of*Δm1pdh*and*Δmtdh*, in *Stagonospora nodorum*, *A. alternata* and *Botrytis cinerea* (Solomon et al., 2005; Vélëz et al., 2007; Dulermo et al., 2010). Single targeted knockouts of *Δm1pdh*resulted in much less accumulation of mannitol than WT strains (Table 3), while the mannitol content of a *Δmtdh* strain was much less affected. Further, *Δmtdh* strains can grow on mannitol as the sole carbon source showing that

Table 3: An overview of roles of mannitol metabolism genes in different fungi.

Species (gene disrupted)	Mannitol content	Ability to use mannitol	Glucose to mannitol	Fructose to mannitol	Sporulation	Pathognicity
S. nodorum (*Δm1pdh*)[a]	+	ND	++	++++	+	++++
A. alternata (*Δm1pdh*)[a,b]	+	+	ND	ND	++++	+
A. alternata (*Δmtdh*)[a,b]	+++	++++	ND	ND	++++	+++
B. cinerea (*Δm1pdh*)[d]	++	++++	++	++++	ND	++++
B. cinerea(*Δmtdh*)[d]	++++	++++	++++	++	ND	++++
A. alternata(*Δm1pdh, Δmtdh*)[a,b]	+	+	ND	ND	++++	+
B. cinerea(*Δm1pdh, Δmtdh*)[d]	++	++++	+	+++	ND	++++

All the values are relative to Wild type (++++) showing decreased activity when specific genes are disrupted. ND- not determined.[a] Solomon et al. (2005); [b] Vélëz et al. (2007); [c] Vélëz et al. (2008); [d] Dulermo et al.(2010).

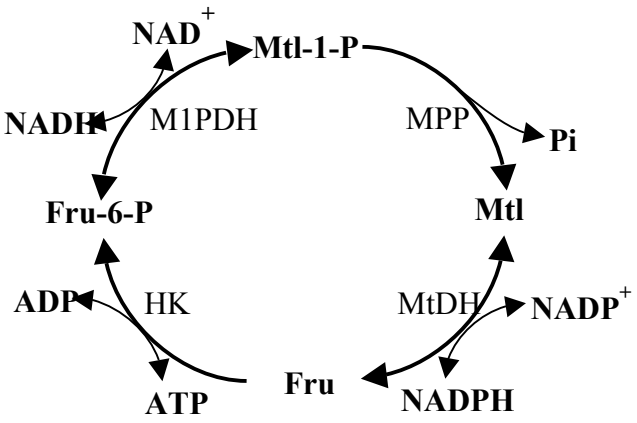

Figure 3: Proposed mannitol cycle in *Alternaria alternata* by Hult and Gatenbeck. HK: hexokinase; M1PDH: mannitol-1-phosphate dehydrogenase; MPP: mannitol-1-phosphate phosphatase; MtDH: mannitol-2-dehydrogenase; Pi: Inorganic phosphate.Fru-6-P: fructose 6-phosphate; Mtl-1-P: mannitol 1-phosphate; Mtl: mannitol; Fru: fructose.

these mutants were still able to catabolize mannitol, while the *A. alternata Δm1pdh* and double mutant (*Δmtdh*/*Δm1pdh*) showed poor growth on mannitol.

From these observations we conclude that M1PDH is the major mannitol biosynthetic enzyme, but it could also be involved in mannitol catabolism. Interestingly, mannitol level was greatly reduced in the double mutant (*Δmtdh*/*Δm1pdh*) of *A. alternata*, while in a similar double mutant in *B. cinerea,* mannitol was still detected. In addition, for *B. cinerea* all mutants had normal growth on mannitol as a sole carbon source. The ability of *B. cinereaΔm1pdh* mutants to synthesize mannitol-1-phosphate from mannitol led the authors to conclude that there may be other single or multiple reactions involved in the phosphorylation of mannitol, or that other pathways/enzymes were present (Dulermo et al., 2010; Figure 4). In fact, *B. cinerea* also has a third mannitol biosynthetic gene (Dulermo et al., 2010), that shares 84% homology with *Tuber borchii mtdh* but only 11% to the previously identified *mtdh* genes in *B. cinerea and A. alternata*.

Roles of mannitol in fungi

Given the remaining unresolved questions on the mechanism of mannitol metabolism in fungi, it is not surprising that the physiological roles of mannitol remain under discussion. Several roles for mannitol have been proposed which range from a carbohydrate reserve, a protectant against osmotic and oxidative stress, roles in sporulation, coenzyme regulation, and storage of reducing power (Lewis and Smith, 1967; Solomon et al., 2007). The role of mannitol as a carbohydrate reserve was initially proposed mainly due to its abundance in

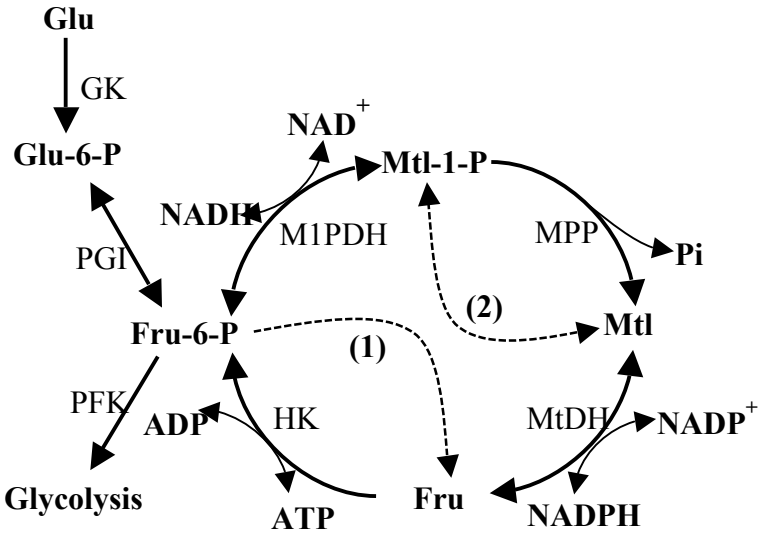

Figure 4: Current understanding of mannitol metabolism in fungi. FK- Fructokinase, GK-Glucokinase, PGI-Phosphoglucose isomerase, PFK-Phosphofructokinase. (1)- Possible reactions due to mannitol-1-phosphate phosphatase (MPP) broad substrate specificity (2)-Unknown single/multiple reaction(s).

fungi. However, reports that mannitol double mutant (*Δmtdh*/*Δm1pdh*) strains of *B. cinerea* and *A. alternata* containing little or no detectable mannitol have normal growth and spore viability raise questions about the importance of mannitol as a carbohydrate reserve (Vélëz et al., 2007; Dulermo et al., 2010). Alternatively, in *Uromyces fabae*, *B. cinerea* and *S. nodorum* mannitol is thought to be a carbohydrate reserve used specifically for spores and required for sporulation (Solomon et al., 2005; Voegele et al., 2005). The capacity of glycerol defective mutants of *Saccharomyces cerevisiae* expressing the bacterial mannitol biosynthetic gene *mtlD* and secreting mannitol to grow on high NaCl and H_2O_2 supports the role of mannitol as a protectant against osmotic and oxidative stress (Chaturvedi et al., 1997). Hult and Gatenbeck (1978) proposed that the cyclic form of mannitol metabolism generates

most of the NADPH required for synthesis of storage lipids. However, studies in *Aspergillus nidulans* and *A. alternata* showed that the fungi were able to grow on different nitrate and carbon sources (Singh et al., 1988; Vélëz et al., 2007), which implies that fungi can generate NAD/NADPH by other metabolic pathways. Finally, mannitol is hypothesized to play a major role in fungal pathogenicity, of which more is discussed in the next section.

ROLES FOR MANNITOL AND MANNITOL DEHYDROGENASE IN PLANT-PATHOGEN INTERACTIONS

General defense and signaling mechanisms in plants

Plant-pathogen interactions form one of the most complex biological interfaces in nature (Baxter et al., 2014). Over evolutionary time, plants and pathogens have each developed extensive layered mechanisms to attack or defend against the other. During this adaptive coevolution, plants have developed a broad range of resistance responses that can be considered in two broad categories; 1) responses that confine or kill the pathogen at the site of infection, and 2) reactions that signal defenses in proximal or distal parts of the plant. The production of Reactive Oxygen Species (ROS) in the apoplast (Levine et al., 1994), including superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), is one of the earliest responses. Pathogen-induced superoxide anions are the first ROS produced *via* activation of preexisting, membrane-localized NADPH oxidases (Doke, 1985; Sagi and Fluhr, 2006). This early, transient burst of $\cdot O_2^-$is followed by the appearance of H_2O_2, likely produced by the dismutation of $\cdot O_2$by an apoplastic Super Oxide Dismutase (SOD) (Cheng et al., 2009a, Kukavika et al., 2005; Vanacker et al., 1998), or by a reverse peroxidase reaction

hypothesized by several groups (Corpas et al., 2001; Bolwell et al., 2002; Bolwell, 1999; Neill et al., 2002). Collectively this set of responses is referred to as the oxidative burst, and occurs in the first minutes after the initiation of the plant-pathogen interaction. H_2O_2 is an effective antimicrobial agent at levels produced during the oxidative burst and is also involved in crosslinking plant cell wall proteins and lignification (Levine et al., 1994; Hammond-Kosack and Jones, 1996). Crosslinking and lignification strengthen the cell wall to confine the pathogen at the infection site, and thus reduce its spread. ROS has also been hypothesized to signal initiation of the hypersensitive response (HR), a localized programed cell death that also serves to limit pathogen spread (Torres, 2010).

The highly reactive nature of the ROS produced during the oxidative burst can result in the production of other reactive molecules that are hypothesized to serve various functions in pathogen defense. One such hypothesized role of ROS is in lipid peroxidation, where the reaction of lipids with ROS produces lipid hydroperoxides (LOOH), lipid peroxyl radicals (LOO·) and lipid radicals (L·) (Farmer and Muller, 2013). Lipid peroxidation is initiated by reaction of the various ROS (Figure 5) with membrane lipids to produce lipid radicals (L·) that in turn react with molecular oxygen to form LOO·. Lipid peroxidation can then propagate in the membrane in a chain reaction with other fatty acids producing LOOH and more L·. In addition, iron catalyzes the conversion of $\cdot O_2^-$ and H_2O_2into the highly reactive hydroxyl radical (·OH, Fenton reaction), which can then react with membrane lipids to form lipid alkoxy radicals (LO·), and LOO·. Many of these lipid radicals are biologically active molecules with known physiological functions. Products of lipid peroxidation are also known as Reactive Electrophile Species (RES) (or lipid electrophiles/ oxylipins) because of their

ability to covalently modify a wide variety of nucleophilic macromolecules including membrane lipids, DNA, and proteins (Farmer and Mueller et al., 2013; Ullery and Marnett, 2012; Jacobs and Marnett, 2009). The reactive nature of RES is reported to give them significant antimicrobial properties (Griffiths et al., 2005), which, together with cell wall lignification, limits the spread of pathogens.

Among the reactive molecular species produced to confine pathogens to the site of infection, some are also thought to act as signal molecules that either initiate localized defense responses or initiate signaling to distal parts of the plant. ROS, primarily H_2O_2, previously were hypothesized to serve as signals that travel long distances in plants to activate defense gene expression in distal tissues via the induction of salicylic acid (SA) and nitric oxide (NO) production (Levine et al., 1994; Alvarez et al., 1998; Torres 2002). This

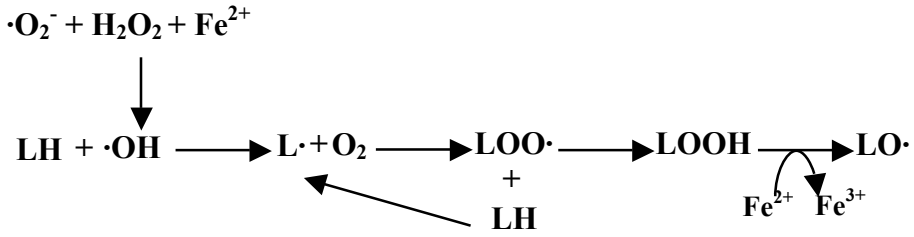

Figure 5: Lipid Peroxidation. LH-Lipid molecule; L·-lipid radical; LOO·-lipid peroxyl radical; LOOH-lipid hydroperoxide; LO·-lipid alkoxy radicals.

results in what is collectively called Systemic Acquired Resistance (SAR). However, more recent findings suggest that lipid radicals formed during the oxidative burst can rapidly

propagate long distances through cell membranes by chain reactions as described above (Farmer and Mueller, 2013). These lipid radicals or RES are also reported to be able to induce expression of pathogenesis-related (PR) genes in *Arabidopsis* (Almeras et al., 2003) as well as being associated with Jasmonic Acid (JA) production and JA-mediated defensive responses (Farmer and Mueller, 2013; Farmer and Davoine, 2007). Interestingly, some induced molecules such as SA are also hypothesized to indirectly control production or turnover of ROS. For instance, SA not only induces PR gene expression, but also is thought to indirectly mediate increased ROS accumulation by binding to catalases and thus inhibiting turnover of H_2O_2 (Conrath et al., 1995). Relative to the complex set of reactive molecular responses in plants during pathogen infection, ROS are the central and first response. Once formed, ROS then directly or indirectly mediate numerous other responses, amongst which RES-mediated responses are startlingly similar to those attributed to ROS. As discussed earlier ROS and RES have both been reported to act as antimicrobial and signal molecules, and acting in concert could create a more effective, integrated defense response. For example, current models suggest that ROS are associated with SA-mediated responses while RES are associated with JA. Although ROS are central to many responses, RES could also be a major mediator with respect to the total set of plant defense responses.

Mannitol as a protective mechanism of pathogens against plant defenses

Given the multiple roles of ROS /RES in plant defenses, if their production or function is blocked, then a whole interacting network of responses could be simultaneously affected. As a result, successful pathogens have evolved mechanisms to counter ROS-

mediated plant mechanisms by molecular, enzymatic and chemical responses that hinder the production or functioning of ROS in plants. For instance, although the precise mechanism is not known, the pathogenicity gene *Defense suppressor 1* (*DES1*) in *Magnaporthe oryzae* was found to mimic inhibition of NADPH-oxidase, suggesting its function limits ROS-mediated defenses in some way (Chi et al., 2009). The tomato pathogen *Pseudomonas syringae*, on the other hand, produces the enzyme catalase to eliminate H_2O_2 produced by the plant oxidative burst (Guo et al., 2012).

