INTRODUCTION

Mannitol is a six-carbon non-cyclic sugar alcohol which appears to be widespread in the biosphere, with the noticeable exception of the animal kingdom. This polyol (i.e., alcohol containing multiple hydroxyl groups) is ubiquitous throughout the fungal kingdom and is considered as being the most abundant of all soluble carbohydrates within mycelia and fruit bodies (Lewis and Smith, 1967; Horer et al., 2001; Dulermo et al., 2009). In fungi, mannitol and its metabolism have been postulated to have a multitude of functions as either a carbohydrate reserve, in NADPH regeneration, in morphogenesis and conidiation or as a protection from environmental stress (Solomon et al., 2007). Furthermore, some studies have reported that mannitol has a role in pathogenicity of plant and animal pathogens (Chaturvedi et al., 1996a; Velez et al., 2008). Levels of mannitol were found to rise dramatically during plant infection by biotrophic or necrotrophic fungi and this accumulation was accompanied by increased expression of genes involved in the mannitol pathway (Voegele et al., 2005; Dulermo et al., 2009). Two hypotheses mainly emerged to explain the pathogenic significance of mannitol production by fungi. Firstly, fungal mannitol may be involved in the sequestration of carbohydrates from host. Since many plants are unable to metabolize mannitol, the conversion of plant hexoses into mannitol seems an ideal strategy for the fungal pathogen or mutualist, providing a means for fungi to store carbohydrates and reducing power in a form not accessible to the host (Ceccaroli et al., 2003; Dulermo et al., 2009). As in planta mannitol accumulation mainly occurred when conidiophores emerged, the latter authors suggest that this polyol could be necessary for spore survival or germination. Similar conclusions were suggested in the case of the biotrophic interaction between the rust fungus *Uromyces fabae* and its host plant *Vicia faba*, or during pathogenesis of *Sclerotinia sclerotiorum*, a *B. cinerea*-related necrotroph (Voegele et al., 2005; Jobic et al., 2007).

Secondly, mannitol is supposed to act as an antioxidant agent and protect fungal cells by quenching reactive oxygen species (ROS) produced by hosts in response to attack. Polyols can thus be powerful radical scavengers *in vitro* (Smirnoff and Cumbes, 1989; Voegele et al., 2005) and *in vivo* (Shen et al., 1997a,b). In the animal pathogen *Cryptococcus neoformans*, a mannitol low-producing mutant was hyper-susceptible to oxidative killing by normal human neutrophils and by cell-free oxidants, and was hypovirulent in mice (Chaturvedi et al., 1996a,b).
Moreover, transgenic tobacco lines constitutively expressing a celery mannitol dehydrogenase (MDH) or a plasma membrane mannitol transporter were shown to have enhanced resistance to pathogenic *Alternaria* species (Jennings et al., 2002; Juchaux-Cachau et al., 2007). These results suggested that both plant-expressed proteins supported the metabolism of fungal secreted mannitol, thus rendering the pathogen more susceptible to reactive oxygen-mediated plant defense. This hypothesis was further strengthened by the fact that the constitutive expression of the MDH transgene did not affect the pathogenicity of the non-mannitol-secreting fungal pathogen *Cercospora nicotianae*.

Mannitol metabolism in fungi was initially thought to be a cyclical process (Hult and Gatenbeck, 1978). In this cycle (Figure 1), mannitol-1-phosphate 5-dehydrogenase (MPD; EC 1.1.1.17) was proposed to reduce fructose 6-phosphate into mannitol-1-phosphate using the NADH cofactor, followed by dephosphorylation by a mannitol-1-phosphate phosphatase (MPP; EC 3.1.3.22), resulting in inorganic phosphate and mannitol. Mannitol would then be oxidized to fructose by mannitol dehydrogenase (MDH; EC 1.1.1.138) using the NADP⁺ cofactor. Finally, fructose would be phosphorylated to fructose 6-phosphate by a hexokinase (HX; EC 2.7.1.1). Dephosphorylation of mannitol-1-phosphate into mannitol via MPP was described as being irreversible. Consequently, the proposed cycle would go in one direction with MPD as the major biosynthetic enzyme and MDH as a catabolic enzyme. However, recent data based on gene disruption experiments indicated that mannitol metabolism is not a cyclical process (Solomon et al., 2006; Velez et al., 2007; Dulermo et al., 2010). According to these reports, mannitol synthesis and degradation were both severely impacted by the loss of MPD, while the deletion of MDH appeared to have a more limited effect. Moreover, the *mdh* strains were found to be able to use mannitol as a sole carbon source, indicating that mannitol was not only catabolized by oxidation to fructose. Dulermo et al. (2010) recently reported the existence of a mannitol phosphorylation pathway in *B. cinerea*, suggesting that mannitol could be metabolized through mannitol-1-phosphate.

The behavior of *mpd* and *mdh* null strains *in planta* also questioned the importance of the mannitol pathway in fungal pathogenicity. Indeed, regardless of the fungus involved (*A. alternata, S. nodorum* or *B. cinerea*), the virulence of the *mpd* and *mdh* strains was not or partially compromised (Solomon et al., 2005, 2006; Velez et al., 2008; Dulermo et al., 2010). Nevertheless, mannitol was shown to be required for *in planta* sporulation, which is a crucial step in a polycyclic pathogen like *S. nodorum* (Solomon et al., 2005, 2006).

In this study, we investigated the role of the mannitol pathway in the plant necrotrophic fungus *Alternaria brassicicola*. This fungus causes black spot disease and is an economically important seed-borne fungal pathogen of Brassicaceae species. We isolated the genes encoding the MPD and MDH enzymes in *A. brassicicola* and used targeted gene disruption to create single and double mutants for each gene. We then explored the physiological functions of mannitol metabolism and, in particular, its involvement...
in A. brassicicola pathogenicity and in the protection of fungal cells against defense compounds [like isothiocyanates (ITC)] and other environmental stresses.

**MATERIALS AND METHODS**

**FUNGAL STRAINS AND GROWTH CONDITIONS**

The A. brassicicola wild-type strain Abra43 used in this study has previously been described (Dongo et al., 2009; Joubert et al., 2011a). For routine culture, A. brassicicola was grown and maintained on potato dextrose agar (PDA) or on Vogel minimal medium (Vogel, 1956). For osmotic stress experiments mycelia were grown on PEG-infused agar plates (Verslues et al., 2006). Colony diameters were measured daily and used for calculation of radial growth. The method based on microscale liquid cultivation (from conidial suspensions) and automated nephelometric recording of growth, followed by extraction of relevant variables (lag time and growth rate), was described by Joubert et al. (2010). To study the susceptibility of fungal strains to ITC, allyl-ITC (AlITC), benzyl-ITC (BzITC) or phenethyl-ITC (PhITC), were diluted from stock solutions prepared in ace-

**ANALYSIS OF CELL VIABILITY**

Propidium iodide (PI) was used as a cell viability marker. Viable cells with intact membranes exclude PI, whereas the mem-

**GENERATION OF TARGETED GENE REPLACEMENT CONSTRUCTS AND FUNGAL TRANSFORMATION**

The gene replacement cassettes were generated using the double-

**NUCLEIC ACID ISOLATION AND ANALYSIS**

Genomic DNA extraction and Southern blot analysis were conducted as previously described (Joubert et al., 2011a). Total RNA extrac-

**INFECTION ASSAYS**

For plant infection assays on Brassica oleracea plants (var. Bartolo), 5 μL drops of A. brassicicola conidia suspension (10^5, 10^4 or 10^3 conidia/mL in water) were inoculated on leaves from 5 weeks-old plants. Inocula were symmetrically deposited on the left and right sides from the central vein. The plants were then maintained under saturating humidity (100% relative humidity). Symptoms were observed and samples collected at 2, 4, 6 days post-inoculation (dpi) for the determination of major soluble carbohydrates contents and AbMpd and AbMdh expression analysis. For in planta sporation assays, symptomatic tissues were sampled and vortexed for 30 s in water containing Tween 20 (0.02%, v/v). The concentration of the resulting conidia suspensions was estimated microscopically using a haemocytometer. For the microscopic analyses, B. oleracea leaf fragments were discolored, cleared and fungal structures were stained with solophenyl flavine 7GFE 500 (Ciba Specialty Chemicals, North Carolina, USA) as described by Hoch et al. (2005). Specimens were observed under a Leica fluorescent microscope (using 480 nm excitation and 527 nm emission).

**SEED CONTAMINATION ASSESSMENT**

Seed contamination assessments were estimated as described by Pochon et al. (2012). Two 2.5 μL drops of an A. brassicicola coni-

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Table 1 | List of primers for the genes used in this study.

| Genes | Use | Primers |
|-------|-----|---------|
| AbMdh | Real-time PCR | F: TTGACACTGACCCTCTCCGAC<br>R: GCCACAGCTTCTGGATGTC |
| AbMpd | Real-time PCR | F: TTCCGAGCAAAACGATTGAG<br>R: CATTGTCGCGCAACGCAC |
| Hph | Transformant validation | F: CGTTGCAAGACCTGCTGAA<br>R: GGATGCCGCTCGAAGTA |
| Nat | Transformant validation | F: TCCGTTCCCTTTCTCCT<br>R: ACATCCACGGACCTGAC |
| AbMdh | 5’ flanking regions for K.O. | F: GCCGAATAGTTGTGCGATT<br>R: TCCTGTGTGAAAATTGTTATCCGCTGGAGC |
| AbMdh | 3’ flanking regions for K.O. | F: GTCGTAATCCTGGAACCTCGGCACACTACGGGTGTGATCT<br>R: CTTTCTGCCATTCACACTA |
| AbMdh | Nested for K.O. | F: GCCATTCACGGCGGAGATT<br>R: GGGGCTGCGTGTTACAGAGGGAAGA |
| AbMpd | 5’ flanking regions for K.O. | F: CGACCTATACCGTTTACGG<br>R: TCCTGTGTGAAAATTGTTATCCGCTGGAGC |
| AbMpd | 3’ flanking regions for K.O. | F: GTCGTAATCCTGGAACCTCGGCACACTACGGGTGTGATCT<br>R: CTTTCTGCCATTCACACTA |
| AbMpd | Nested for K.O. | F: TGGGCTTCTTTTGCTGTGTA<br>R: GGGGCTGCGTGTTACAGAGGGAAGA |
| Hph or Nat | Complementary tail for pcb1636 or pnr2 | F: GTCGTAATCCTGGAACCTCGGCACACTACGGGTGTGATCT<br>R: CTTTCTGCCATTCACACTA |
| AbMpd | 5’ flanking regions for fusion GFP | F: ACATATATACCCCGCCAACG<br>R: CTTCCTGCCCTTTGCTCATGCAGTCGGGC |
| AbMpd | 3’ flanking regions for fusion GFP | F: TCCTGTGTGAAAATTGTTATCCGCTGATTACCTCAGTAAACCTGAG<br>R: AAGCGGATTTGGGAGGTGCAA |
| AbMpd | Nested for fusion GFP | F: TTCTCAACCCACTTCCCTCCAAC<br>R: AAGCGGATTTGGGAGGTGCAA |
| AbMdh | 5’ flanking regions for fusion GFP | F: CTCCACATACCGCTTCTCAT<br>R: CTTCCTGCCCTTTGCTCATGCAGTCGGGC |
| AbMdh | 3’ flanking regions for fusion GFP | F: TCCTGTGTGAAAATTGTTATCCGCTGATTACCTCAGTAAACCTGAG<br>R: CACGCATCGCGTAGTTCGTTT |
| AbMdh | Nested for fusion GFP | F: CCCAAACCTTCTACTCCCTCAAC<br>R: GTACCACGACGGTTCACTCC |
| actin | Real-time PCR | F: GGCAATGTTATCGTGTTCTCCT<br>R: GAGCGAAGCAAGAATGGAAC |
| Gfp and Hph | Complementary tail for pCT74 | F: CTCCCTGCCCTTTGCTCATGCAGTCGGGC<br>R: CTTTCTGCCATTCACACTA |

F: forward primer; R, reverse primer.

with 0.01% (v/v) Tween 20 were placed on the five youngest siliques (one drop at the silique base and one in the middle) from 1-month-old A. thaliana (Ler) plants. At least five plants per fungal genotype were inoculated and the experiment was repeated twice. As a control for all experiments, two 2.5 μL drops of a 0.01% (v/v) Tween 20 solution were placed on five siliques of one plant. The plants were then maintained under saturating humidity for 2 days in the dark. Contaminated siliques were harvested 10 dpi. Inoculated or control siliques were dissected with sterile forceps and seeds were carefully harvested to avoid contact with the fungus potentially present on the outer surface of siliques. Seeds were incubated separately on PDA medium for 2 days.
A seed was considered as contaminated when incubation resulted in typical *A. brassicicola* colony development.