In turn, to counteract plant-produced ROS, a number of fungi appear to secrete mannitol to protect against ROS-mediated defense responses. Fungal pathogens such as *A. alternata, B. cinerea, U. fabae and Cladosporium fulvum* secrete mannitol when they encounter plants or aqueous plant extracts (Jennings et al., 2002; Williamson et al., 2013; Voegele et al., 2005; Joosten et al., 1990). As described above, mannitol is hypothesized to have anti-ROS properties. Thus secretion of mannitol by the pathogens during infection has been proposed to quench ROS-mediated plant defenses (Jennings et al., 1998). Mannitol's role as a necessary pathogenicity factor is supported by the observation that double knockout mutants (*Δmtdh*/*Δm1pdh*) of *A. alternata* which were deficient in mannitol synthesis, were no longer virulent on tobacco (Vélëz et al., 2008). In addition, mannitol was present in the apoplast of tomato leaves infected with a virulent strain of *C. fulvum,* but was absent when leaves were inoculated with an avirulent strain (Joosten et al., 1990). Studies by Dulermo et al., (2010) further underscore the importance of mannitol production in fungal pathogenesis. In the Dulermo et al., (2010) study, the observation that double mutant strains (*Δmtdh*/*Δm1pdh*) of *B. cinerea* were still pathogenic led to the discovery that *B. cinerea* has a

third, as yet unnamed, mannitol biosynthetic gene. This gene shares 84% homology with *Tuber borchii mtdh* but only 11% to the previously identified *B. cinerea* and *A. alternata mtdh* genes. Finally, a study by Juchaux-Cachau et al. (2007) found that ectopic expression of*AgMaT2*, a celery mannitol transporter, in tobacco (a plant normally lacking mannitol transporters) conferred increased resistance to *Alternaria longipes*. This increased resistance is hypothesized to result at least partly from the transgenic plant's newly acquired ability to import pathogen-produced mannitol from the apoplast into the symplast, thereby removing mannitol from the apoplast by transport rather than catabolism.

Current hypotheses suggest that secretion of mannitol by pathogens during infection could serve two distinct purposes; first, as a self-defense for the pathogen against biotic and abiotic stresses associated with plant defense responses, and secondly as a means of interfering with the plant's defense signaling pathways by blocking the signal itself (i.e. ROS/RES) (Jennings et al., 1998). For pathogens such as *B. cinerea*, however, induction of HR is actually thought to facilitate infection (Gorvin and Levine, 2000), because it provides both access and releases nutrients. This rather unexpected role for HR was a result of research on HR-deficient *Arabidopsis* mutants that showed they were actually less susceptible to infection by *B. cinerea* than plants with normal HR(Yu et al., 1998). Interestingly, *A. alternata* has *nox* (NADPH oxidase) genes (Morita et al., 2013; Yang et al., 2012), while *B. cinerea* has both *nox* and *sod* (superoxide dismutase) genes (Segmüller et al., 2008; Rolke et al., 2004). Morita et al. (2013) showed that the *A. alternata* Nox proteins are localized at appressoria and that a *Δnox* knockout mutant had reduced lesion development when compared to WT *A. alternata*. Similarly, gene knockout studies on *B. cinerea Δnox*

(Segmüller et al., 2008) and *Δsod* (Rolke et al., 2004) showed that both genes made a significant contribution to virulence. This suggests that ROS produced by these pathogens might also play a part in augmenting HR induction. If these pathogens do produce ROS to strengthen HR, then a general suppression or quenching of ROS by mannitol secretion would be counter-productive, at least at the site of infection. In fact, as discussed above, the amounts of mannitol secreted by the pathogen relative to the amounts required for effective ROS quenching at the site of infection (Jennings et al., 1998; Williamson et al., 2013) are in any case likely to be inadequate. We propose instead that *B. cinerea* secretes mannitol primarily to protect itself against the direct antimicrobial effects of ROS produced by the oxidative burst as well as its own NADPH oxidase/SOD. High localized concentrations of mannitol around the fungal structures and spores themselves could quench ROS and protect *B. cinerea* during the early stages of infection. This is consistent with reports (Jennings et al., 2002; Williamson et al., 2013, Patel, 2014) that removing fungal mannitol during the infection process by overexpressing MTD significantly reduces fungal infection, presumably because the plant's antimicrobial ROS can now act directly on the fungus. In addition, being a compatible solute, mannitol can interact with and form a protective shell of hydration around plant as well as fungal structures. Thus, if lipid radical proliferation is a signaling mechanism (Almeras et al., 2003; Farmer and Mueller, 2013), mannitol could also hinder signaling by interacting with membrane lipids and thus prevent lipid radical formation. The resulting decrease in signaling efficiency could then allow the pathogen sufficient time to overcome plant defenses.

Mannitol dehydrogenase (MTD) and mannitol interaction.

As indicated above, plants have evolved numerous mechanisms to combat attacking pathogens and to neutralize the pathogen's defenses. For instance, while secretion of catalases by bacterial pathogens presumably inhibits H_2O_2-mediated plant defenses, the production of SA by the plant induces PR-gene expression in addition to binding to and thus inhibiting catalase, which presumably maximizes accumulation of H_2O_2 and associated defenses (Conrath et al., 1995; Guo et al., 2012). Similarly, plants appear to have also evolved a means of neutralizing the effects of mannitol secreted by fungal pathogens by repurposing a preexisting enzyme mannitol dehydrogenase (MTD). As discussed earlier, many plants use mannitol as a major metabolite, and thus have specific mannitol catabolic enzymes. In these plants, after transport to sink tissues, mannitol is converted to mannose (Figure 1) by the MTD and subsequently enters central metabolism. Interestingly, MTD homologs had been reported to be elicitor induced PR proteins in both parsley and *Arabidopsis* (ELI3) (Williamson et al., 1995). Subsequent studies showed that the PR-protein inducer, SA also induced expression of MTD in celery suspension cells suggesting MTD itself was a PR protein (Williamson et al., 1995). Remarkably, in addition to the identification of active MTD homologs in tobacco (Jennings et al., 1998), MTD homologs have also been found in a number of other plants that do not make mannitol such as *Arabidopsis* and tomato (Williamson et al., 1995; Lauter, 1996;)[*Arabidopsis* (X67816)-85%, tobacco (ABD73289)-82%; tomato (X92855)-80%]. Further, Jennings et al., (1998) reported the induction of MTD by INA (2,6-dichloroisonicotinic acid) and fungal elicitors in tobacco leaf discs and cell suspension studies. The presence of MTD in non-mannitol plants and its

induction by plant defense inducers seemed to support the hypothesis *Mtd* is a PR gene with a role in plant pathogen response.

It was initially hypothesized that, given the large amount of mannitol in plants like celery, pathogen-induced MTD metabolized the plant-produced mannitol to prevent quenching of ROS-mediated defense responses (Jennings et al., 1998). However, while the oxidative burst is apoplastic (and the resulting ROS is extracellular) (Levine et al., 1994) mannitol in these plants is symplastic (inside the cell) (Rumpho and Edwards, 1983; Loescher et al., 1995), and thus could not impact ROS-mediated responses. This model is consistent with the fact that neither parsley nor celery is notably susceptible to mannitol-secreting fungal pathogens. However, observations that plants that do not produce mannitol, like tobacco, have pathogen-induced MTD, and that at least some fungal pathogens secrete mannitol as a part of the infection process suggest that MTD might instead protect a plant by oxidizing pathogen-produced mannitol. As noted above, however, MTD production and localization in uninfected celery cells is symplastic (Yamamoto et al., 1997; Zamski et al., 1996). How then can symplastic MTD metabolize pathogen-produced mannitol that is localized in the apoplast? Surprisingly, although MTD has no recognized signal peptide to mediate secretion via the Golgi (Cheng et al., 2009b), both MTD expression and MTD secretion was found to be induced by SA in tobacco (Cheng et al., 2009b) and celery (Blackburn et al., 2010). This work suggests that mechanisms not only exist to co-localize MTD to the apoplast with pathogen-produced mannitol, but that other normally cytosolic enzymes that have pathogenesis related roles outside the cell such as SOD can also be secreted by a leaderless or signal peptide independent mechanism (Cheng et al., 2009a).

Therefore, our current hypothesis suggests that 1) when pathogens and plants interact, an apoplastic oxidative burst occurs that initiates multiple resistance responses in the plant; 2) at least one class of fungal pathogens secrete mannitol to interfere with or buffer these ROS mediated responses; and 3) plants produce and localize MTD to the apoplast to oxidize mannitol to mannose, thereby potentiating these ROS-mediated responses.

If this hypothesis for MTD's role in plant-pathogen interaction is so, and if plants like tomato and tobacco have functional MTD homologs, why are they still susceptible to mannitol secreting fungi like *A. alternata* and *B. cinerea*? When compared to mannitol synthesizing plants like celery and parsley, currently documented MTD expression in plants that do not produce mannitol is relatively low in both induced and non-induced plants (Cheng et al., 2009b; Jennings et al., 2002). This suggests that either the amount of MTD induced in these plants is inadequate to oxidize mannitol at effective rates, or that the timing of *Mtd* induction and expression is not fast enough to successfully intercept the mannitol secreted by the pathogen. For instance, if MTD is not expressed very early in the infection process, fungal mannitol could overwhelm defenses before the antimicrobial activity of ROS produced by the oxidative burst becomes effective. Further, because the oxidative burst is transient, the continuous secretion of mannitol by the pathogen might simply saturate ROS-mediated defenses over time. These scenarios all suggest that continuous, high levels of MTD would be most effective in providing maximum resistance to mannitol secreting pathogens. Indeed, this hypothesis is supported by reports that transgenic tobacco plants continuously overexpressing celery *MTD* cDNA were resistant to *A. alternata*, but were not resistant to *Cercospora nicotianae,* a pathogen that does not secrete mannitol (Jennings et al.,

2002). Additional validation for this hypothesis comes from a recent report that geranium plants constitutively over-expressing MTD were resistant to *B. cinerea* (Williamson et al., 2013).

CONCLUSIONS

Although the occurrence of mannitol metabolism in organisms as diverse as plants and fungi clearly demonstrates its importance, our understanding of such fundamental processes as mannitol transport in plants and the interaction of mannitol metabolism in plants and fungal pathogens are still unclear. In addition, even though the importance of mannitol and its metabolic enzymes during salt, osmotic and oxidative stresses in both in plant and fungi have been well documented, our current understanding of mannitol's molecular roles in the associated reactive molecular chemistry is still in its infancy. Finally, among the emergent and dynamic areas of discovery addressed here, the integration of fungal mannitol synthesis and secretion with plant mannitol catabolism and protein transport during infection is a novel example of the complex interactions that can develop over evolutionary time.

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CHAPTER 2

Title

Tomato plants overexpressing a celery mannitol dehydrogenase (MTD) have decreased susceptibility to *Botrytis cinerea.*

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Abbreviations: MTD- mannitol dehydrogenase enzyme; *Mtd*- mannitol dehydrogenase gene; *mtlD*- bacterial mannitol dehydrogenase gene.

ABSTRACT

The oxidative burst is a critical early event in plant-pathogen interactions that leads to a localized, programmed cell death (PCD) called the hypersensitive response (HR). The HR and associated PCD retard infection by biotrophic pathogens, but in fact, can enhance infection by necrotrophic pathogens like *Botrytis cinerea*. In addition to signaling the HR, reactive oxygen species (ROS) produced during the oxidative burst are antimicrobial but pathogens such as *B. cinerea* survive, presumably by secreting the antioxidant mannitol during infection. Previous work supports the notion that overexpression of the catabolic enzyme mannitol dehydrogenase (MTD) can decrease a plants susceptibility to mannitol-secreting pathogens like *B. cinerea*. To extend the above hypothesis, and test the general utility of this approach in an important horticultural crop, we overexpressed celery MTD in tomato (*Solanum lycopersicum* cv. 'Moneymaker'). In these studies, we observed a significant increase (up to 90%) in resistance to *B. cinerea* in transgenic 'Moneymaker' tomatoes expressing high amounts of MTD.

INTRODUCTION

Botrytis cinerea is a necrotrophic fungus with a documented host range of more than 200 plant species (van Kan, 2005; Nakajima and Akutsu, 2014). This broad host range is likely due, at least in part, to the wide range of virulence factors that have been described for *B. cinerea*, including enzymes and toxins that aid in penetrating and killing host cells (Nakajima and Akutsu, 2014). Normally, the hypersensitive response (HR), a localized, programmed cell death (PCD), is one of the plants primary defenses against pathogen attack.

The HR is initiated by the rapid, post-infection production of reactive oxygen species (ROS) and subsequent induction of PCD that limits the spread of infection (Levine *et al*., 1994; Torres, 2010). Interestingly, while HR is an important defense against biotrophic pathogens, HR facilitates infection by necrotrophic pathogens like *B. cinerea* (Gorvin and Levine, 2000). This is supported by the observation that HR-deficient *Arabidopsis* mutants are less susceptible to *B. cinerea* (Yu *et al*., 1998). Further, *B*. *cinerea* is itself reported to have an NADPH oxidase and a superoxide dismutase (SOD) (Rolke *et al*., 2004; Segmuller *et al*., 2008) that are activated, and thus presumably produce additional ROS as part of the infection process. We hypothesize that the additional ROS produced by *B. cinerea* could amplify the host plant HR/PCD and thus increase physical access for infection. Although induction of HR by the oxidative burst provides *B. cinerea* access to the plant, ROS are also very effective antimicrobial/ antifungal agents (Torres, 2010). To successfully infect a plant, *B. cinerea* must, therefore, not only survive the effects of ROS produced as part of the plant's oxidative burst, but also its own ROS. This implies that *B. cinerea* has very effective protective mechanisms against ROS.

Previous work found that *B. cinerea,* as well as other fungal pathogens such as *Alternaria alternata*, *Uromyces fabae* and *Cladosporium fulvum* (Williamson *et al*., 2013; Jennings *et al*., 2002; Voegele *et al*., 2005; Joosten *et al*., 1990) secrete mannitol during the infection process, presumably to buffer the effects of ROS. This is based on the hypothesis that mannitol is, amongst other things, an antioxidant. Smirnoff and Cumbes (1989), for instance, initially reported that mannitol quenched hydroxyl radicals (·OH) generated in an *in vitro* assay. *In vivo, Saccharomyces cerevisiae* mutants that lacked endogenous ROS

quenchers but produced mannitol via expression of a bacterial *mtlD* were able to grow on media with high concentrations of H_2O_2(Chaturvedi *et al*., 1997). The role of mannitol as a necessary pathogenicity factor is further supported by number of *in vivo* plant studies. For instance, Joosten *et al*. (1990) observed that one observed difference between virulent and avirulent strains of *C. fulvum*, was the presence of mannitol in the apoplast of tomato leaves during infection by the virulent strain. More direct evidence from Vélëz *et al*. (2008) showed that mutant strains of *A. alternata* deficient in mannitol production became avirulent. Finally, mannitol secretion by both *B. cinerea* and *A. alternata* was induced in the presence of plant extracts (Williamson *et al*., 2013; Jennings *et al*., 2002), suggesting that mannitol is an important extracellular pathogenicity factor for these fungi.

In response to infection by mannitol-secreting fungal pathogens, a number of plants have been found to secrete the mannitol catabolic enzyme, mannitol dehydrogenase (MTD). In the plant kingdom there are over 100 species that use mannitol as a carbon and energy source. In these mannitol-utilizing plants, the catabolic enzyme MTD, a 1-oxidoreductase, oxidizes mannitol to mannose for entry into central metabolism (Stoop and Pharr, 1992; Stoop *et al*., 1996). *Mtd* homologs surprisingly are also found in plants that do not make or utilize mannitol, such as Arabidopsis, tobacco, and tomato (Williamson *et al*., 1995; Jennings *et al*., 1998; Lauter, 1996), although expression of these genes is typically low. Increased MTD expression is also triggered in these plants, as well as in celery, by inducers of the pathogenesis response (PR gene inducers) such as salicylic acid (SA), INA (2,6-dichloroisonicotinic acid) and fungal elicitors (Jennings *et al*., 1998). This implies that, in addition to its metabolic role in plants like celery (Jennings *et al*., 1998; Williamson *et al*.,

1995; Kiedrowski *et al*., 1992), *Mtd* is also a PR-gene in both mannitol and non-mannitol plants. Interestingly, SA not only induces MTD expression; it also induces the secretion of MTD into the extracellular space or apoplast (Cheng *et al*., 2009; Blackburn *et al*., 2010). This presumably facilitates co-localization of the normally cytosolic/symplastic MTD with the apoplastically localized mannitol secreted by fungal pathogens.