**CONSERVATION OF CONIDIA ON SEEDS**

*B. oleracea* seeds were artificially inoculated by incubation (1 h) in a conidia suspension (5 mL at 10^5 conidia/mL). After removing the solution, the seeds were air-dried for 2 h and separated into two batches. The initial contamination rate was determined on one seed batch before storage. One seed per microplate well was placed in 300 μl of PDB and fungal growth was recorded in a laser-based nephelometer. The mean lag time was calculated and representative of the initial seed infection rate. The second seed batch was stored in a dry dark place at 24 °C for 6 months and processed as above. As the lag time was found to be directly proportional to the number of germinating conidia, the viability rate was estimated from the ratio between lag times before and after storage. This experiment was repeated twice for each fungal genotype.

**ENZYME ASSAYS**

Three-day-old cultures grown in PDB medium were harvested, submerged in liquid nitrogen for 5 min and stored at −80 °C until use. Extraction procedures for cell-free extracts were carried out at 4 °C and MPD and MDH enzymatic activities were measured as described by Velez et al. (2007). For both enzymes, specific activities were defined as the μmole of NADP(H) or NAD(H) oxidized per minute per mg of protein. Three independent experiments were done for each sample.

**SUGAR AND SUGAR ALCOHOL EXTRACTION AND ANALYSIS**

Ethanolic extractions of cells from each sample used for hexose sugar and polyol measurements were performed as described by Stoop and Pharr (1993) with minor modifications. Dry powdered samples were suspended in 80% ethanol solution and incubated at 80°C for 5 min. After 5 min of centrifugation at 1000 g, the supernatants were recovered and pellets were re-extracted twice. Pooled ethanolic solutions were evaporated using a vacuum concentrator (speedVac UNIEQUIP), and residues were dissolved in sterile water or D_2O for analysis. High performance liquid chromatography (HPLC) was performed on a Carbopac PA-1 column (Dionex Corp., Sunnyvale, CA, USA) as described by Rosnoblet et al. (2007). For each sample, three independent experiments were done from separate cultures.

**DETECTION OF BRASSICICOLIN A FROM FUNGAL EXTRACTS**

Brassicicolin A was extracted as described by (Gloer et al., 1988) from cultures (minimal medium plus thiamine, Pedras et al., 1997) of Abra43 and ΔΔabmpd-abmdh strains. Each filtered culture broth (1 L) was extracted with ethyl acetate (3 × 300 ml), and the organic phase was dried over MgSO_4 and evaporated to generate 25 mg and 13.5 mg of crude extracts from Abra43 and ΔΔabmpd-abmdh strains, respectively. Liquid chromatography-mass spectrometry (LC/MS) was performed on each extract using a Bruker Esquire 3000 Plus electrospray ionization-ion trap mass spectrometer coupled with a Waters 2790 high performance liquid chromatography (HPLC-ESI-MS^2). Elution was carried out on an Hypersil RP18 column (250 × 4.6 mm, 5 μm, Termo) using the following gradient: initial mobile phase AcN/H_2O 0.01% formic acid 15/85 reaching 60/40 in 35 min and maintained for 10 min before reaching 100/0 (v/v) until 46 min, with a flow rate of 1 mL/min. Only HPLC grade solvents were used. All samples were diluted in a solution of acetonitrile and filtered (UptiDisc™ PVDF 0.22 μm) prior to HPLC injection. They were analysed at 10 mg/ml concentration. The ESI parameters were as follows: solvent split ratio 1:9; nebulizer: 30 psi; dry gas (N_2): 7 L/min; dry temperature: 340°C; skim: 40 V; trap drive: from 90 to 178, octopole RF amplitude: from 144 to 210 Vpp; capillary exit: from −156 to −240, capillary voltage 4500 V. The ion trap mass spectrometer was run in negative ion scanning mode for m/z ranging from 80 to 1500. MS^n was performed at a fragmentation amplitude ranging from 0.8 to 2.0 V depending on the samples. Preparative thin-layer chromatography (PTLC) was carried out on silica gel 60F254 (0.25 mm, Merck) using MeOH/CHCl_3 (5/95) as eluent. This experiment was done twice from separate cultures of each fungal genotype.

**NMR ANALYSIS**

For nuclear magnetic resonance (NMR) metabolite analysis, 500 μl of the D_2O-samples were transferred to a 5 mm NMR tube, then analyzed on a BRUKER Advance DRX 500 MHz
spectrometer equipped with a multinuclear QNP probe (Bruker, Wissembourg, France). Proton-decoupled $^{13}$C NMR spectra (sweep width = 31 450 Hz) were recorded at 125 MHz excitation frequency, 30-degrees pulse angle (6.5 μs pulse duration) at 2 s intervals. The free induction decays were collected as 32 K data points and processed with a 1–2 Hz exponential line broadening for $^{13}$C NMR. Maleic acid ($\delta_{CH}130.4$ ppm) was the external reference for chemical shifts. Identifications were made by comparison with spectra of pure known standards. For brassicicolin A, $^1$H (500 MHz) and 2D NMR spectra (HMQC and COSY) were recorded in CDCl$_3$ in a capillary probe (Bruker TXI 1.7 mm) with chloroform resonances ($\delta_H$ 7.28, $\delta_C$ 77.0 ppm) as internal references. For each sample, NMR analysis was done twice.

**RESULTS**

CHARACTERIZATION OF Mpd AND Mdh GENES IN A. brassicicola AND GENERATION OF REPLACEMENT MUTANTS

The presumed Mpd and Mdh loci were identified by a homology search against the A. brassicicola genome assembly (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html) with genes previously described in A. alternata (Velez et al., 2007). AbMdh and AbMpd sequences (GenBank accession No JX403801 and JX403800, respectively) consisted of 851 and 1173 nucleotides, respectively. Blast search on the whole genome sequence and Southern analyses suggested the presence of only one copy of each gene (Figure 3B). Among the putative regulatory elements identified on sequences upstream of the ATG, consensus sequences for the binding of transcription factors involved in response to thermal, osmotic and oxidative stresses (Msn2p/Msn4p, HsF2) were found on the two genes.

The resulting AbMdh protein belongs to the short-chain group of the dehydrogenase/reductase superfamily (Jornvall et al., 1995) and has 95, 88, and 75% identity to the corresponding proteins described in A. alternata, S. nodorum, and B. cinerea, respectively. The AbMpd amino acid sequence shares 92, 82, and 57% identity with that of A. alternata, S. nodorum, and B. cinerea, respectively, and contains both NAD-interacting domains and a specific mannitol-1-phosphate dehydrogenase motif.

For each targeted gene, 2 replacement mutants (called Δabmdh1-2, Δabmpd1-2) were generated by replacing the targeted ORF with a hygromycin B resistance cassette. Two Δabmpd-Δabmdh1-2 double deletion mutants were then constructed by transforming the Δabmpd genotype with an AbMdh-replacement cassette containing a

**FIGURE 3 | Verification of deletion mutants.** (A) Schematic representation of the AbMpd and AbMdh loci (gray boxes), replacement constructs with the HygB resistance gene (Hph) or the nourseotricin resistance gene (Nat) cassettes (white boxes) and flanking sequences (dark gray boxes). The positions of HindIII and SacI sites are indicated. (B) Southern hybridization of genomic DNA from wild-type Abra43 and transformants (for each targeted gene, two replacement mutants were generated and analyzed). Each DNA was digested with SacI for the blot hybridized with the Abmdh probe or with HindIII for the blot hybridized with the Abmpd probe. Probes used are shown in (A).
nourseothricin-resistance marker (Figure 3). In all further experiments, the phenotypic characters for transformants of the same genotype were not found to be significantly different.

**BIOCHEMICAL CHARACTERIZATION OF REPLACEMENT MUTANTS**

To confirm that gene inactivation impacted the enzyme activity, the transformants were grown in liquid culture and analysed for their ability to reduce fructose using NADPH as cofactor, or for fructose-6-phosphate conversion to mannitol-1-phosphate. Enzymatic assays (Table 2) confirmed that AbMdh and AbMpd deletions abolished mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase activities, respectively.

### Table 2 | Enzyme activities related to mannitol metabolism in mycelia of Abra43 wild-type and mutant strains.

| Strains         | MDH activity \( \mu \text{mol min}^{-1} \text{mg of protein}^{-1} \) | MPD activity \( \mu \text{mol min}^{-1} \text{mg of protein}^{-1} \) |
|-----------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Abra43          | 3.7 ± 0.16                                                    | 5.1 ± 0.85                                                    |
| \( \Delta abmpd \) | 3.7 ± 0.03                                                    | 0                                                             |
| \( \Delta abmdh \) | 0                                                             | 5.6 ± 0.35                                                    |
| \( \Delta abmpd-abmdh \) | 0                                                             | 0                                                             |

MDH activity was measured in extracts as the rate of mannitol-dependent conversion of NAD\(^+\) to NADH and MPD activity was measured in extracts as the rate of mannitol-dependent conversion of NADP\(^+\) to NADPH (U). Data represent the means of three independent experiments.

The effects of the \( \Delta abmdh \), \( \Delta abmpd \), and \( \Delta abmpd-abmdh \) mutations on the accumulation of sugars and sugar alcohols were estimated by \(^{13}\)C NMR. Ethanolic extracts of sporulating mycelium grown in synthetic Vogel medium with glucose (2\%) were obtained and analysed (Figure 4A). The wild-type and \( \Delta abmpd \) extracts exhibited similar sugar profiles. However, lower amounts of mannitol and higher amounts of trehalose were found in the latter genotype. Conversely, the \(^{13}\)C-NMR spectra of \( \Delta abmdh \) mutants were dominated by mannitol resonances. Mannitol was absent in extracts from \( \Delta abmpd-abmdh \) mutants in which trehalose and glycerol appeared to be the major compounds. Quantitative estimations of the mannitol content in the different genotypes during *in vitro* development were obtained by HPLC analysis of extracts from mature conidia and young non-sporulating mycelia (Figure 4B). While the wild-type accumulated nearly the same amount of mannitol in conidia and mycelia, this polyol was exclusively detected in conidia of the \( \Delta abmpd \) mutants. By contrast, the \( \Delta abmdh \) mutants preferentially accumulated mannitol in mycelia. No mannitol was detected in either conidia or young mycelia (30 h post germination) from the \( \Delta abmpd-abmdh \) mutants. However, traces of mannitol were detected from the double deletion strains in 1-week-old cultures (data not shown).

**SUSCEPTIBILITY OF REPLACEMENT MUTANTS TO STRESS CONDITIONS**

Mannitol has been proposed to act as a potent protective metabolite against oxidative stress. As the mannitol contents of conidia

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**FIGURE 4 | Sugar and polyol content during *in vitro* development of A. brassicicola wild-type and mutant strains.** (A) \(^{13}\)C-NMR spectra obtained from ethanolic extracts of 1-week-old fungal colonies. The peaks were identified and labeled as follows: g, glucose; gly, glycerol; m, mannitol; t, trehalose. The spectra scales are identical and the chemical shift was expressed in parts per million (ppm). This experiment was done twice and representative spectra are presented here. (B) Mannitol content [expressed in \( \mu \text{g/mg DW} \)] obtained by HPLC analysis of extracts from mature conidia and young non-sporulating mycelium. Mannitol was extracted from at least three independent biological replicates. Error bars indicate standard deviations. Asterisks indicate a significant difference between the mutant and the parental isolate (Student test, \( P < 0.01 \)).
and mycelia from the different mutant genotypes were significantly different from that of the wild-type, the effects of AbMpd and AbMdh inactivation in A. brassicicola on conidia germination and initial mycelium growth in the presence of oxidative stressors were examined. Analysis of nephelometric growth curves revealed that under non-stress conditions (PDB medium), no significant phenotypic differences in conidia germination (based on the lag time parameter) or mycelium growth (based on the maximum growth rate parameter) were detected in any of the tested mutants as compared to the wild-type (data not shown). By contrast, Δabmdh and Δabmpd mutants, and to a lesser extent ΔΔabmpd-abmdh mutants, were far more susceptible than the wild-type to 1 mM \( \text{H}_2\text{O}_2 \) treatments (Figure 5A). The Δabmpd and Δabmpd-abmdh mutants were also more susceptible to exposure to the superoxide-generating compound menadione than the wild-type and Δabmdh genotypes (Figure 5B). As some brassicaceous defense metabolites have antifungal properties that might be at least partially linked to their capacity to generate oxidative stress (Sellam et al., 2007a), the susceptibility to allyl-ITC (Al-ITC), benzyl-ITC (Bz-ITC), and phenethyl-ITC (Ph-ITC) and brassinin were investigated (Figures 5C–F). A genotype susceptibility pattern similar to that obtained with menadione was observed in the presence of 5 mM Al-ITC, 5 mM Bz-ITC, 10 mM Ph-ITC and 200 µM brassinin. Note that after 24 h of exposure to ITC, the mannitol content of wild-type mycelia was 1.4-fold higher than in control and a 3-fold increased expression

![Figure 5](image-url)
of AbMpd was recorded in cultures after 3 h of ITC treatment, while the AbMdh expression level remained unchanged (data not shown).