Our hypothesis is that the survival of *B. cinerea* during the oxidative burst, and thus its ability to infect, is at least partly due to its ability to secrete mannitol and thus protect itself against the effects of antimicrobial ROS. Thus, removal of this protective mannitol could allow destruction of invading *B. cinerea* early during the oxidative burst. If MTD is produced and secreted by plants to counter mannitol secreting fungal infection, why are plants that do not produce mannitol like tomato with pathogen-induced *Mtd* homologs still susceptible to pathogens like *A. alternata* and *B. cinerea*? Given that MTD overexpression protects tobacco and zonal geranium against *A. alternata* and *B. cinerea*, respectively (Jennings *et al*., 2002, Williamson *et al*., 2013), and that native MTD expression in plants that do not produce mannitol can be quite low (Jennings *et al*., 1998), it is thought that the MTD protective mechanism in these plants is saturated by high levels of fungal mannitol secretion.

Taken together, this suggests that, while MTD induction and secretion could be an important resistance mechanism against mannitol-secreting fungi, relatively high amounts of MTD might be required with the implication that overexpression of MTD could be an effective means of providing broad resistance against mannitol-secreting necrotrophic fungi. In the following study we test and extend the hypothesis that overexpressing MTD in tomato

(*Solanum lycopersicum*cv. 'Moneymaker') increases resistance to mannitol secreting fungal pathogens, we assess potential changes in resistance to the mannitol-secreting pathogen *B. cinerea* in first and second generation transgenic plants overexpressing MTD.

RESULTS

Leaves of transgenic tomatoes expressing high amounts of MTD are more resistant to *B. cinerea*

MTD levels in wild type (WT), vector transformed (VT) and plants transformed with a MTD-encoding transgene, were determined using protein blot analyses. Transgenic tomato plants expressed MTD in varying amounts, ranging from high to low. If MTD protects against fungi that secrete mannitol as a pathogenicity factor, increased MTD in transformed plants should provide increased resistance to a mannitol secreting pathogen such as *B. cinerea*. To test this hypothesis, detached leaf assays were used to assess potential differences in symptom development between WT and transgenic plants expressing different amounts of MTD. Susceptible reactions were observed as expanding necrotic lesions around the site of inoculation, which was predominant on both the WT and low MTD expressing leaves (Figure 1a). In contrast, resistant reactions ranged from little, or no visible, necrosis to decreased rate of lesion development when compared to susceptible reactions (Figure 1b). Lesion development was assessed every 24 hr until day 4 post-inoculation, with lesion area determined as described above.

Leaves from transgenic plants expressing high amounts of MTD had significantly reduced lesion development compared to leaves from WT plants (Plants 2, 25, 33, 42 etc.;

Figure 2). Plants expressing intermediate levels of MTD (e.g. 29, 14) also showed reduced lesion size, although not reduced as much as the high MTD expressers (e.g. 2, 33). Finally, plants like 17 and 20 with MTD levels similar to those of WT plants, showed lesion development similar to WT plants. Although higher levels of MTD generally correlated with decreased lesion size (increased resistance), plants 44 and 41 were exceptions. However, it must be noted that plant 44, which expressed extremely high levels of MTD was no more resistant than WT, suggesting that there is an optimal level of MTD required for resistance to *B. cinerea*. Possible reasons for this are addressed in the discussion.

The results for fully expanded leaves (4 to 6 cm) are shown in Figure 2. The same assay was used to test immature leaves (2.5 to 3.5 cm),which showed a similar gain in resistance, although, when normalized against WT, the difference was not as great that seen for the mature leaves. Younger tissues are often reported to be more resistant to pathogens (e.g. Barna and Györgyi, 1992) and higher resistance to *B. cinerea* was, indeed, observed in younger leaves both from transgenic plants and non-transgenic controls. Nonetheless, resistance gain in young and mature leaves showed a strong positive correlation (R=0.92) (Supplementary Figure 2).

Second generation transgenic seedlings retain resistance to *B. cinerea*

Seedlings (T_1) grown from seed of selfed, primary transformants (T_0) were also tested for resistance to *B. cinerea*, to assess the effect of MTD overexpression in a whole-plant assay, and to see whether MTD resistance is heritable. Seeds from both a selfed high MTD expresser (plant 25), and a selfed medium MTD expresser (plant 14), along with vector

transformed VT and WT controls were selected for seedling assays. As seen in Figure 3,whole plant infection rates for both medium and high expressing plants were lower than for either WT or VT, with reduction in symptoms being particularly significant for seedlings in the high expressing group (group 25; Figure 3). WT and VT seedlings were both ca. 40% infected by day 8, although at this point infection had not yet reached a maximum. In contrast, high expressing group 25 plants had reached a maximum infection of 10-15% by day 4;infectiondid not increase thereafter. At this point, new uninfected growth was observed on seedlings from group 25, but not on other plants in the test.

DISCUSSION

As reported previously for the effect of MTD overexpression on pathogen resistance in tobacco and zonal geranium (Jennings *et al*., 2002; Williamson *et al*., 2013), results here show a similar relationship between MTD overexpression and decreased susceptibility to mannitol secreting fungal pathogens, in this case *B. cinerea*. However, while most of the transgenic plants in this study that expressed high levels of MTD had decreased *B. cinerea* lesion formation in detached leaf assays, there were two exceptions. Transgenic plant 44 had the highest MTD expression but had lesion size and development nearly identical to WT (Figure 2). A similar, exception was found for plant 41, which had the second highest level of MTD accumulation, but only had a moderate reduction in lesion development. Although plant 44 was phenotypically normal, any number of things could be occurring that would cause the observed response. The might range from defects in the MTD protein itself that effect enzyme activity or transport, to previously proposed detrimental effects of extreme

protein overexpression. These can include the combined effects of diverting cellular resources from critical processes, or overtaxing cellular mechanisms such as RNA translation (reviewed in Glick, 1995;Howell, 2013).

Results of second-generation seedlings assays suggest that; first, resistance to *B. cinerea* resulting from MTD overexpression is stable and heritable. Second, whole plant seedling resistance correlates well with results of the detached mature leaf assays of the respective T_0 plants. Third, whole seedlings of the high MTD expressing genotype (25) are nearly 75% less susceptible than non-transgenic seedlings, and that this resistance extends to the whole plant level. This increase in whole plant resistance should have a substantial impact on disease severity and ultimately crop yield.

Secretion of mannitol during infection is important for full virulence in fungi such as *A. alternata* and *B. cinerea*. In addition to previous transgenic studies from our lab (Jennings *et al*., 2002; Williamson *et al*., 2013), a recent study by Dulermo *et al*. (2010) illustrates the importance provide compelling evidence of mannitol for *B. cinerea* pathogenicity. Dulermo *et al*. (2010)observed that, unlike the dramatic loss of virulence reported by Vélëz *et al*. (2008) for mannitol double knockout mutants of *A. alternata*, a mutant of *B. cinerea* disrupted for the same two mannitol metabolic enzymes still produced mannitol, and was still virulent. This observation led to the discovery of a third mannitol metabolic gene in *B. cinerea* that was only 11% homologous to the previously described mannitol dehydrogenase (*Bcmtdh*), but 84% homologous to *Tuber borchii* (truffle) mannitol dehydrogenase (*Tbmtdh*) (Ceccaroli *et al*., 2007). The presence of this third mannitol biosynthetic gene homolog

combined with the continued synthesis of mannitol in the double mannitol biosynthetic mutant support the hypothesis that mannitol is important for *B. cinerea* virulence.

Although data on the virulence of triple knockout mutants for mannitol synthesis is not available, the gain in resistance observed here for MTD overexpressing plants supports a role for mannitol in *B. cinerea* virulence. The exact mechanism whereby mannitol serves as a virulence factor is, however, still a matter of debate. We had previously hypothesized that mannitol, being an efficient antioxidant, quenched ROS that signaled induction of all plant defense responses. Unfortunately, this model is not consistent with a number of recent observations concerning the role of ROS and the induction of HR in the *B. cinerea* infection process (Gorvin and Levine, 2000, Nakajima and Akutsu, 2014; Rolke *et al*., 2004; Segmuller *et al*., 2008). These studies indicate that, because ROS and the associated induction of the HR enhance infection by *B. cinerea*, a general quenching of ROS would not be advantageous for a necrotrophic pathogen.

We propose instead that *B. cinerea* secretes mannitol primarily to protect itself against the direct antimicrobial effects of the oxidative burst. Because the oxidative burst is transient, high localized concentrations of mannitol around the fungal structures and spores themselves could quench ROS and protect *B. cinerea* during the oxidative burst. Consequently, if fungal mannitol is removed during the initial period of infection, i.e. during the oxidative burst, then the plant's antimicrobial ROS could act on *B. cinerea* at an early enough stage that infection is suppressed prior to the induction of significant HR. This is consistent with the observation that some leaves showed virtually no necrosis in detached

leaf assays (Figure 1; plants 2 and 33). Both plant 2 and 33 are high MTD expresser, hence in this case mannitol was removed at initial infection by MTD.

Our results, together with those of other researchers, support the hypothesis that mannitol is an important pathogenicity factor and that the pathogen-induced expression and secretion of MTD is a common defense response even in plants like *Arabidopsis*, tobacco and tomato. In many cases, however, these plants are still susceptible to mannitol secreting pathogens such as *B. cinerea*, perhaps because MTD is typically induced both too late and in insufficient quantities to effectively counter the infection process. For maximum effectiveness pathogen produced mannitol should be metabolized prior to induction of the oxidative burst, and remain high enough to prevent mannitol accumulation thereafter. Thus, the constitutive overexpression of MTD used here provides a more an effective resistance strategy than induced expression of endogenous MTD. Our results not only confirm, but also extend the idea that MTD overexpression can be an effective global strategy to engineer resistance.

EXPERIMENTAL PROCEDURES

Tomato transformation

Seeds of tomato (*Solanum lycopersicum*) 'Moneymaker' (Thompson and Morgan, Aurora, IN) were surface sterilized with 70% ethanol for 1 min, followed by 50% Clorox (commercial bleach) for 30 min and then rinsed 3 times with sterile distilled water. Sterilized seeds were then allowed to germinate in Magenta™ GA-7 boxes on agar (12 g/L) (Caisson Laboratories Inc., North Logan, UT) medium containing MS salts (Murashige and Skoog,

1962) with Gamborg's vitamins (Gamborg *et al*., 1968) and sucrose (10 g/L) at pH 5.6. The seeds were germinated at 26°C in the light with a 16/8 hr light/dark cycle. Cotyledons and hypocotyls of 7-day old seedlings were used as a source of explants for transformation.

A 500 µl aliquot of a frozen stock of *Agrobacterium tumefaciens* strain GV3101 harboring the plasmid construct pTN214N8 (Williamson *et al*., 2013) was inoculated into 25 ml liquid Yeast Extract Peptone (YEP) with 50 mg/L gentamycin, 100 mg/L spectinomycin and 10 mg/L rifampicin and grown overnight on a shaker at 220 rpm at 28°C. This overnight culture was centrifuged and the resulting bacterial pellet resuspended in 25 ml liquid suspension (RL) medium containing MS salts with Gamborg's vitamins, sucrose (30 g/L), glucose (2 g/L), and 40 mg/L acetosyringone at pH 5.6, and grown for another 1-2 hr at 28°C. For explant inoculation this culture was diluted to achieve a cell density that gave an OD_{600} of ~ 0.3.

Seedling explants with hypocotyls and well-shaped (not curly) cotyledons were collected in a sterile Petri dish containing a small amount of RL medium. Explants were then placed on a solid co-cultivation medium (TCC) [MS salts, Gamborg's vitamins, sucrose (30 g/L), glucose (2 g/L), 1-naphthaleneacetic acid (NAA) (0.1 mg/L), benzyladenine (BA) (1 mg/L), 2-(N-morpholino)ethanesulfonic acid (MES) (700 mg/L), acetosyringone (40 mg/L) and agar (10 g/L) at pH 5.6] where cotyledons were wounded several times with a needle and hypocotyls cut into 3-5 mm segments. Both types of explants (cotyledons and hypocotyls) were then submerged in 40 ml of the *Agrobacteriun* suspension described above, and shaken for 15 min at 50 rpm at room temperature. Following inoculation, cotyledon and hypocotyl pieces were blotted dry on sterile paper towels and placed on TCC medium for 48 hr in the

dark at 25°C.

Following 48 hr co-cultivation, cotyledon and hypocotyl explants were washed 3 times with liquid RL medium containing 300 mg/L timentin, blotted dry on sterile paper towels and placed on shoot regeneration (TSR) medium [MS salts, Gamborg's vitamins, sucrose (30g/L), indole-3-acetic acid (IAA) (0.1 mg/L), zeatin (2 mg/L), timentin (300 mg/L), kanamycin (100 mg/L), acetosyringone (40 mg/L), agar (6 g/L) at pH 5.6]. After one week all explants were re-positioned to achieve full contact with the medium. Three weeks later all explants were transferred to fresh TSR medium for continued incubation under 20 watt cool-white fluorescent lights with a 16/8 hr light/dark photoperiod.

As soon as shoots had grown to 1-2 cm they were excised and placed on rooting medium (TR) containing MS salts, Gamborg's vitamins, sucrose (30 g/L), timentin (300 mg/L), and kanamycin (100 mg/L) for secondary selection. Plants with well-developed roots were transferred into small plastic pots with soil and covered with clear plastic covers for a week in a chamber at 25°C under 20 watt cool-white fluorescent lights with a 16/8 hr light/dark photoperiod for acclimatization. After acclimatization plants are transferred to the greenhouse for further growth, fruiting and seed collection.

Verifying the presence of the *Mtd* transgene

Kanamycin-resistant shoots were screened for the presence of the 35S-*Mtd* transgene using PCR primers that amplify a 1.88 kb fragment extending from inside the 35S promoter to the interior of the nos terminator of the *Mtd* transgene as previously described (Williamson *et al*., 2013). Primers were: 35S-FWD1 and 35S-FWD2; (gaactcgccgtaaagactgg and

aaacctcctcggattccatt, respectively), and nosT-REV1 and nosT-REV2 (agggaagaaagcgaaaggag and ttgcgcgctatattttgtt, respectively). A total of forty-four kanamycin-resistant shoots were screened, of which thirty-five primary transformants were found to have the *Mtd* transgene, while 9 lacked the 35S-*Mtd* transgene although they were Km^R (i.e. presumed vector transformants).

Protein extraction and MTD blot analyses

For analysis of *Mtd* transgene expression, leaf tissue from plants [transformed, untransformed (WT) and vector transformed (VT) controls] was harvested and ground in Bio-Rad (Richmond, CA) SDS sample buffer (1:2 w/v) and protein concentrations determined by the method of Bradford (1976). Proteins were 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 plants containing the *Mtd* transgene that were assessed, 8 expressed high levels of MTD protein, while the rest expressed intermediate to low amounts, although higher than the WT controls.