Based on these susceptibility patterns, comparing the response of the four genotypes to H2O2 on one hand and to menadione or ITC on the other suggested different modes of action for these oxidants. This was further supported by comparing the effects on the cell viability of non-germinated and germinated conidia of low-time exposure to H2O2 and Al-ITC at their respective IC50 (Figure 6). On germinated conidia, treatment with Al-ITC resulted in PI-positive hyphae suggesting a loss of cell viability. No PI staining was observed in non-germinated conidia exposed to Al-ITC. Conversely, H2O2 caused cell death in non-germinated or germinated conidia.

Besides its potential role as antioxidant molecule, mannitol has been proposed to provide protection against drought stress (Dijksterhuis and de Vries, 2006). Media with different water potentials (from −0.25 MPa to −1.7 MPa) were prepared using a PEG-infuse plate protocol (Verslues et al., 2006), inoculated with the different A. brassicicola genotypes and radial growth was recorded after 15 days of incubation. As shown in Figure 7, strains lacking functional MPD (Δabmpd and ΔΔabmpd-abmdh) were much more susceptible to low water potential treatments than the wild-type. Conversely, Δabmdh mutants were more tolerant than the wild-type.

MANNITOL METABOLISM DURING PLANT COLONIZATION

The HPLC profiles of the major soluble carbohydrates present over the course of A. brassicicola infection on B. oleracea leaves were established (Figure 8A). At 2 dpi, the wild-type strain produced characteristic appressoria-like structures at the tips of germ tubes in contact with the leaf epidermis (Figure 8B). Small necrotic symptoms were observed at 4 dpi, and they continued to expand into large typical necrotic areas surrounded by chlorotic halos at 6 dpi. At this infection stage, necrotic spots exhibited dense conidia formation on the surface. The HPLC analysis showed that, while the mannitol level remained below the detection limit in control plants, mannitol accumulated throughout infection and revealed a twenty-fold increase from 2 dpi to 4 dpi. A small decrease in mannitol was then observed at 6 dpi when sporulation was abundant. However, the mannitol level at this stage was still significantly higher than that detected at 2 dpi. Trehalose was only detected at low levels at 4 dpi and 6 dpi. Sucrose, which was the major carbohydrate in control non-inoculated plant samples, quickly decreased to undetectable levels at 4 dpi.

The expression of AbMdh and AbMpd during infection was also examined (Figure 8C). At 2 dpi, the expression level of AbMpd increased and remained higher than during in vitro growth over the time course of the experiment. Increased AbMdh expression was observed at a later stage and reached 3.5-fold its basal expression level at 6 dpi. These results are consistent with the metabolic profiling, which indicated a prevalence of mannitol production during plant infection. In order to follow the in vitro and in planta spatial expression patterns of both genes, strains expressing AbMpd and AbMdh, under the control of their own promoter and fused at their carboxy-terminal end to SGFP, were engineered. None of the transformants showed visible phenotypic changes discernible from the wild-type except for expression of green fluorescence (data not shown). In both strains, the fluorescence signal was detected in mature conidia and in the young germ tubes (Figure 9). In the in vitro-produced mature hyphae, the signal was still detectable but was much weaker in the AbMpd-GFP strain than in the AbMdh-GFP strain.
Consistent with the *in planta* expression patterns, GFP fluorescence appeared to be stronger in the mutant during host plant infection. In AbMpd- and AbMdh–GFP fusion mutants, fluorescence intensity increased dramatically during the *in vitro* or *in planta* conidiation process, reaching a maximum in young conidia (Figure 9).

To test the hypothesis that host plants would elicit changes in fungal mannitol production, the *A. brassicicola* wild-type strain was cultured for 7 days in the presence and absence of leaf extracts from host (*B. oleracea*) or non-host (*Solanum lycopersicum*) plants. The amount of mannitol in the fungal mycelia was then determined by HPLC (Figure 10). Fungal growth was essentially unaffected by plant extracts (data not shown). *A. brassicicola* responded to the presence of plant extracts by accumulating significantly higher levels of mannitol as compared to the amount detected in control culture or in the presence of non-host extract.

**PATHOGENIC BEHAVIOR OF REPLACEMENT MUTANTS ON VEGETATIVE ORGANS**

The accumulation of mannitol throughout infection of *B. oleracea* by *A. brassicicola* and the increased susceptibility of mannitol biosynthesis mutants to oxidative stress prompted us to comparatively evaluate the pathogenicity of the different fungal genotypes. The wild-type and *Δabmdh*, *Δabmpd*, and *Δabmpd-abmdh* mutants were all able to produce typical symptoms (Figure 11A). However, as judged from the lesion sizes at low inoculum charge, significant decreases in aggressiveness (up to 85% that of the wild-type at 10^3 conidia per ml) were recorded for the *Δabmpd-abmdh* mutants and to lesser extent for the *Δabmdh* and *Δabmpd* mutants. Closer inspection of symptoms suggested that weak *in planta* sporulation occurred on necrosis obtained after inoculation with *AbMdh* deficient mutants (Figure 11B). This was confirmed by measuring the quantity of conidia produced per mm^2 of necrotic tissue. All genotypes produced significantly fewer conidia *in planta* than the wild-type, with a 90% reduction for *Δabmdh* mutants.

To check whether the lower aggressiveness of mutants could be correlated with *in planta* spore germination and/or plant tissue penetration defects, observation of solophenyl stained samples at 1 dpi was performed to quantify germinated conidia and appressoria-like structures at the plant surface. Although nearly 95% of conidia from all genotypes were germinated at this time, only 5 and 20% had differentiated infection structures in samples inoculated with the *Δabmpd-abmdh* and the *Δabmpd* mutants, respectively, vs. 40% for samples inoculated with the wild-type or *Δabmdh* mutants. Following host penetration, the fungus has to produce necrotic factors to progress within infected tissues. Brassicicolin A, a fungal metabolite considered as being a mannitol derivative, represents a potent necrotic toxin produced by *A. brassicicola* (Pedras et al., 2009). We hypothesized that the weak virulence in mutants lacking mannitol may also be explained by the absence of brassicicolin A synthesis. To test this hypothesis, ethyl acetate extracts of culture filtrates from submerged cultures of *A. brassicicola* wild-type and *Δabmpd-abmdh* strains were analysed by HPLC-UV-MS. The resulting total ion chromatograms (TIC) of both extracts revealed a major metabolite at *R_t* = 39 min (Figures 12A,B) exhibiting the quasi-molecular ion [M-H]^- (m/z 683) of brassicicolin A (C_{32}H_{48}N_{2}O_{14}), whereas typical MS/MS fragments (1 V) were recorded at m/z, 565 (loss
FIGURE 8 | Mannitol metabolism during plant colonization. (A) Major soluble carbohydrate concentrations (expressed in μg/mg DF) measured by HPLC during the infection kinetics of the A. brassicicola wild-type strain on Brassica oleracea. Undetected sugars are represented by the symbol Ø. Three independent experiments were done. (B) Progression of symptoms on a B. oleracea leaf inoculated with the Abra43 strain and microscopic observations of the infection structures at 2 dpi. Inoculated plant tissue fragments were collected at 2 dpi and stained with solophenyl flavine for fluorescence microscopy observations. Appressoria-like structures are indicated by white arrows. (C) Quantitative RT-PCR results for the expression of AbMpd (white bars) and AbMdh (dark gray bars) during the infection kinetics of A. brassicicola wild-type strain on B. oleracea. For each gene, expression induction is represented as a ratio of its relative expression at 2, 4, and 6 dpi (studied gene transcript abundance/actin transcript abundance) in each inoculated sample to its relative expression in free-living fungal control cultures. The experiment was performed twice on biologically independent samples with three technical replicates. Error bars indicate standard deviations and asterisks indicate a relative expression significantly different from 1 (Student test, \( P < 0.01 \)).

of two acetyl units) and 473 (loss of one \( \alpha \)-hydroxyisovaleryl unit together with one \( \alpha \)-isocyanooisovaleryl unit) (data not shown). The corresponding compound, which seems to accumulate in the same amounts in both strains, was purified through preparative TLC from the wild-type extract and analyzed (\( ^1 \)H NMR, COSY and HMQC) in a capillary NMR probe (500 MHz). The resulting data (data not shown) were also in full agreement with former measurements obtained by Gloer et al. (1988) for this mixture of stereoisomers and confirmed that the isolated compound was brassicicolin A. We concluded that brassicicolin A was present in organic extracts from the culture broths of both the wild-type strain and the \( \Delta \Delta abmpd-abmdh \) mutant and that the attenuated virulence of mannitol-deficient mutants could not be linked to the loss of brassicicolin A production.
FIGURE 9 | Developmental phase-specific expression of AbMpd and AbMdh. All panels are microscopy images of GFP expression in A. brassicicola strains expressing AbMdh (panels A, B, C, and G) and AbMpd (panels D, E, F, and H), under the control of their own promoter and fused at their carboxy-terminal end to SGFP. (A) and (D) Early germination stage 5 h after transfer to a solid PDA medium. Scale bars = 10 μm. (B, C, E, and F) Mycelia grown for 72 h on a PDA medium. At this stage, hyphae started to differentiate into conidiophores, leading to the production of young (arrow) and mature (arrowhead) conidia. Scale bars = 10 μm. (G) and (H) Fungal growth 6 days after inoculation of B. oleracea leaves. At this time, the fluorescence signal increased in aerial hyphae during the conidiation process. The right part corresponds to fluorescence microscopy and the left part to bright-field microscopy. Scale bars = 30 μm.

PATHOGENIC BEHAVIOR OF REPLACEMENT MUTANTS ON REPRODUCTIVE ORGANS

A. brassicicola is a seed-borne pathogen and has potential for long-term survival on dry seeds. As the differential abilities of the mutants to overcome a water potential stress have been observed, their capacity to survive on artificially contaminated seeds during storage was examined. After 6 months of storage, the percentage of viability was estimated using laser nephelometry growth.
curves on the basis of Δlag time values. As shown in Figure 13A, ΔΔabmpd-abmdh mutant survival was significantly lower than that of other tested genotypes. Similarly, the ability of A. brassicicola to efficiently feed seeds has been correlated with their capacity to cope with severe stress conditions consecutive to gradual dehydration of maturing reproductive organs (Jacomi-Vasilescu et al., 2008). Using the model pathosystem recently described for investigating seed transmission in Arabidopsis plants (Pochon et al., 2012), the abilities of the mutants to transmit to seeds were compared with that of the wild-type. As shown in Figure 13B, the transmission capacity to A. thaliana seeds was significantly affected in Δabmpd and Δabmdh mutants and almost completely abolished in the ΔΔabmpd-abmdh mutants.

**DISCUSSION**

The main goal of this study was to specify the roles of C6-polyl mannitol in the parasitic cycle of the fungal necrotroph A. brassicicola. We showed here that fungal mannitol over-accumulated in B. oleracea leaves during the interaction with A. brassicicola, as previously reported in Arabidopsis during A. brassicicola infection (Botanga et al., 2012) or for other necrotrophic (Jobic et al., 2007; Dulumero et al., 2009) and biotrophic (Voegelle et al., 2005) interactions. In the latter type of interaction, accumulated mannitol might provide a means for the fungus to store carbohydrate in a form that is not accessible to the host and maintain a gradient of metabolites in favor of the pathogen (Voegelle et al., 2005). Similarly to the finding of previous studies involving fungal necrotrophs (Jobic et al., 2007; Dulumero et al., 2009), sucrose dramatically decreased during host colonization by A. brassicicola and this plant specific carbohydrate was below the detection level at 6 dpi, suggesting that mannitol biosynthesis could be a general fungal strategy to rapidly mobilize plant sugars. As proposed by Solomon et al. (2006), accumulated mannitol may then provide the necessary substrates and energy required for conidiogenesis.

Mannitol dehydrogenase (MDH), which is mainly involved in mannitol mobilization, and mannitol-1-phosphate dehydrogenase (MPD) are two key enzymes of mannitol metabolism in fungi (Krahulec et al., 2011). The corresponding A. brassicicola encoding genes (AbMpd and AbMdh, respectively) have been identified and their expression monitored in B. oleracea infected leaves. The AbMpd- and AbMdh-GFP fusion analysis showed that the expression pattern is closely related to the conidiation and germination processes in A. brassicicola. GFP fluorescence indicated that both proteins exhibited a relatively similar expression pattern during host infection or in vitro growth: the maximum fluorescence intensity was reached in young conidia during the conidiation process. A high fluorescence intensity was also detected in mature conidia and into the young germ tubes, whereas the signal was weaker or undetectable in mature hyphae. Our results differ from previous observations in Aspergillus niger (Aguilar-Osorio et al., 2010), reporting a spatial differential expression of the expression of these two proteins: expression of MDHA and MDH activity were detected only in spores, while expression of MPDA and MPD activity were detected only in hyphae. These conflicting results may reflect functional differences among fungal enzymes involved in mannitol metabolism.