Fungal growth, maintenance and spore preparation

Mycelial cultures of the fungal pathogen *Botrytis cinerea* (a field isolate from the lab of Dr. Michael Benson, Department of Plant Pathology, North Carolina State University) were maintained at room temperature on Potato Dextrose Agar (PDA) (Benton, Dickinson

Co., Sparks, MD). A plug from the edge of a spreading culture was transferred to fresh media every 2 weeks for maintenance. To initiate sporulation, a mycelial plug or spore stock was transferred to the center of an oatmeal agar (Benton, Dickinson Co., Sparks, MD) plate and maintained at 21°C under an approximately 8/16 hour light/dark photoperiod in a humidity chamber made from a lidded plastic box with wet paper towels lining the bottom. Significant sporulation was present by 10 to 14 days. Sporulating cultures were overlaid with 10 mL sterile 0.01% 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 the desired concentration. For long-term storage spores were stored at -20°C in 15% glycerol, 0.01% Tween 80.

Detached leaf assay

A detached leaf assay similar to that described by Spurr (1973) was used to assess resistance to *B. cinerea*. Both young and mature leaves from each transformed genotype were harvested and placed abaxial side up on wire mesh suspended above water in a transparent plastic container with a lid. Five µl of *B. cinerea* frozen stock spore suspension (containing $4x10^6$ spores·mL^{-1}) were spotted onto each leaf at 2 to 6 locations (depending on leaf size) and allowed to dry briefly. These spots were then pierced with a sterile needle to assist infection. In addition to leaves from *Mtd*-transformed plants, leaves from WT plants were included in each experimental repetition (i.e. in each box) as a susceptible control. Boxes were then closed and placed inside another plastic box at room temperature with an approximately 16/8 hr light/dark photoperiod. Two to six treatments (inoculations) per leaf

with 3 experimental units per treatment were replicated at least three times. Symptom development was assessed as described by Floryszak-Wieczorek *et al*. (2007), with the area of each lesion being recorded at 24 hr intervals for 4 days or until lesions merged. Lesion areas were scored using the area function of the Adobe Photoshop™ software suite. Lesion areas normalized against those of wild type controls in each box were used for comparison of resistance.

Seedling assay

Seeds from one high and one medium MTD expresser, as well as from VT and WT plants were selected for the seedling assay. Seed from selfed, primary transformants (T_o) were grown on medium containing ½MS salts, kanamycin (100 mg/L), agar (6 g/L) at pH 5.6 to produce T_1 generation (primary transformants are designated T_0) tomato seedlings and plants. Because this population was still segregating for the *Mtd* transgene, only plants/seedlings with the *Mtd* transgene grew on kanamycin selection. All seedlings that grew on kanamycin media had the *Mtd* transgene (Supplementary Figure 1). WT and VT seeds were grown on similar media without kanamycin. Seedlings (6-8 cm) were transferred to small plastic pots with soil and covered with clear plastic covers at 25°C for a week to acclimate. After acclimation, 12 to 15 cm seedlings were arranged in a random complete block design for 8 replications and sprayed with *B. cinerea* spores (10^7 spores·mL^{-1}). This spore concentration was selected by inoculating wild type seedlings with a range of spore concentrations (10^3-10^8 spores·mL^{-1}) and selecting the lowest concentration that produced robust symptoms on these susceptible WT controls. Symptom development was assessed as

the percentage of infected leaf tissue area, with the total seedling leaf area being 100 %. Infected area was recorded every 24 hr through day 8 post inoculation, or until seedlings started to show new uninfected growth. A minimum of six seedlings per group were used as experimental units in eight replications. Differences in resistance were compared using repeated measure analysis and ANOVA in Statistical Analysis Software (SAS, version 9.3, Cary, NC).

ACKNOWLEDGEMENT

This work was funded by a specialty crops block grant from NCDA to JW, GA and DP. We thank Dr. Heather Olson, Department of Plant Pathology, NCSU for *B. cinerea* cultures. We also thank Dr. Emily Griffith and Mr. Andrew Wilcox, Department of Statistics, NCSU for their help with statistical analysis of the seedling assay. Tomato transformation was carried out at the NCSU Plant Transformation Laboratory.

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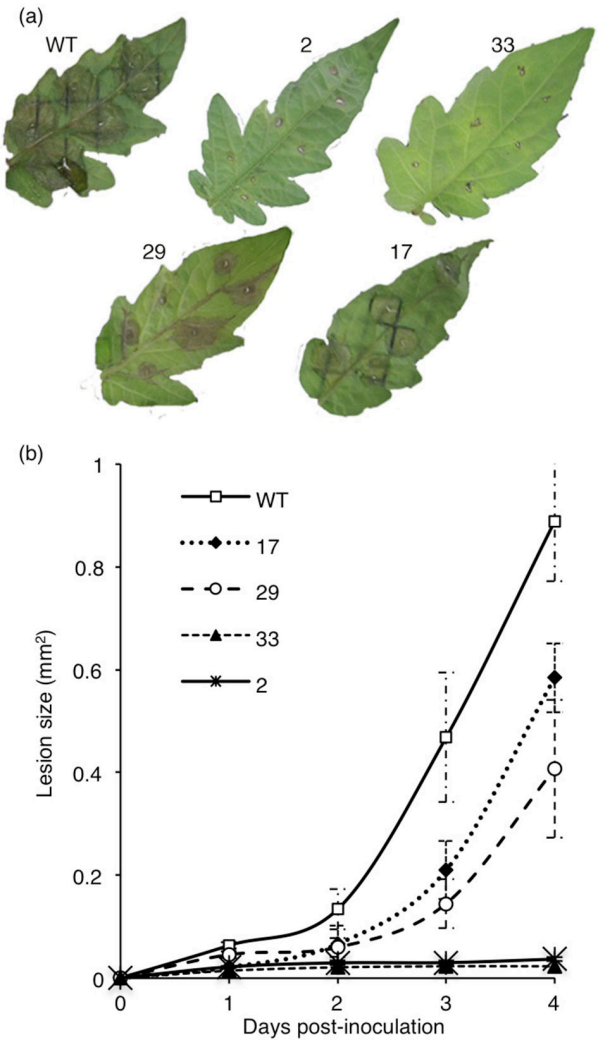


Figure 1: Rate of *B. cinerea* disease development on leaves of *Mtd* over-expressing tomato. Mature leaves from *Mtd*-transformed (2, 33, 29, 17) and untransformed plants (WT) were inoculated at 5 or 6 spots with *B. cinerea* spores. (a) *B. cinerea* necrotic lesion development on leaves of transformed and untransformed plants with various amounts of MTD protein expression [2, 33-high MTD expression; 29, 17-low MTD expression] at 4 days post-inoculation. (b) Lesion size was scored as the area of necrosis at each inoculation site each day over a period of 4 days. Data are means ± SE for 5 to 6 lesion on the leaf shown.

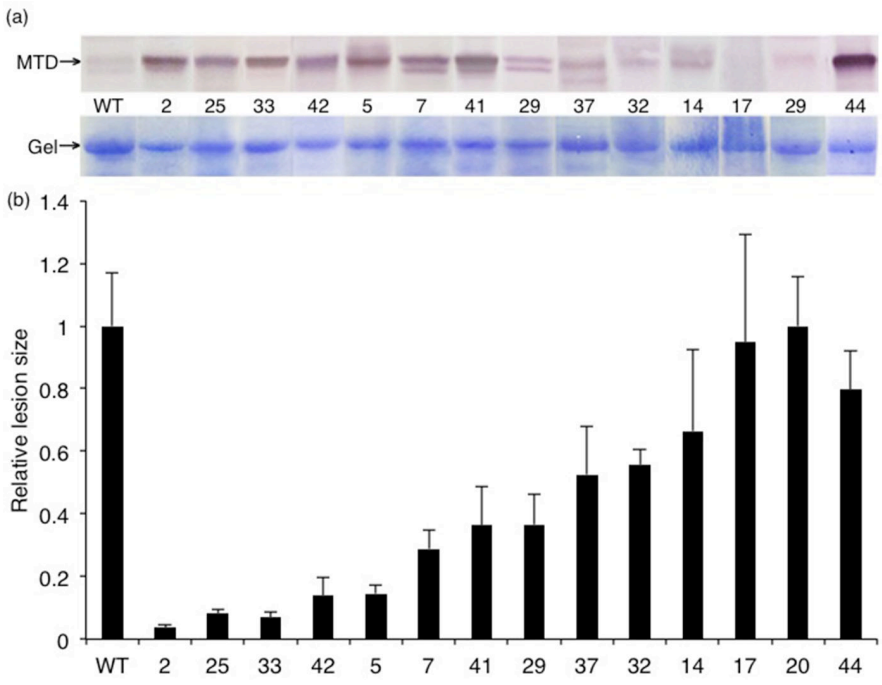


Figure 2: *B. cinerea* susceptibility of detached leaves from MTD over-expressing tomato. (a) Blot analysis of MTD protein in *Mtd*-transformed and untransformed (WT) controls. Lanes are a composite reordered to correspond to leaf assay results arranged from high to low resistance. Coomassie stained lanes are shown to document relative protein loading (Gel), and allow comparison of MTD expression and relative loading. (b) *B. cinerea* infection was scored as lesion area on day 4 post-inoculation for each inoculation site. Lesion sizes are normalized against lesion size on untransformed (WT) leaves. Data shown are means $\pm$ SE from at least three independent replications for each transformed plant (2, 25, 33 etc.).

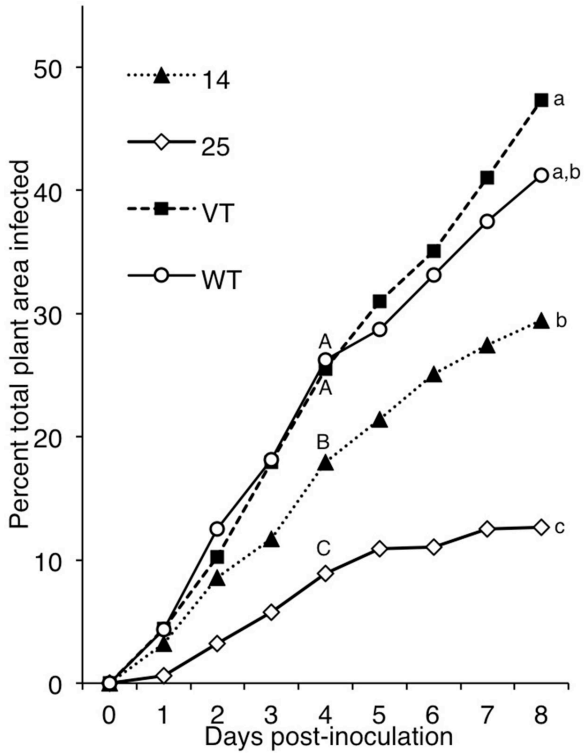
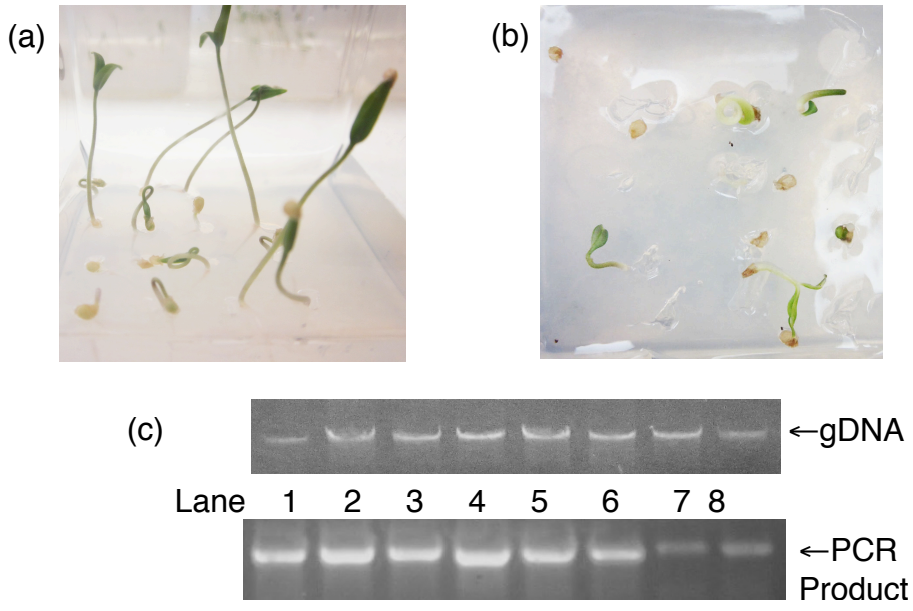
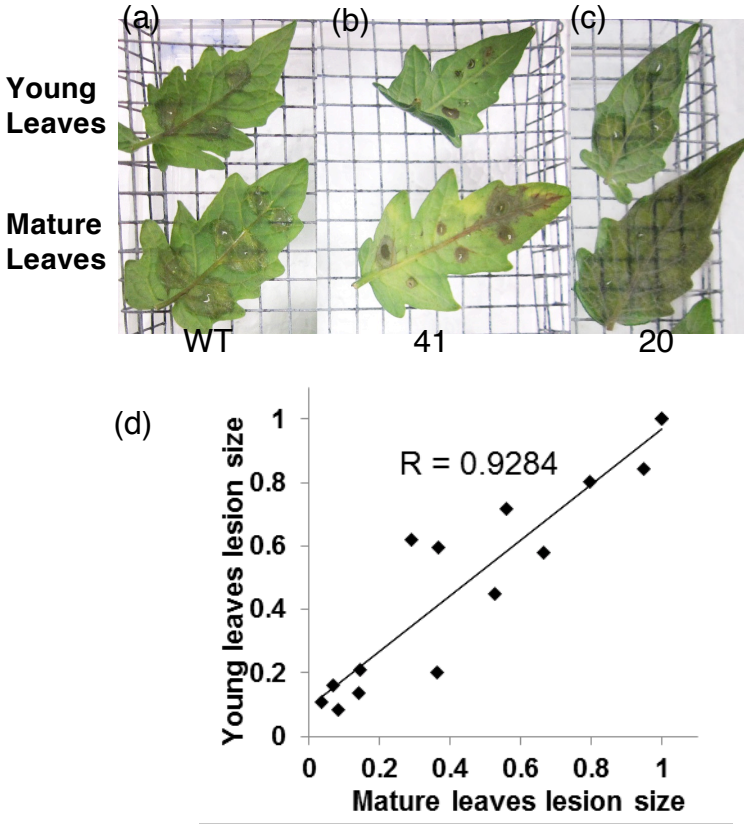


Figure 3: Resistance to *B. cinerea* in second-generation MTD-over-expressing transgenic tomato plants. Seedlings from one high (25) and one medium (14) MTD expresser, vector transformed (VT) and untransformed (WT) tomato plants were assayed. Seedlings were arranged, assessed and inoculated as described in Experimental Procedures. Disease development was scored over a period of 8 days as a relative percentage of the total leaf tissue area, with total plant leaf area being 100 percent. Means for each seedling group are shown. Differences in resistance among the four groups for each day were compared by ANOVA. Means with different letters are significantly different from each other on day 4 and day 8 at $p < 0.05$.

SUPPORTING INFORMATION



Supplementary Figure 1: PCR analysis of second-generation *Mtd* transgenic seedlings. Seed from selfed T_0 plants were grown on kanamycin-containing media as described in materials and methods. (a) Only seedlings having at least one copy of the *Mtd* gene were able to grow. Seedlings are shown on day 3 after imbibition. (b) Seeds with no *Mtd* gene did not germinate or root properly by day 10. (c) Presence of an *Mtd* transgene was confirmed in seedlings that grew on kanamycin media. Upper and lower gels represent isolated genomic DNA (gDNA) and the amplified PCR product from the *Mtd* transgene, respectively.



Supplementary Figure 2: Correlation between resistance in young and mature leaves. (a-c) Younger leaves were observed to be relatively more resistant with lower absolute lesion sizes; this was observed for all plants including untransformed (WT) controls. The apparent or relative resistance of the young transgenic leaves then was less than mature leaves because data was normalized against a more resistant "susceptible" control. (d) Correlation between the lesion sizes on young and mature leaf data was analyzed by simple linear regression. Although not individually labeled, each point on the graph represents lesion size for young and mature leaves for a specific plant (e.g. plant 2, 33 and etc). Although young leaves were innately more resistant, a correlation coefficient (R)of 0.9284 indicates a strong positive correlation between resistance of young and mature leaves.

CHAPTER 3

Title

Overexpression of a celery mannitol dehydrogenase (MTD) in tomato('NC1-Grape') decreases infection to *Alternaria solani*

ABSTRACT

Reactive Oxygen Species (ROS) produced by fungal and plant NADPH oxidases (Nox) are an important part of plant-pathogen interactions. Characterization of Nox in *Alternaria alternata* (Morita et al., 2013; Yang and Chung, 2012) found 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. As a result, necrotrophic pathogens like *A. alternata* need to survive the antimicrobial effects of the ROS produced both by themselves and the host plant. Intracellular transcription factors like *YAP1* (Lin et al., 2009; Yang et al., 2009) have been hypothesized to help *Alternaria* spp. tolerate ROS by mediating global induction of resistance responses. One specific resistance mechanism reported, however, involves secretion of mannitol, an antioxidant (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997) during plant infection. *A. alternata* mutants defective in mannitol production and secretion were less virulent than wildtype strains on tobacco (Vélëz et al., 2008). Conversely, tobacco plants expressing a mannitol transporter gene (*AgMaT2*) (Juchaux-Cachau et al., 2007) were reported to be more resistant to *Alternaria longipes*, presumably due to relocation of pathogenic mannitol away from the site of infection.

INTRODUCTION

In response to pathogen infection, plants in turn secrete an enzyme, mannitol dehydrogenase (MTD) that oxidizes mannitol to mannose, which is not an antioxidant.

Earlier work in this lab showed that transgenic tobacco, geranium and tomato (cv. 'Moneymaker') overexpressing MTD were more resistant to the mannitol-secreting pathogens A. alternata and Botrytis cinerea (Jennings et al., 2002; Williamson et al., 2013; Second chapter of this thesis). Here we transformed a tomato field/breeding variety *Solanum lycopersicum*, cv. 'NC1-grape' with a celery *Mtd* cDNA and assessed the effect on *Alternaria solani* resistance.

RESULTS AND DISCUSSION

Second-generation seedlings from groups 12 and 19, as well as non-transformed (WT) controls were assessed for A. solani resistance. Second-generation group 12 seedlings showed significantly less infection than seedlings in the other groups (Figure 1), with infection rates less than 65% and new growth appearing on day 7. The seedlings from group 19 had intermediate resistance, with infection rates less than 75%, while the WT controls had a terminal disease index of 95% by the end of day seven.

Although the data set here is much smaller than in previous studies, these results are still consistent with the hypothesis that mannitol secretion by necrotrophic pathogens like A. solani is important for survival from the effects of ROS occurring during the infection process. Mannitol is hypothesized to be a quencher of ROS and is thus very important during the infection process. We hypothesize that the timely/immediate removal of pathogenic

mannitol by MTD exposes fungal structures to the antimicrobial effects of ROS. Although Mtd homologs are present in tomato (Lauter, 1996), the amount of MTD expressed naturally is typically low (Figure 1A, WT). Further, salicylic acid, INA (2,6-dichloroisonicotinic acid) and fungal elicitors also induce MTD expression in other non-mannitol plants (Jennings et al., 1998; Williamson et al., 1995, Kiedrowski et al., 1992) although the induced amounts reported are also low. If this were also true for MTD induction in tomato, levels would also likely 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 (group 12) supports the hypothesis that the interaction of mannitol and MTD forms an important and dynamic interface during infection by mannitol secreting fungal pathogens.

EXPERIMENTAL PROCEDURES

Tomato transformation and Verifying the presence of the Mtd transgene

Tomato 'NC1-grape' was transformed using Agrobacterium tumefaciens strain GV3101 containing the plasmid pTN214N8 (Williamson et al., 2013) as described in Patel (2014). Resulting kanamycin resistant regenerants were screened for the presence of 35S-Mtd transgene using PCR with forward primers gaactcgccgtaaagactgg and aaacctcctcggattccatt, (35S-FWD1 and 35S-FWD2, respectively) and reverse primers gaactcgccgtaaagactgg and aaacctcctcggattccatt, (nosT-REV1 and nosT-REV2, respectively). A total of 15 kanamycin resistant shoots were screened, of which 9 plants had the Mtd transgene.

Protein extraction and MTD blot analyses

To analyze MTD expression, proteins were first extracted from leaf tissue of primary transformants (T0's) as well as untransformed (wild type, 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 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).

Fungal growth, maintenance and spore preparation

Spores of the fungal pathogen Alternaria solani (a field strain isolated by Dr. Tika Adhikari in the lab of Dr. Frank Louws, Department of Plant Pathology, NC State University) were stored at -20°C in 15% glycerol. To initiate sporulation, spore stock was transferred onto a Potato Dextrose Agar (PDA) (Benton, Dickinson Co., Sparks, MD) plate pH 5 and maintained at 25°C with an approximately 8/16 hr light/dark photoperiod in a controlled humidity chamber (Percival Scientific, Inc., IA, USA). Significant sporulation was observed by 14 to 18 days. Sporulating cultures were overlaid with 10 mL sterile 0.01% 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 the desired concentration.

Seedling assay

Seed from selfed, primary transformants (To) were grown on medium containing ½MS salts, kanamycin (100 mg/L), and agar (6 g/L) at pH 5.6 to produce second generation (T1) tomato seedlings and plants. Seedlings from one high (plant 12) and one low (plant 19) MTD expresser, as well as from untransformed control plants were selected for analysis. This population was still segregating for the Mtd trangene, so only plants/seedlings with the Mtd transgene grew on kanamycin selection. Seedlings (6-8 cm) were transferred to small plastic pots with soil and covered with clear plastic covers at 25°C for a week to acclimate. After acclimation, seedlings were arranged in a random complete block design and sprayed with A. solani spores (1.8×10^6 spores/mL). The spore concentration was selected in preliminary experiments by titrating wild type seedling with a range of spores concentrations (10^4-10^7 spores/mL). Based on symptom development observed on WT plants, 1.8×10^6spores/mL was used. Symptom development was assessed as the percentage of infected leaf tissue area, with the total seedling leaf area being 100 %. Infected area was recorded every 24 hr through day 7 post inoculation, or until seedlings began to die. Eight (8) to 16 seedlings per type were used as experimental units in 8 replications. Differences in resistance were compared using repeated measure analysis and ANOVA in Statistical Analysis Software (SAS, version 9.3, Cary, NC)

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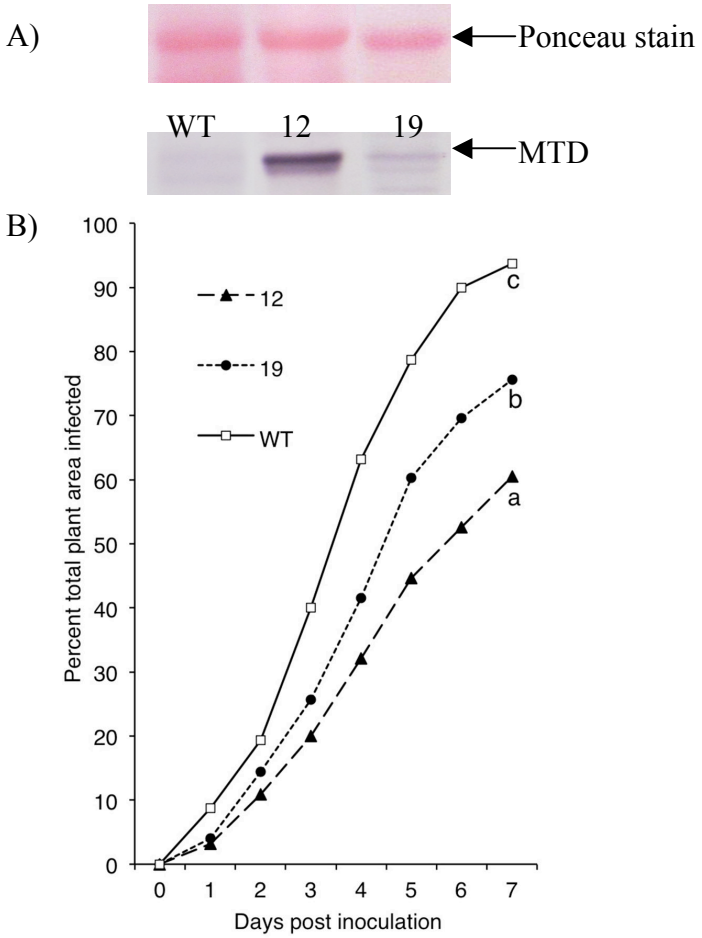


Figure 1. Resistance to *A. solani* in second-generation of MTD over-expressing transgenic tomato plants. MTD protein from T_0 plants of one high (12) and one intermediate (19) MTD expressing lines, and untransformed (WT) plants were quantifiedby protein blot analyses. Segregating seedlings from groups 12, 19 and WT controls were assessed for resistance. Seedlings were arranged, inoculated and scoredfor resistance as indicated in materials and methods. A)Blot analysis of MTD protein in *Mtd*-transformed and untransformed (WT) controls. 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 percent. Means from8 to 16 seedlings for each seedling group are shown. Differences in resistance for day 7 were compared by ANOVA for differences among the three groups. Means with different letters are significantly different from each other on day 7 at $p< 0.05$.

APPENDICES

APPENDIX A

PROTOCOLS

Genetic transformation of Tomato with celery *Mtd* cDNA

Agrobacterium tumefaciens culture

1. Add 500 µl aliquot of frozen stock of *Agrobacterium tumefaciens* strain GV3101 harboring the plasmid construct pTN214N8 in 25 ml liquid Yeast Extract Peptone (YEP).
2. Place the inoculated culture overnight on a shaker at 220 rpm at 28°C.
3. Next day centrifuge the culture to pellet *Agrobacterium* cells.
4. Resuspended the pellet in 25 ml of Re-suspension liquid (RL) medium for another 1-2 hr at 28°C.
5. For explant inoculation, dilute this culture to achieve a density that gives an OD_{600} of ~ 0.3.

Seed germination

1. Put tomato seeds in 15 ml falcon tube.
2. Add 70% ethanol for 1 min.
3. Replace ethanol with 50% Clorox (commercial bleach) for 30 min.
4. Rinse 3 times with sterile distilled water.
5. Place sterilized seeds on MS media in magenta boxes at 26°C under 16 hrs of light in growth chamber.
6. Seedlings will be ready in 7-10 days.

Explant inoculation with *Agrobacterium* shoot and root initiation.

1. Collect seedling explants with hypocotyls and well-shaped (not curly) cotyledons in a sterile petri dish containing a small amount of RL medium.
2. Place explants on a solid co-cultivation medium (TCC).
3. Wound the cotyledons several times with a needle and cut hypocotyls into 3-5 mm segments.
4. Submerge both types of explants (cotyledons and hypocotyls) in 40 ml of the *Agrobacteriun* suspension described above, and shake for 15 min at 50 rpm at room temperature.
5. Following inoculation, dry the cotyledon and hypocotyl pieces on sterile paper towels and placed on TCC medium for 48 hrs in the dark at 25°C.
6. After 48 hr co-cultivation, wash cotyledon and hypocotyl explants 3 times with liquid RL medium containing 300 mg/l timentin.
7. Dry on sterile paper towels and placed on shoot regeneration (TSR) medium.
8. Reposition all explants after one week to achieve full contact with the medium.
9. After three weeks transfer all explants were to fresh TSR medium for continued incubation under 20 watt cool-white fluorescent lights with a 16/8 hrs light/dark photoperiod.
10. As soon as shoots grow to 1-2 cm, excise them and place on rooting medium (TR) for secondary selection.
11. Transfer plants with well-developed roots into small plastic pots with soil and cover with clear plastic covers for a week in a chamber at 25°C under 20 watt cool-white fluorescent

lights with a 16/8 hrs light/dark photoperiod for acclimatization.

12. After acclimatization, transfer plants to greenhouse for further growth, fruiting, and seed collection.

Yeast Extract Peptone (YEP) media

Yeast extract	10 g
Bactro peptone	10 g
Gentamycin	50 mg
Spectinomycin	100 mg
Rifampicin	10 mg
Distilled H_2O	1000 ml
pH	7

MS media

MS salts + Gamborg's vitamins	4.4 g
Agar	12 g
Sucrose	10 g
Distilled H_2O	1000 ml
pH	5.6

Re-suspension Liquid (RL) media

MS salts + BS salts	4.4 g
Sucrose	30 g
Glucose	2 g
Acetosyringone	40 mg
Distilled H_2O	1000 ml
pH	5.6

Cocultivation (TCC) media

MS salts	4.4 g
Sucrose	30 g
Glucose	2 g
1-naphthaleneacetic acid	0.1 mg
Benzyladenine	1 mg
2-(N-morpholino) ethanesulfonic acid	700 mg
Acetosyringone	40 mg
Agar	10 g
Distilled H_2O	1000 ml
pH	5.6

Shoot Regeneration (TSR) media

MS salts	4.4 g
Sucrose	30 g
Indole-3-acetic acid	0.1 mg
Zeatin	2 mg
Timentin	300 mg
Kanamycin	100 mg
Acetosyringone	40 mg
Agar	6 g
Distilled H_2O	1000 ml
pH	5.6

Rooting (TR) media

MS salts	4.4 g
Sucrose	30 g
Timentin	300 mg
Kanamycin	100 mg
Timentin	300 mg
Kanamycin	100 mg
Distilled H_2O	1000 ml
pH	5.6

DNA extraction

1. Place 1-3 young folded leaves in centrifuge tube.
2. Freeze the tube in liquid nitrogen or -20°C then -80°C.
3. Grind sample to fine powder by either metal ball or small sterile pestle.
4. Put the sample back in liquid nitrogen; do not allow the sample to thaw.
5. Add 800 µl of extraction buffer (EB) and immediately vortex the sample for 10-15 seconds.
6. Incubate at 65°C for 30 min.
7. Add 650 µl of Phenol:Choloroform:Isoamyalcohol (25:24:1) into the same tube.
8. Constantly mix for 10-15 min.
9. Centrifuge at 13,500 rpm for 10 min.
10. Transfer top layer to a new centrifuge tube containing 600 µl of cold isopropanol (Stored at -20°C)
11. Mix and incubate at -20°C for 10 min to precipitate DNA.
12. Spin at 15,000 rpm for 10 min.
13. Remove supernatant by pipetting.
14. Wash pellet with 70% ethanol, flick to dislodge the pellet.
15. Spin at 15,000 rpm for 5-10 min.
16. Remove supernatant.
17. Dry the tube to remove ethanol.
18. Suspend the pellet in 25µl sterile water.
19. Store at -20°C.

EB (250 ml)

Tris	1 M	25 ml	pH 8
NaCl	5 M	70 ml	
EDTA	0.5 M	10 ml	pH 8
CTAB	2%		

1M Tris -121.1 g Tris for 1000ml
0.5 EDTA -186.12g of EDTA for 1 L, adjust pH with NaOH pellets
5M NaCl- 292.2g NaCl for 1000 ml.