Gradual induction of AbMdh gene expression during infection was observed, as previously shown in the U. fabae-V. faba interaction (Voegelle et al., 2005). The highest expression level measured was at 6 dpi, i.e., coinciding with decreased accumulation of mannitol, suggesting the mobilization of the polyol at this stage of infection may be a consequence of massive germination of newly formed conidia. It has indeed been shown in B. cinerea that mannitol rapidly degrades during spore germination and that such a catabolic process likely involves MDH activity (Dulumero et al., 2010). However, there is accumulating evidence that, contrary to what was postulated by Hult and Gatenbeck (1978), mannitol metabolism is not a cyclical process in fungi (Solomon et al., 2007; Dulumero et al., 2009). In line with this, MPD deficient A. brassicicola mutants still produced mannitol and thus AbMdh may also contribute to mannitol biosynthesis during infection. Abmpd expression was found to be induced at the earliest stages of the interaction and remained at a higher level than in control mycelia throughout the infection. According to the well-documented involvement of the MPD-dependent pathway in mannitol biosynthesis in fungi (Solomon et al., 2007), such Abmpd over-expression during tissue infection might be necessary for asexual sporulation and pathogen propagation. In line with this hypothesis, mpd-deficient A. brassicicola mutants that failed to accumulate mannitol in hyphae were significantly compromised in their ability to develop conidia in planta. However, conidiation was also severely impaired in the mdh-deficient mutant that still accumulated high levels of intra-hyphal mannitol, thus questioning the direct relationship between mannitol content and asexual spor differentiation. Note that, as observed in B. cinerea (Dulumero et al., 2010), trehalose was detected by 13C NMR in all the studied genotypes except the mdh-deficient mutant. This sugar might therefore also be required to promote normal conidiation in A. brassicicola, in line with its demonstrated involvement in S. nodorum sporulation (Lowe et al., 2009).
The role of stored mannitol in conidia germination also seems unclear. In conidia of all tested *A. brassicicola* mutants, a drastic decrease in mannitol to below the detectable level for the ΔΔabmpd-abmdh strain was observed, but normal spore germination kinetics were recorded. Similar observations were reported for *A. niger* and *S. nodorum* (Ruijter et al., 2003; Solomon et al., 2005, 2006). In contrast, the capacity to accumulate mannitol in hyphae could be correlated with the ability to differentiate penetration (i.e., appressoria-like) structures as revealed by microscopic observation of plant tissue inoculated with *mdp*-deficient mutants in which no mannitol could be detected in young hyphae. This inability to efficiently produce penetration structures by strains lacking a functional MPD-dependent pathway was not observed in *A. alternata* (Velez et al., 2007), but probably at least partially explained the reduced aggressiveness of *A. brassicicola* *mpd*-deficient mutants.

Besides a possible role in this pathogenesis-related developmental process, mannitol may have other functions...
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FIGURE 12 | Detection of the phytotoxin brassicicolin A in organic extracts from the culture broths of both the wild-type strain and the ΔΔabmpd-abmdh mutant. LC-MS total ion chromatograms (TICs) of Abra43 (A) and ΔΔabmpd-abmdh (B) culture filtrate EtOAc extracts. Arrow indicates brassicicolin A. This experiment was done twice.

during plant-fungus interactions. One earlier reported function attributed to mannitol is protection against oxidative stress generated by the host plant defense system (Jennings et al., 1998). Oxidative burst is a general plant defense mechanism that occurs at a very early stage of the interaction (Parent et al., 2008). It is characterized by rapid accumulation of hydrogen peroxide in the extracellular space of plant tissues exposed to biotic stress (Wojtaszek, 1997). This ROS, besides its potential antimicrobial activity, might regulate induced cell death at the infection site, as shown in the A. thaliana–A. brassicicola pathosystem (Pogany et al., 2009). In our study, A. brassicicola was found to be relatively tolerant of physiologically compatible H$_2$O$_2$ concentrations. By contrast, mannitol metabolism mutants were all characterized by increased susceptibility to this ROS and lower mannitol content in conidia. As propidium iodide staining revealed that H$_2$O$_2$ induced cell death, even in non-germinated conidia, mannitol may accumulate in this organ and have a major protective role against oxidative stress generated by H$_2$O$_2$. The decreased aggressiveness on B. oleracea and the lower capacity to be transmitted to Arabidopsis seeds via siliques observed for all mutant genotypes could therefore be related to their increased susceptibility to oxidative burst during the early leaf or silique infection stage. At a later infection stage, i.e., during leaf or silique tissue colonization, A. brassicicola is also exposed to glucosinolate-derived ITC that induce intracellular ROS accumulation in fungal cells (Sellam et al., 2007a). The results of the present study strongly suggested that ITC cell toxicity is mainly exerted on germ-tubes and young hyphae rather than on conidia, thus confirming previously published observations (Sellam et al., 2007b). Interestingly, Δabmpd and ΔΔabmpd-abmdh strains that failed to accumulate mannitol in young hyphae were found to be hyper-susceptible to allyl-, benzyl- and phenetyl-ITC and also to menadione, a reference superoxide–generating molecule. Conversely, Δabmdh strains that accumulated normal mannitol levels in hyphae were found to be as tolerant as the wild-type genotype. In planta assays were conducted on leaves of Brassica oleracea var. Bartolo and fruits of A. thaliana ecotype Ler that both accumulated various glucosinolates. Thus, in addition to their increased susceptibility to extracellular ROS, the low aggressiveness and seed colonization capacity of MPD-deficient mutants may also be related to their failure to overcome the intracellular oxidative stress caused by ITC during leaf or silique colonization.

Besides the capacity to colonize fruit and seed tissues, efficient seed transmission required long-term survival of the seed-borne fungus on dry seeds teguments. Ruijter et al. (2003) proposed that the alcohol functions of mannitol could enable this polyol to mimic water molecules and participate in cell tolerance to water stress. In line with this, A. brassicicola ΔΔabmpd-abmdh mutants, i.e., with no detectable hyphal and conidial mannitol, were found to be highly susceptible to matricial stress generated by PEG and showed low viability rates after 1 month of storage under on dry seeds.