Mix Tris, NaCl and EDTA together in a beaker, then add 5g of CTAB powder and mix slowly on a hot block with medium heat to dissolve CTAB as much as possible. Autoclave for 20 min.

Verification of MTD transgene

1. Prepare 20-25 µl PCR mixture.
2. Run the PCR cycle on thermo cycler.
3. Prepare agarose gel in TAE buffer for electrophoresis.
4. Load 10-15 µl of PCR sample.
5. Run the gel in TAE buffer at 80-100 V for 45-60 min.
6. Visualize gel using UV light.
7.

PCR Master mix (20 µl)

Apex master mix	10 µl
DNA	1-3 µl
Primer 1 (Forward)	1 µl
Primer 2 (Reverse)	1 µl
Sterile H_2O	5-7 µl

PCR machine cycles

Step	Temperature	Time
1	95°C	3 min
2	94°C	45 sec
3	55°C	1 min
4	72°C	2 min
5	Go to step 2, 15 times	
6	94°C	45 sec
7	56°C	1 min
8	72°C	2 min
9	Go to step 6, 10 times	
10	94°C	45 sec
11	58°C	1 min
12	72°C	2 min
13	Go to step 10, 10 times	
14	72°C	5 min
15	4°C	Forever

TAE Buffer

Tris base	4.84 g
Glacial acetic acid	1.142 ml
EDTA	10 mM
Distill H_2O	1000 ml
pH	8

Protein extraction, quantification, electrophoresis and Protein Immunoblotting Blotting Protocol for MTD in Moneymaker (Tomato)

Protein Extraction

1. Collect leaf tissue, weigh around 150-200 mg for each plant.
2. Flash freeze leaves with liquid nitrogen.
3. Homogenize sample using mortar and pestle, add 900µl of extraction buffer to the homogenized sample and grind using pestle.
4. Transfer tissue lysate to 1.5ml centrifuge tube and keep it on ice until the next step.
5. Boil sample at 95°C for 5 min.
6. Centrifuge for 5 to 10 min at 10,000 rpm.
7. Collect supernatant in new centrifuge tube.
8. Check the protein concentration of the sample.

Protein Concentration

1. Make different concentrations of Bovine Serum Albumin (BSA), such as 1, 2, 3, 4…. µg per ml of Bio-Rad protein assay solution.
2. Prepare a standard graph in the computer, or you can make manually (more preferable).
3. Add around 1 or 2µl of each sample in 1 ml of Bio-Rad protein assay solution.
4. Measure the OD and concentration for each sample.
5. Load around 15-20 µg of each protein.

SDS-PAGE Electrophoresis

1. Use Bio-Rad mini-protein tetra system with mini protein TGX precast PAGE gels.
2. Usetris-glycine running buffer to run gel.

3. Amount of volume to be loaded for various samples is determined from the graph.
4. Run gel at 130-180 V for approximately 40 min to 1 hr. Higher voltage causes smiling of gel.

Protein immunoblotting (Western Blotting).

1. Soak the gel in Towbin buffer after electrophoresis for 15 min.
2. Prepare Bio-Rad semidry transfer system.
3. Cut nitrocellulose membrane and filter paper and soak in Towbin buffer.
4. Assemble the sandwich with nitrocellulose membrane below the PAGE gel, with 3 filter papers on top and below the arrangement.
5. Run it for 15 min at 12V and 500 mA.
6. Stain the nitrocellulose membrane with Ponceau red until bands develop.
7. Stain gel with Coomassie blue for overnight and add de-staining solution next day for 4-5 hours.
8. Wash Ponceau red with distilled water and add milk for 30min.

Antibody treatment

1. Prepare solution of primary antibody (Ab), using NC32, PB32,at ratios of 1:2400 or 1:3000Ab:TBST solution, respectively.
2. Pour off milk, wash nitrocellulose membrane with TBST, and add primary Ab and keep on shaker for 1 hr.
3. Wash the NC membrane with TBST for 3 times, 5 min each.

4. Prepare solution of secondary Ab (Anti rabbit conjugate). Add the secondary Ab and keep on shaker for 1 hr.
5. Wash the NC membrane with TBST for 3 times, 5 min each.
6. Wash with TBS (not TBST) for 5 min.
7. Prepare Alkaline Phosphate solution. (10 ml of Alkaline phosphate, 66µl NBT and 33 µl BCIP)
8. Add alkaline phosphate solution and keep it until the bands develop.

Extraction Buffer

Biorad2× Lamelli Buffer	8ml
Distill H_2O	4ml
DTT	0.432g

Keep on ice while using. Store at -20^{o}C.

Towbin Buffer

Tris base	3.03 g
Glycine	14.4g
Methanol	200 ml
Distill H_2O	800 ml
pH	8.6

Tris glycine running buffer

Tris base	3 g
Glycine	14.4 g
SDS	1 g
Distill H_2O	1000 ml
pH	8.3

TBS (Tris-buffered Saline) buffer

Tris base	6.05 g
NaCl	8.76 g
Distill H_2O	1000 ml
pH	7.6

TBST- Add 1 ml of Tween-20 to 1000 ml TBS buffer.

Destaining Solution
H_2O: methanol: acetic acid = 5:4:1(v/v/v)

Fungal Culture maintenance and sporulation

Botrytis cinerea

1. Inoculate *B. cinerea* spores on oatmeal agar plate.
2. Maintain plates at 21°C with 8/16 hrs of light/dark photoperiod in a humidity chamber.
3. Do not seal the plates with parafilm.
4. Greyish mycelium is an indication of good sporulation. If greenish growth is seen, then it is not *B. cinerea.*
5. Significant sporulation should be present by 10 to 14 days.
6. Now overlay the sporulating plates with 10-12 ml sterile 15% glycerol with 0.01% Tween 80.
7. Use a sterile spreader and mix the mycelium with the solution. Collect the resulting spore suspensions.
8. Filter the spore suspension through two layers of sterile cheesecloth to separate spores from mycelia.
9. Quantify spores using a hemocytometer and dilute to the desired concentration using sterile 15% glycerol with 0.01% Tween 80.

10. For long-term storage keep the spores at -20°C.
11. Thaw the spores when needed and discard the thawed culture once used. Do not refreeze and reuse.

Alternaria solani

1. Inoculate *A. solani* spores on Potato Dextrose Agar (PDA) plate at pH-5. As *A. solani* spores wells in acidic conditions.
2. Maintain plates at 25°C with 8/16 hrs light/dark photoperiod in a chamber. Temperatures below 23°C will lead to no sporulation.
3. Seal the plates with parafilm.
4. Blackish mycelium is indication of good sporulation. If whitish growth is seen, then *A. solani* is not sporulating.
5. Significant sporulation should be present by 14 to 21 days.
6. Now overlay the sporulating plates with 10-12 ml sterile 15% glycerol with 0.01% Tween 80.
7. Use a sterile spreader to mix the mycelium with the solution and collect the resulting spore suspensions.
8. Filter the spore suspension through two layers of sterile cheesecloth to separate spores from mycelia.
9. Quantify spores using a hemocytometer and dilute to the desired concentration using sterile 15% glycerol with 0.01% Tween 80.
10. For long-term storage keep the spores at -20°C.

11. Thaw the spores when needed and discard the thawed culture once used. Do not refreeze and reuse.

Detached leaf assay

1. Collect young and/or mature healthy leaves from plants.
2. Wash them tenderly without damaging the tissues. Dry using paper towels.
3. Place abaxial sides up on wire mesh suspended above water in a transparent plastic container with a lid.
4. Put 5 µl of fungal frozen stock spore suspension of desired concentration on leaf at least 6-7 mm apart from other spots to be inoculated.
5. Allow the spore suspension to dry briefly.
6. Pierce these spots with a sterile needle to assist infection.
7. Close the box and place it inside another plastic box containing wet paper towels at bottom.
8. Keep the bigger box at room temperature with an approximately 16/8 hrs light/dark photoperiod.
9. Decide a time line for symptom development assessment, mainly based on merging of two lesions *B. cinerea* on tomato takes around 5 days.
10. Record the area of each lesion at 24 hrs intervals until lesions have merged.
11. Score lesion areas using the area measurement functions of software like Adobe Photoshop™ software suite (Image > Analysis > Set measurement scale > Record measurement).

12. Keep a real physical measurement unit inside the photographs, as most software requires setting a standard measurement unit.

Fungal resistance seedling assay

Growing transgenic seeds.

1. Prepare media with ½MS salts, kanamycin (100 mg/L), and agar (6 g/L) at pH 5.6.
2. Incubate surface sterile transgenic seeds on the media. Seed sterilization method can be in genetic transformation protocol.
3. Grow non-transgenic and vector transformed controls on non-kanamycin media.
4. Keep the media boxes at 24-25°C under white fluorescent lights.
5. Mark the seeds as they germinate, at the bottom of the container.
6. Within 7-10 days 6-8 cm transgenic seedlings will be ready.
7. Transfer the seedlings to small plastic pots with soil and cover with clear plastic cover at 25°C for a week to acclimate. Keep the humidity high during acclimatization.
8. Seedlings will acclimate within 7-10 days.
9. Remove the cover and let seedlings grow up to 12-15 cm.
10. These seedlings can be used for assay.

Seedling assay

1. Arrange seedlings in random complete block design.
2. Spray with the desired fungal spore concentration. Do not overspray; this may kill all seedlings irrespective of the transgene.

3. Assess symptom development for each day post inoculation until either seedlings start dying of infection or new growth appears on less infected seedlings.
4. Symptoms can be recorded as percent seedling area infected with total canopy area as 100 %.

APPENDIX B

STATISTICAL ANALYSIS REPORT FOR SECONG GENERATION SEEDLING ASSAY

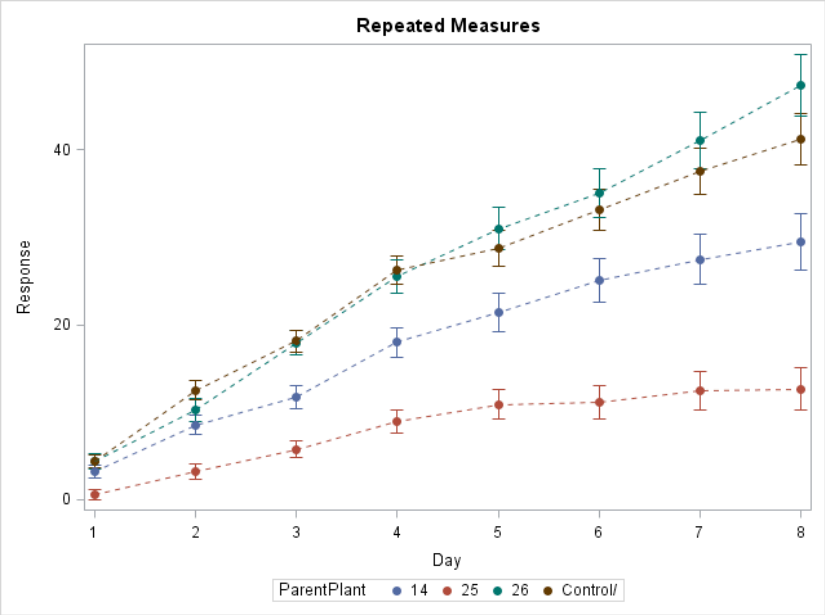
All the statistical analyses in these reports were carried out using Statistical Analysis Software (SAS, version 9.3, Cary, NC).

Statistical Report 1: 'Moneymaker' second generation transgenic seedlings Vs. *Botrytis cinerea*

Linear model for first data set with gene numbers combined.

	Day															
Line	1		2		3		4		5		6		7		8	
14	3.20	A, B	8.56	A	11.75	B	17.97	B	21.40	B	25.08	A	27.45	B	29.48	B
25	0.58	B	3.23	B	5.77	C	8.94	C	10.92	C	11.08	B	12.49	C	12.65	C
26/VT	4.43	A	10.25	A	17.95	A	25.52	A	30.99	A	35.05	A	41.05	A	47.34	A
Control/WT	4.38	A	12.50	A	18.13	A	26.25	A	28.75	A, B	33.13	A	37.50	A, B	41.25	A, B

Means with different letters are statistically different for the respective days



The GLM Procedure

Class Level Information

Class	Levels	Values
ParentPlant	4	14 25 26 Control/WT
Tray	8	1 2 3 4 5 6 7 8

Number of Observations Read	34
Number of Observations Used	34

The GLM Procedure
Repeated Measures Analysis of Variance

Repeated Measures Level Information

Dependent Variable	Response1	Response2	Response3	Response4	Response5	Response6	Response7	Response8
Level of Day	1	2	3	4	5	6	7	8

MANOVA Test Criteria and Exact F Statistics for the Hypothesis of no Day Effect
H = Type III SSCP Matrix for Day
E = Error SSCP Matrix

S=1 M=2.5 N=7.5

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.04538193	51.09	7	17	<.0001
Pillai's Trace	0.95461807	51.09	7	17	<.0001
Hotelling-Lawley Trace	21.03520329	51.09	7	17	<.0001
Roy's Greatest Root	21.03520329	51.09	7	17	<.0001

MANOVA Test Criteria and F Approximations for the Hypothesis of no Day*ParentPlant Effect
H = Type III SSCP Matrix for Day*ParentPlant
E = Error SSCP Matrix

S=3 M=1.5 N=7.5

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.12501345	2.50	21	49.365	0.0042
Pillai's Trace	1.18907197	1.78	21	57	0.0438
Hotelling-Lawley Trace	4.71616573	3.61	21	29.447	0.0007
Roy's Greatest Root	4.24516216	11.52	7	19	<.0001

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

MANOVA Test Criteria and F Approximations for the Hypothesis of no Day*Tray Effect
H = Type III SSCP Matrix for Day*Tray
E = Error SSCP Matrix

S=7 M=-0.5 N=7.5

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.06063106	1.36	49	90.729	0.1008
Pillai's Trace	2.08739185	1.40	49	161	0.0637
Hotelling-Lawley Trace	4.03827271	1.30	49	42.435	0.1943
Roy's Greatest Root	1.84832788	6.07	7	23	0.0004

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

The GLM Procedure
Repeated Measures Analysis of Variance
Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ParentPlant	3	15462.86408	5154.28803	34.08	<.0001
Tray	7	4343.74950	620.53564	4.10	0.0046
Error	23	3478.64633	151.24549		

The GLM Procedure
Repeated Measures Analysis of Variance
Univariate Tests of Hypotheses for Within Subject Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F	AdjPr> F	
						G - G	H-F-L
Day	7	21264.20195	3037.74314	222.70	<.0001	<.0001	<.0001
Day*ParentPlant	21	3882.81872	184.89613	13.56	<.0001	<.0001	<.0001
Day*Tray	49	1379.43331	28.15170	2.06	0.0004	0.0319	0.0264
Error(Day)	161	2196.08753	13.64030				

Greenhouse-Geisser Epsilon	0.2912
Huynh-Feldt-Lecoutre Epsilon	0.3198

The GLM Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

Day_N represents the nth successive difference in Day

Contrast Variable: Day_1

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	849.2770703	849.2770703	94.55	<.0001
ParentPlant	3	145.4856889	48.4952296	5.40	0.0058
Tray	7	173.6106889	24.8015270	2.76	0.0308
Error	23	206.5976445	8.9825063		