The altered mannitol metabolism observed in MPD- and MDH-deficient mutants may have other pleiotropic effects on A. brassicicola pathogenicity. During tissue invasion, necrotrophic pathogens such as A. brassicicola, synthesize phytotoxins that facilitate their spread within the infected organs (Thomma, 2003). Gloer et al. (1988) proposed that mannitol could be a precursor for the biosynthesis of brassicicolin A, which was later described as being the major host-selective phytotoxin produced by A. brassicicola (Pedras et al., 2009). Surprisingly, brassicicolin A was
identified in organic extracts from the culture broths of both the wild-type strain and the ΔΔabmpd-abmdh mutant. Our HPLC profile analysis revealed the presence of traces of mannitol in 1-week-old ΔΔabmpd-abmdh cultures, suggesting that mannitol was still produced in minute amounts despite complete alteration of both mannitol biosynthesis pathways. As already suggested by Dulermo et al. (2010), this latter finding challenges the existence of one additional yet undescribed pathway which could participate in mannitol metabolism and, more specifically, in A. brassicicola, in brassicicolin A synthesis. We identified several candidate enzymes to perform mannitol synthesis through potentially other metabolic routes. First, a BlastP search pointed out an A. brassicicola sequence (AB1271) sharing homology with the NADP+-dependent D-mannitol dehydrogenase TbMDH, described in Tuber borchii and belonging to a distinct subfamily among the polyol dehydrogenase family (Ceccaroli et al., 2007). Dulermo et al. (2010) have already suggested its involvement in B. cinerea mannitol metabolism. Secondly, we also found potential homologs of mannose-6-phosphateductase (M6PR), a key enzyme that is involved in mannitol biosynthesis in higher plants (Everard et al., 1997).

In conclusion, these results highlight the importance of mannitol metabolism with respect to the ability of A. brassicicola to efficiently accomplish key steps of its pathogen life cycle. At the earliest stages of plant infection, the differentiation of infection structures and fungal protection against extracellular ROS generated by oxidative burst were correlated with mannitol accumulation in hyphae and conidia, respectively. During tissue colonization, although rapid conversion of plant sugars into mannitol through hyphae invasion may not be directly linked to necrotic toxin production, the polyol probably participates in fungal protection against intracellular ITC-derived oxidative stress. It may also constitute a carbohydrate store that could be remobilized during late infection stages for the in planta conidiation necessary for efficient horizontal transmission of the pathogen. Lastly, mannitol could also be involved in vertical transmission (i.e., seed transmission) of the pathogen by conferring protection against dehydration and allowing long-term survival of the fungus on stored seeds.

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REFERENCES

Aguilar-Osorio, G., Vankuyk, P. A., Seiboth, B., Blom, D., Solomon, P. S., Vinck, A., et al. (2010). Spatial and developmental differentiation of mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase in Aspergillus niger. Eukaryot. Cell 9, 1398–1402.

Botanga, C. J., Bethke, G., Chen, Z., Gallie, D. R., Fiehn, O., and Glazebrook, J. (2012). Metabolite profiling of Arabidopsis inoculated with Alternaria brassicicola reveals that ascobole reduces disease severity. Mol. Plant Microbe Interact. 25, 1628–1638.

Ceccaroli, P., Saltarelli, R., Guescini, M., Polidori, E., Buffalini, M., Menotta, M., et al. (2007). Identification and characterization of the Tuber borchii D-mannitol dehydrogenase which defines a new subfamily within the polyol-specific medium chain dehydrogenases. Fungal Genet. Biol. 44, 965–978.

Chaturvedi, V., Flynn, T., Niehaus, W. G., and Wong, B. (1996a). Stress tolerance and pathogenic potential of a mannitol mutant of Cryptococcus neoformans. Microbiology 142(Pt 4), 937–943.

Chaturvedi, V., Wong, B., and Newman, S. L. (1996b). Oxidative killing of Cryptococcus neoformans by human neutrophils. Evidence that fungal mannitol protects...
Role of mannitol metabolism in fungal pathogenicity

Dongo, A., Bataille-Simoneau, N., Calmes et al. (2007). Characterization of AgMaT2, a plasma membrane mannitol transporter from celery, expressed in pho21 mutant cells, including phloem parenchyma cells. Plant Physiol. 145, 62–74. (www.frontiersin.org/May 2013|Volume 4|Article 131|17)

Krahulec, S., Armao, G. C., Klimacek, M., and Niederzky, B. (2011). Enzymes of mannitol metabolism in the human pathogenic fungus Aspergillus fumigatus–kinetic properties of mannitol-1-phosphate 5-dehydrogenase and mannitol 2-dehydrogenase, and their physiological implications. FERS J. 278, 1264–1276.

Lewis, D., and Smith, D. (1967). Sugar alcohols in fungi and green plants. New Phytol. 66, 143–184.

Solomon, P. S., Waters, O. D., and Oliver, R. P. (2005). Mannitol protects against oxidation by hydroxyl radicals. Plant Physiol. 115, 527–532.

Snirnoff, N., and Cumbes, Q. J. (1989). Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060.

Solomon, P. S., Waters, O. D., Jorgens, C. I., Lowe, R. G., Rechberger, J., and Trengove, R. D. (2006). Decoding the mannitol enigma in filamentous fungi. Trends Microbiol. 15, 257–262.

www.frontiersin.org
Stoop, J., and Pharr, D. M. (1993). Effect of different carbon sources on relative growth rate, internal carbohydrates, and mannitol 1-oxidoreductase activity in celery suspension cultures. *Plant Physiol.* 103, 1001–1008.

Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. (1995). Identification, cloning, and characterization of PWL2, a gene for host species specificity in the rice blast fungus. *Plant Cell* 7, 1221–1233.

Thomma, B. P. (2003). Alternaria spp.: from general saprophyte to specific parasite. *Mol. Plant Pathol.* 4, 1211–1233.

Velez, H., Glassbrook, N. J., and Daub, M. E. (2007). Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata*. *Fungal Genet. Biol.* 44, 258–268.

Velez, H., Glassbrook, N. J., and Daub, M. E. (2008). Mannitol biosynthesis is required for plant pathogenicity by *Alternaria alternata*. *FEMS Microbiol. Lett.* 285, 122–129.

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., and Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J.* 45, 523–539.

Voegele, R. T., Hahn, M., Lohaus, G., Link, T., Heiser, I., and Mendgen, K. (2005). Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. *Plant Physiol.* 137, 190–198.

Vogel, H. J. (1956). A convenient growth medium for *Neurospora crassa*. *Microbial Genet. Bull.* 13, 42–43.

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochem. J.* 322(Pt 3), 681–692.

Ya, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Dominguez, Y., and Scaggs, T. E. (2004). Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* 41, 973–981.

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