Contrast Variable: Day_2

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	639.7591019	639.7591019	77.12	<.0001
ParentPlant	3	123.3677580	41.1225860	4.96	0.0085
Tray	7	178.9927580	25.5703940	3.08	0.0192
Error	23	190.7989087	8.2956047		

Contrast Variable: Day_3

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	1107.800848	1107.800848	93.75	<.0001
ParentPlant	3	142.394474	47.464825	4.02	0.0195
Tray	7	73.019474	10.431353	0.88	0.5352
Error	23	271.772193	11.816182		

Contrast Variable: Day_4

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	315.0059444	315.0059444	32.36	<.0001
ParentPlant	3	48.6069383	16.2023128	1.66	0.2024
Tray	7	107.3569383	15.3367055	1.58	0.1927
Error	23	223.8930617	9.7344809		

Contrast Variable: Day_5

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	265.7138837	265.7138837	35.93	<.0001
ParentPlant	3	114.0508853	38.0169618	5.14	0.0072
Tray	7	95.5092186	13.6441741	1.84	0.1265
Error	23	170.1157814	7.3963383		

Contrast Variable: Day_6

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	352.4845005	352.4845005	43.97	<.0001
ParentPlant	3	96.8658469	32.2886156	4.03	0.0193
Tray	7	61.4491802	8.7784543	1.10	0.3987
Error	23	184.3841531	8.0167023		

Contrast Variable: Day_7

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	263.8014556	263.8014556	20.67	0.0001
ParentPlant	3	157.3397532	52.4465844	4.11	0.0179
Tray	7	134.6314199	19.2330600	1.51	0.2143
Error	23	293.4935801	12.7605904		

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

ParentPlant	Response1 LSMEAN	LSMEAN Number
14	3.20177918	1
25	0.57711158	2
26	4.43186560	3
Control/WT	4.37500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response1

i/j	1	2	3	4
1		0.0582	0.7086	0.6957
2	0.0582		0.0072	0.0029
3	0.7086	0.0072		1.0000
4	0.6957	0.0029	1.0000	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

		Response1 LSMEAN	ParentPlant	LSMEAN Number
	A	4.43186560	**26**	3
	A			
	A	4.37500000	**Control/WT**	4
	A			
B	A	3.20177918	**14**	1
B				
B		0.57711158	**25**	2

ParentPlant	Response2 LSMEAN	LSMEAN Number
14	8.5603267	1
25	3.2332297	2
26	10.2502814	3
Control/WT	12.5000000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response2

i/j	1	2	3	4
1		0.0059	0.7511	0.0877
2	0.0059		0.0009	<.0001
3	0.7511	0.0009		0.5497
4	0.0877	<.0001	0.5497	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

	Response2 LSMEAN	ParentPlant	LSMEAN Number
A	12.500000	**Control/WT**	4
A			
A	10.250281	**26**	3
A			
A	8.560327	**14**	1
B	3.233230	**25**	2

ParentPlant	Response3 LSMEAN	LSMEAN Number
14	11.7533349	1
25	5.7746521	2
26	17.9488774	3
Control/WT	18.1250000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response3

i/j	1	2	3	4
1		0.0060	0.0168	0.0079
2	0.0060		<.0001	<.0001
3	0.0168	<.0001		0.9997
4	0.0079	<.0001	0.9997	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

	Response3 LSMEAN	ParentPlant	LSMEAN Number
A	18.125000	**Control/WT**	4
A			
A	17.948877	**26**	3
B	11.753335	**14**	1
C	5.774652	**25**	2

ParentPlant	Response4 LSMEAN	LSMEAN Number
14	17.9705621	1
25	8.9377202	2
26	25.5220232	3
Control/WT	26.2500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response4

i/j	1	2	3	4
1		0.0015	0.0283	0.0085
2	0.0015		<.0001	<.0001
3	0.0283	<.0001		0.9910
4	0.0085	<.0001	0.9910	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

	Response4 LSMEAN	ParentPlant	LSMEAN Number
A	26.25000	**Control/WT**	4
A			
A	25.52202	**26**	3
B	17.97056	**14**	1
C	8.93772	**25**	2

ParentPlant	Response5 LSMEAN	LSMEAN Number
14	21.4001859	1
25	10.9162611	2
26	30.9868654	3
Control/WT	28.7500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response5

i/j	1	2	3	4
1		0.0043	0.0312	0.0956
2	0.0043		<.0001	<.0001
3	0.0312	<.0001		0.8960
4	0.0956	<.0001	0.8960	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

		Response5 LSMEAN	ParentPlant	LSMEAN Number
	A	30.98687	**26**	3
	A			
B	A	28.75000	**Control/WT**	4
B				
B		21.40019	**14**	1
	C	10.91626	**25**	2

ParentPlant	Response6 LSMEAN	LSMEAN Number
14	25.0758875	1
25	11.0841162	2
26	35.0505270	3
Control/WT	33.1250000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response6

i/j	1	2	3	4
1		0.0008	0.0519	0.1106
2	0.0008		<.0001	<.0001
3	0.0519	<.0001		0.9500
4	0.1106	<.0001	0.9500	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

	Response6 LSMEAN	ParentPlant	LSMEAN Number
A	35.05053	**26**	3
A			
A	33.12500	**Control/WT**	4
A			
A	25.07589	**14**	1
B	11.08412	**25**	2

ParentPlant	Response7 LSMEAN	LSMEAN Number
14	27.4479566	1
25	12.4879233	2
26	41.0458479	3
Control/WT	37.5000000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response7

i/j	1	2	3	4
1		0.0018	0.0175	0.0760
2	0.0018		<.0001	<.0001
3	0.0175	<.0001		0.8302
4	0.0760	<.0001	0.8302	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

		Response7 LSMEAN	ParentPlant	LSMEAN Number
	A	41.04585	**26**	3
	A			
B	A	37.50000	**Control/WT**	4
B				
B		27.44796	**14**	1
	C	12.48792	**25**	2

ParentPlant	Response8 LSMEAN	LSMEAN Number
14	29.4769092	1
25	12.6526546	2
26	47.3401030	3
Control/WT	41.2500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response8

i/j	1	2	3	4
1		0.0015	0.0041	0.0547
2	0.0015		<.0001	<.0001
3	0.0041	<.0001		0.5578
4	0.0547	<.0001	0.5578	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant

LS-means with the same letter are not significantly different.

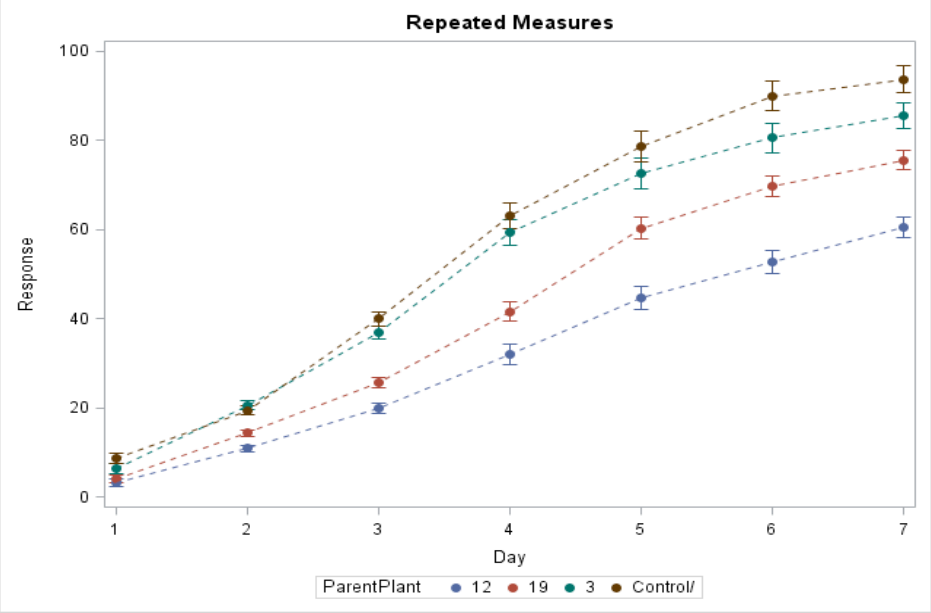
		Response8 LSMEAN	ParentPlant	LSMEAN Number
	A	47.34010	**26**	3
	A			
B	A	41.25000	**Control/WT**	4
B				
B		29.47691	**14**	1
	C	12.65265	**25**	2

Statistical Report 2: 'NC1-Grape' second generation transgenic seedlings Vs. *Alternaria solani*

Linear model for second data set with gene numbers combined.

Day														
Line	1		2		3		4		5		6		7	
12	3.18	B	10.87	C	20.05	C	32.14	C	44.60	C	52.6	C	60.54	C
19	4.06	B	14.38	B	25.63	B	41.56	B	60.31	B	69.6	B	75.63	B
3/VT	6.25	A,B	20.63	A	36.88	A	59.38	A	72.50	A	80.6	A, B	85.63	A
Control/WT	8.75	A	19.38	A	40.00	A	63.13	A	78.75	A	90	A	93.75	A

Means with different letters are statistically different for the respective days



The GLM Procedure

Class Level Information

Class	Levels	Values
ParentPlant	4	12 19 3 Control/WT
Tray	8	1 2 3 4 5 6 7 8

Number of Observations Read	46
Number of Observations Used	46

The GLM Procedure
Repeated Measures Analysis of Variance

Repeated Measures Level Information

Dependent Variable	Response1	Response2	Response3	Response4	Response5	Response6	Response7
Level of Day	1	2	3	4	5	6	7

MANOVA Test Criteria and Exact F Statistics for the Hypothesis of no Day Effect
H = Type III SSCP Matrix for Day
E = Error SSCP Matrix

S=1 M=2 N=14

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.00999463	495.27	6	30	<.0001
Pillai's Trace	0.99000537	495.27	6	30	<.0001
Hotelling-Lawley Trace	99.05375338	495.27	6	30	<.0001
Roy's Greatest Root	99.05375338	495.27	6	30	<.0001

MANOVA Test Criteria and F Approximations for the Hypothesis of no Day*ParentPlant Effect
H = Type III SSCP Matrix for Day*ParentPlant
E = Error SSCP Matrix

S=3 M=1 N=14

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.11980344	5.30	18	85.338	<.0001
Pillai's Trace	1.38011961	4.54	18	96	<.0001
Hotelling-Lawley Trace	3.79492102	6.13	18	54.267	<.0001
Roy's Greatest Root	2.83961737	15.14	6	32	<.0001

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

MANOVA Test Criteria and F Approximations for the Hypothesis of no Day*Tray Effect
H = Type III SSCP Matrix for Day*Tray
E = Error SSCP Matrix

S=6 M=0 N=14

Statistic	Value	F Value	Num DF	Den DF	Pr > F
Wilks' Lambda	0.03844800	3.44	42	144.16	<.0001
Pillai's Trace	2.22009596	2.94	42	210	<.0001
Hotelling-Lawley Trace	5.36840106	3.67	42	80.092	<.0001
Roy's Greatest Root	2.70280624	13.51	7	35	<.0001

NOTE: F Statistic for Roy's Greatest Root is an upper bound.

The GLM Procedure
Repeated Measures Analysis of Variance
Tests of Hypotheses for Between Subjects Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ParentPlant	3	25895.88542	8631.96181	40.94	<.0001
Tray	7	15353.63627	2193.37661	10.40	<.0001
Error	35	7379.59077	210.84545		

The GLM Procedure
Repeated Measures Analysis of Variance
Univariate Tests of Hypotheses for Within Subject Effects

Source	DF	Type III SS	Mean Square	F Value	Pr > F	Adj Pr > F	
						G - G	H-F-L
Day	6	203760.4258	33960.0710	1333.19	<.0001	<.0001	<.0001
Day*ParentPlant	18	6438.5532	357.6974	14.04	<.0001	<.0001	<.0001
Day*Tray	42	6529.5226	155.4648	6.10	<.0001	<.0001	<.0001
Error(Day)	210	5349.3039	25.4729				

Greenhouse-Geisser Epsilon	0.3866
Huynh-Feldt-Lecoutre Epsilon	0.4157

The GLM Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

Day_N represents the contrast between the nth level of Day and the last

Contrast Variable: Day_1

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	223197.6007	223197.6007	3247.46	<.0001
ParentPlant	3	4685.2894	1561.7631	22.72	<.0001
Tray	7	2537.9828	362.5690	5.28	0.0004
Error	35	2405.5440	68.7298		

Contrast Variable: Day_2

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	162545.2711	162545.2711	2848.89	<.0001
ParentPlant	3	3317.2164	1105.7388	19.38	<.0001
Tray	7	3073.1391	439.0199	7.69	<.0001
Error	35	1996.9502	57.0557		

Contrast Variable: Day_3

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	96632.14464	96632.14464	1866.19	<.0001
ParentPlant	3	1099.35185	366.45062	7.08	0.0008
Tray	7	1771.61376	253.08768	4.89	0.0006
Error	35	1812.31481	51.78042		

Contrast Variable: Day_4

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	36952.48061	36952.48061	816.85	<.0001
ParentPlant	3	407.51157	135.83719	3.00	0.0435
Tray	7	879.84788	125.69255	2.78	0.0208
Error	35	1583.32176	45.23776		

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	9146.959459	9146.959459	272.25	<.0001
ParentPlant	3	41.562500	13.854167	0.41	0.7451
Tray	7	353.303571	50.471939	1.50	0.1988
Error	35	1175.937500	33.598214		

Contrast Variable: Day_6

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Mean	1	1323.668199	1323.668199	87.50	<.0001
ParentPlant	3	98.015046	32.671682	2.16	0.1103
Tray	7	606.452546	86.636078	5.73	0.0002
Error	35	529.484954	15.128142		

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

ParentPlant	Response1 LSMEAN	LSMEAN Number
12	3.17708333	1
19	4.06250000	2
3	6.25000000	3
Control/WT	8.75000000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response1

i/j	1	2	3	4
1		0.8790	0.1627	0.0025
2	0.8790		0.4140	0.0102
3	0.1627	0.4140		0.4230
4	0.0025	0.0102	0.4230	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter are not significantly different.

		Response1 LSMEAN	ParentPlant	LSMEAN Number
	A	8.7500000	**Control/ WT**	4
	A			
B	A	6.2500000	**3**	3
B				
B		4.0625000	**19**	2
B				
B		3.1770833	**12**	1

ParentPlant	Response2 LSMEAN	LSMEAN Number
12	10.8680556	1
19	14.3750000	2
3	20.6250000	3
Control/	19.3750000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response2

i/j	1	2	3	4
1		0.0126	<.0001	<.0001
2	0.0126		0.0001	0.0020
3	<.0001	0.0001		0.8275
4	<.0001	0.0020	0.8275	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter are not significantly different.

	Response2 LSMEAN	ParentPlant	LSMEAN Number
A	20.62500	**3**	3
A			
A	19.37500	**Control/ WT**	4
B	14.37500	**19**	2
C	10.86806	**12**	1

ParentPlant	Response3 LSMEAN	LSMEAN Number
12	20.0520833	1
19	25.6250000	2
3	36.8750000	3
Control/	40.0000000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response3

i/j	1	2	3	4
1		0.0060	<.0001	<.0001
2	0.0060		<.0001	<.0001
3	<.0001	<.0001		0.4703
4	<.0001	<.0001	0.4703	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter are not significantly different.

	Response3 LSMEAN	ParentPlant	LSMEAN Number
A	40.00000	**Control/ WT**	4
A			
A	36.87500	**3**	3
B	25.62500	**19**	2
C	20.05208	**12**	1

ParentPlant	Response4 LSMEAN	LSMEAN Number
12	32.1354167	1
19	41.5625000	2
3	59.3750000	3
Control/ WT	63.1250000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response4

i/j	1	2	3	4
1		0.0232	<.0001	<.0001
2	0.0232		0.0001	<.0001
3	<.0001	0.0001		0.8130
4	<.0001	<.0001	0.8130	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter
are not significantly different.

	Response4 LSMEAN	ParentPlant	LSMEAN Number
A	63.12500	**Control/ WT**	4
A			
A	59.37500	**3**	3
B	41.56250	**19**	2
C	32.13542	**12**	1

ParentPlant	Response5 LSMEAN	LSMEAN Number
12	44.6006944	1
19	60.3125000	2
3	72.5000000	3
Control/ WT	78.7500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response5

i/j	1	2	3	4
1		0.0006	<.0001	<.0001
2	0.0006		0.0324	0.0006
3	<.0001	0.0324		0.5812
4	<.0001	0.0006	0.5812	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter are not significantly different.

	Response5 LSMEAN	ParentPlant	LSMEAN Number
A	78.75000	**Control/ WT**	4
A			
A	72.50000	**3**	3
B	60.31250	**19**	2
C	44.60069	**12**	1

ParentPlant	Response6 LSMEAN	LSMEAN Number
12	52.6388889	1
19	69.6875000	2
3	80.6250000	3
Control/ WT	90.0000000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response6

i/j	1	2	3	4
1		0.0001	<.0001	<.0001
2	0.0001		0.0541	0.0001
3	<.0001	0.0541		0.2162
4	<.0001	0.0001	0.2162	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter are not significantly different.

		Response6 LSMEAN	ParentPlant	LSMEAN Number
	A	90.00000	**Control/ WT**	4
	A			
B	A	80.62500	**3**	3
B				
B		69.68750	**19**	2
	C	52.63889	**12**	1

ParentPlant	Response7 LSMEAN	LSMEAN Number
12	60.5381944	1
19	75.6250000	2
3	85.6250000	3
Control/WT	93.7500000	4

Least Squares Means for effect ParentPlant
Pr> |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: Response7

i/j	1	2	3	4
1		0.0001	<.0001	<.0001
2	0.0001		0.0415	<.0001
3	<.0001	0.0415		0.2247
4	<.0001	<.0001	0.2247	

Tukey-Kramer Comparison Lines for Least Squares Means of ParentPlant
LS-means with the same letter
are not significantly different.

	Response7 LSMEAN	ParentPlant	LSMEAN Number
A	93.75000	**Control/WT**	4
A			
A	85.62500	**3**	3
B	75.62500	**19**	2
C	60.53819	**12**	1

APPENDIX C

DESIGN PROTOCOLS FOR TRANSGENIC FIELD TRIALS

Design Protocols for MTD-1

PerformanceStandard1. To prevent dissemination during transit, transgenic seed will be packaged for transport in screw-top, heavy-duty plastic tubes, placed in a padded cardboard box (meeting requirements of 7 CFR 340.8), taped shut, labeled "MTD transgenic tomato seed" and hand-carried by state vehicle from the NCSU campus to Mills River. At the destination facility the labeled package will be stored in a locked cabinet in Dr. Dilip Panthee's Laboratory (itself a locked, controlled access facility) until planting. To prevent release in transit to the field the material will be transported in the unopened shipping tubes to the planting site, again in a state vehicle.

Performance Standard2. Although all plants will be deflowered to prevent mixing, plots used for these experiments will be separated from adjacent plots by 50 feet and planted with 18"x45" plant-to-plant and row-to-row spacing, respectively. Plants are weeded, deflowered and harvested by hand so no mechanical mixing of plant materials will occur. All plant materials removed from the plot (trimmings, flowers etc.) will be placed in an autoclave bag returned to the lab and autoclaved. Bags will be prominently labeled as transgenic material.

Performance Standard3. The planting site is isolated from other tomato planting sites. It will be flagged with yellow flags so that people are aware of the plants. A sign with a label 'Transgenic plants being grown, do not handle' will be placed at the four corners of the plot.

At the conclusion of the tests, all material will be destroyed by using non-selective herbicide and tilled under

PerformanceStandard4. To ensure that no viable vector agent is associated with the regulated material, tissues/cells during transformation/regeneration were selected on 100 mg/l carbenicillin or 300 mg/l timentin.

PerformanceStandard5. To prevent pollen release and possible cross contamination, plants will be scouted daily and deflowered as necessary. Sample size is intentionally kept small to allow emasculation and weeding. Area to be planted is fenced to prevent animal incursion, or public access.

PerformanceStandard6. Although plants will be scouted daily and deflowered as necessary to prevent persistence in the field, plots will be planted in an alternate crop (that can be readily differentiated from the regulated material) the following year and monitored weekly to ensure no "volunteers" appear. The release will be terminated by destruction of the plants using a nonselective herbicide and tilled under. Destruction/devitalization of plant and plant material remaining at the field release site will occur on or before the expiration date of this notification.

APPENDIX D

TRANSGENIC FIELD TRAIL REPORTS

‘Moneymaker’ and ‘NC1Grape’ Transgenic field trial 2013.

Duration:	May (1st week) 2013 to July (4th week) 2013.
Place:	Mountain Horticultural Crops Research and Extension Center, 455 Research Drive, Mills River, NC 28759.
Advisors:	Dr. John Williamson and Dr. Dilip Panthee (on-site).
Student:	Mr. Takshay Patel.
Gene of Interest:	Celery Mannitol dehydrogenase (*Mtd*).
Transgene:	Celery *Mtd* cDNA.
Lines:	T_1 (Second) generation of MTD high expressing T_0 (First) plants.
Total plants:	T_1 generation of four ‘Moneymaker’ (MM) and four ‘NC1Grape’ (NCG), with 50 plants for each T_0 plant. Total 400 transgenic plants and 96 controls (38 Non-transgenic MM, 35 Non-transgenic NCG and 23 non-transgenic NC1CELBR).
Pathogens:	*Alternaria solani* and Late Blight (*Phytophthora infestans*). *Alternaria* was inoculated at 20,000 conidia/ml. Late blight was expected to infect from naturally occurring population.
Objectives:	1) Confirm the presence of *Mtd* transgene in the segregating T_1 population by DNA primers. 2) Evaluate pathogen development on T_1 plants with the transgene

3) Follow regulations for transgenic field trials. Avoid pollen escape by picking flower at very early stage of development. Collect all transgenic plant material produced by pruning and harvest and autoclave it before discarding.

Field trial on transgenic MM and NCG second-generation (T_1) was carried out. The main purpose of this experiment was to see whether *Mtd* provides field resistance to *Alternaria*. Seeds were collected from selfed T_0 transgenic plants that expressed high levels of MTD and showed high resistance to *Botrytis cinerea* during laboratory detached leaf assays (MM only). Planting of plot with nearly 500 plants including transgenic and control plants was designed. MM T_1 plants were from MM25, MM33, MM2, MM7 lines and NCG T_1 plants were from NCG3, NCG13, NCG12, and NCG11 lines. The line NC1CELBR (ELBR = Early and Late Blight Resistant) was included as a resistant control in the trial.

During the 2nd week of May, 50 seeds of each transgenic line were germinated in shell trays. In 2nd week of June, plants were transplanted from trays to the field. 50 plants for each T_1 line were planted in rows, with a total of 8 randomly allotted rows for 8 T_1 lines and 2 rows of three control lines. Flowers were collected in the early stage of development (well before opening). Flower collection was started as soon as transgenic plants began flowering and regularly continued twice per week till the end of the trial.

From the segregating T_1 population, leaf samples were collected on 9th July. DNA was isolated by a high throughput protocol on 12th July. Further, PCR was carried out on all T_1 plants to confirm the presence of the *Mtd* transgene using appropriate DNA primers. Each

50 plant T_1 population had from 12 to 19 verified transgenic *Mtd* plants. Only plants showing *Mtd* bands in PCR analyses were to be considered for assessing the pathogen resistance.

Parallel preparation of *Alternaria* spores was made in Dr. Panthee's laboratory. On 19th June, a field isolate of *Alternaria* was inoculated onto 50 large Petri dishes containing potato dextrose agar (PDA) (Table 1). On 15th July *Alternaria* spores were isolated from the plates and plants were inoculated on the same day at 7.30 am. The spore concentration used was 20,000 conidia/ml. No laboratory preparations were made for late blight (LB), because LB occurs naturally in that region around the 1st week of August.

Disease scoring commenced the day following *Alternaria* inoculation. Unfortunately, LB occurred early that season (around the 2nd week of July) and infected the transgenic plot the same week *Alternaria* was inoculated. Relative to *Alternaria.,* LB infection is more rapid so within a week the plot was totally infected by LB. Very few plants had developed *Alternaria* symptoms by that time, and those that had were not present in sufficient numbers to make any definite conclusions about *Mtd* resistance.

In the last week of July the plot was sprayed with Roundup® and all the plants were killed. The field trial was concluded.

During the whole trial, NCDA and USDA regulations for transgenic field trial were followed. All plant materials including flowers and stems were collected in biohazard bags. The bags were then autoclaved and discarded properly. The transgenic plot was isolated and more than 50 feet away from other research plots. Plot will not be replanted with tomato next year.

Observation and Results:

Due the early occurrence of LB it was impossible to conclude anything regarding the *Alternaria* resistance in the transgenic plants. No resistance against LB was observed in any of the *Mtd* containing T1 plants. Lack of resistance to late blight was expected, because the MTD enzyme is effective only against pathogens secreting mannitol (1). The LB pathogen *Phytophthora infestans*is an oomycete and does not secrete mannitol (2). Hence the only conclusion to be drawn from this experiment is that MTD does not work against non-mannitol secreting pathogens (in this case against LB).

References:

Jennings D, Daub M, Pharr D, Williamson J (2002) Constitutive expression of a celery mannitol dehydrogenase in tobacco enhances resistance to the mannitol-secreting fungal pathogen *Alternaria alternata*. *The Plant Journal: For Cell and Molecular Biology,* **32**: 41-49.

Pfyffer G, Boraschi-Gaia C, Weber B, Hoesch L, Orpin C, &Rast D (1990) A further report on the occurrence of acyclic sugar alcohols in fungi. Mycol Res **94**: 219-222.

Table 1. *Alternaria* growth requirements:

Media	Potato Dextrose Agar
pH	5
Temperature	25^{o}C
Light period	16 hours/day
Spore isolation	Sterile water with 0.1% Tween-20
Strain	Field Isolate from HCREC provided by Dr. Frank Louis, NCSU

‘NC1Grape’ Transgenic field trial 2014.

Duration:	May (3rd week) 2014 to August (2nd week) 2014.
Place:	Mountain Horticultural Crops Research and Extension Center, 455 Research Drive, Mills River, NC 28759.
Advisors:	Dr. John Williamson and Dr. Dilip Panthee (on-site).
Student:	Mr. Takshay Patel.
Gene of Interest:	Celery Mannitol dehydrogenase (*Mtd*).
Transgene:	Celery *Mtd* cDNA.
Lines:	T_1 (Second) generation of MTD high expressing T_0 (First) plants.
Total plants:	T_1 generation of four ‘NC1Grape’ (NCG), with around 60 plants for each T_0 plant. Total 228 transgenic plants and 86 controls (34 NC1CELBR resistant controls, 52 Non-transgenic susceptible NCG).
Pathogens:	*Alternaria solani*. *A. solani* was inoculated at 25,000 conidia/ml.
Objectives:	1) Confirm the presence of *Mtd* transgene in the segregating T_1 population by DNA primers. 2) Evaluate pathogen development on T_1 plants with the transgene 3) Follow regulations for transgenic field trials. Avoid pollen escape by picking flower at very early stage of development. Collect all transgenic plant material produced by pruning and harvest and autoclave it before discarding.

Field trial on transgenic NCG second-generation (T_1) was carried out. The main purpose of this experiment was to see whether *Mtd* provides field resistance to *Alternaria*. Seeds were collected from selfed T_0 transgenic plants that expressed high levels of MTD and showed high resistance to *Botrytis cinerea* during laboratory detached leaf assays (MM only). Planting of plot with nearly 300 plants including transgenic and control plants was designed. NCG T_1 plants were from NCG11, NCG12, NCG13 and NCG19 lines. The line NC1CELBR (ELBR = Early and Late Blight Resistant) was included as a resistant control in the trial.

During the 3rd week of May seeds of each transgenic line were germinated in shell trays. In 4th week of June, plants were transplanted from trays to the field. Transgenic plants were divided into two blocks in the same plot. Plants for each T_1 line were planted in rows, with a total of 8 randomly allotted two rows each for 4 T_1 lines and 4 rows of for two control lines. Flowers were collected in the early stage of development (well before opening). Flower collection was started as soon as transgenic plants began flowering and regularly continued twice per week till the end of the trial.

From the segregating T_1 population, leaf samples were collected on last week July. DNA was isolated by a high throughput protocol. Further PCR was carried out on all T_1 plants to confirm the presence of the *Mtd* transgene using appropriate DNA primers.

A. solani spores were grown in Dr. Williamson's laboratory at Raleigh. On 9th July, a field isolate of *Alternaria* was inoculated onto 50 large Petri dishes containing potato dextrose agar (PDA) (Table 1). On last week of July *A. solani* spores were isolated from the plates and plants were inoculated on the same day at 7.30 pm. The spore concentration used

was 25,000 conidia/ml. Plants were sprayed with specific late blight fungicides in order to control late blight on the plot.

Disease scoring commenced the day following *A. solani* inoculation. Unfortunately, LB again infected the transgenic plot. Relative to *Alternaria,* LB infection is more rapid so within a week the plot was totally infected by LB. Very few plants had developed *Alternaria* symptoms by that time, and those that had were too few to make any definite conclusions about *Mtd* resistance.

In the third week of August the plot was sprayed with Roundup® and all the plants were killed. The field trial was concluded.

During the whole trial, NCDA and USDA regulations for transgenic field trial were followed. All plant materials including flowers and stems were collected in biohazard bags. The bags were then autoclaved and discarded properly. The transgenic plot was isolated and more than 50 feet away from other research plots. Plot will not be replanted with tomato next year.

Observation and Results:

Due the early occurrence of LB it was impossible to conclude anything regarding the *Alternaria* resistance in the transgenic plants. No resistance against LB was observed in any of the *Mtd* containing T1 plants. Lack of resistance to late blight was expected, because the MTD enzyme is effective only against pathogens secreting mannitol (1). The LB pathogen *Phytophthora infestans*is an oomycete and does not secrete mannitol (2). Hence the only conclusion to be drawn from this experiment is that MTD does not work against non-mannitol secreting pathogens (in this case against LB). Although *A. solani* infection was

observed, all the plants were had nearly equal symptoms. This was likely mostly due to less late blight uninfected plant material.

Late blight infected plot

***A. solani* symptoms**

References:

Jennings D, Daub M, Pharr D, Williamson J (2002) Constitutive expression of a celery mannitol dehydrogenase in tobacco enhances resistance to the mannitol-secreting fungal pathogen *Alternaria alternata*. *The Plant Journal: For Cell and Molecular Biology,* **32**: 41-49.

Pfyffer G, Boraschi-Gaia C, Weber B, Hoesch L, Orpin C, &Rast D (1990) A further report on the occurrence of acyclic sugar alcohols in fungi. Mycol Res **94**: 219-222.