Research Article

Trehalose phosphate synthase 5-dependent trehalose metabolism modulates basal defense responses in *Arabidopsis thaliana*

Running Title: Trehalose metabolism in plant basal defense

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Abstract

Despite the recent discovery that trehalose synthesis is important for plant development and abiotic stress tolerance, the effects of trehalose on biotic stress responses remain relatively unknown. In this study, we demonstrate that TREHALOSE PHOSPHATE SYNTHASE 5 (TPS5)-dependent trehalose metabolism regulates Arabidopsis thaliana defenses against pathogens (necrotrophic Botrytis cinerea and biotrophic Pseudomonas syringae). Pathogen infection increased trehalose levels and up-regulated TPS5 expression. The application of exogenous trehalose significantly improved plant defenses against B. cinerea, but increased the susceptibility of plants to P. syringae. We demonstrated that elevated trehalose biosynthesis, in transgenic plants over-expressing TPS5, also increased the susceptibility to P. syringae, but decreased the disease symptoms caused by B. cinerea. The knockout of TPS5 prevented the accumulation of trehalose and enhanced defense responses against P. syringae. Additionally, we observed that a TPS5-interacting protein (multiprotein bridging factor 1c) was required for induced expression of TPS5 during pathogen infections. Furthermore, we show that trehalose promotes P. syringae growth and disease development, via a mechanism involving suppression of the plant defense gene, Pathogenesis-Related Protein 1. These findings provide insight into the function of TPS5-dependent trehalose metabolism in plant basal defense responses.
INTRODUCTION

Pathogen infections of crops result in considerable economic losses, worldwide. *Botrytis cinerea* is a ubiquitous pathogen that causes severe losses in many agriculturally important crops because of its broad host range. As a necrotrophic fungus, *B. cinerea* destroys plant tissues and defense mechanisms using a series of toxic molecules prior to feeding (Dean et al. 2012; Windram et al. 2012). *Pseudomonas syringae* is a gram-negative bacterial plant pathogen responsible for various diseases, including blights and leaf spots, and has been used as a model system in plant molecular pathology research (Cui et al. 2013).

Plants have developed efficient systems to defend against pathogens. Several plant hormones, including jasmonic acid (JA) (Pré et al. 2008; Wild et al. 2012; Kazan and Manners 2013; Nakata et al. 2013; Wild and Achard 2013), salicylic acid (SA) (Argueso et al. 2012; Gimenez-Ibanez and Solano 2013), ethylene (ET) (Lorenzo et al. 2003; Berrocal-Lobo and Molina 2004), auxin (Naseem and Dandekar 2012), and abscisic acid (ABA) (Xu et al. 2013) have been implicated in plant basal defenses.

Responses against biotrophic pathogens are generally regulated by the SA signaling pathway. These responses have been studied with mutant and transgenic plant lines exhibiting abnormal SA biosynthesis, perception, or signaling. Meanwhile, responses to necrotrophic pathogens are mainly mediated by JA and ET (Spoel et al. 2003). Previous studies revealed that JA-activated genes, including *Plant Defensin1.2* (*PDF1.2*) which encodes a protein that is inhibitory toward necrotrophic pathogens (Mengiste et al. 2003; Shim et al. 2013). Accumulation of SA up-regulates the expression of defense-related genes, including *PR* genes (e.g., *PR1*, *PR2*, and *PR5*), ultimately enhancing disease resistance against biotrophic pathogens (Zheng et al. 2006; Shim et al. 2013). The SA pathway often suppresses the JA
pathway (van der Does et al. 2013).

In addition to hormones, sugar signals may also activate immune responses against pathogens. Moreover, sugars are likely to function as priming molecules leading to pathogen-associated molecular pattern-triggered and effector-triggered immunity in plants. These putative roles also depend on an intricate network of coordinated interactions with hormones and the light status (Moghaddam and Van den Ende 2012). Sucrose is a potential signaling molecule contributing to plant innate immunity (Gómez-Ariza et al. 2007). Hence, characterizing the role of sugars related to plant defenses against pathogens is warranted.

Trehalose, which is a non-reducing disaccharide composed of two glucose units, is present in various organisms (Satoh-Nagasawa et al. 2006; Schluepmann et al. 2012). In plants, trehalose is synthesized in two steps by the otsA–otsB pathway (also referred to as the TPS–TPP pathway) (Paul et al. 2008). First, trehalose 6-phosphate (T6P) is formed from UDP-glucose and glucose 6-phosphate in a reaction catalyzed by T6P synthase (TPS). The generated T6P is then converted to trehalose by T6P phosphatase (TPP).

Trehalose can be degraded to glucose by trehalase. *Arabidopsis thaliana* has a single trehalase-encoding gene (*TRE1*). A search of the complete *A. thaliana* genome revealed 11 *TPS* and 10 *TPP* homologs (Kolbe et al. 2005). The 11 *A. thaliana* genes encoding TPS enzymes have been classified into two classes (Leyman et al. 2001; Avonce et al. 2006; Paul et al. 2008). The class I genes (*TPS1–4*) are closely related to the yeast *TPS1* gene, whereas the class II genes (*TPS5–11*) contain sequences corresponding to phosphatase and synthase domains (Schluepmann and Paul 2009; Vandesteene et al. 2010).

In plants, trehalose is an important metabolic signal that regulates gene expression and is associated with diverse processes (Wingler 2002; Eastmond and Graham 2003; Fernandez et al. 2010), including various developmental pathways (Fritzius et al. 2001; Eastmond et al. 2002; Chary et al. 2008; Van Houtte et al. 2013; Wahl et al. 2013), senescence (Wingler et al. 2012), and
responses to biotic stresses (Reignault et al. 2001; Brodmann et al. 2002; Foster et al. 2003; Singh et al. 2011; Govind SR et al. 2016) and abiotic stresses (Avonce et al. 2004; Zhang et al. 2006; Li et al. 2011; Van Houtte et al. 2013; Henry C et al. 2015). TREHALOSE PHOSPHATE SYNTHASE 11-dependent trehalose metabolism mediates Arabidopsis thaliana defenses against the green peach aphid by regulating the expression of PAD4, which encodes phytoalexin deficient 4 (Singh et al. 2011). However, little is known about the molecular mechanisms underlying trehalose effects on plant immune responses.

Multiprotein bridging factor 1 (MBF1) is a highly conserved transcriptional co-activator (Kim et al. 2007). There are three known Arabidopsis thaliana MBF1 genes (MBF1a, MBF1b, and MBF1c), with MBF1a and MBF1b belonging to plant group I and MBF1c classified in plant group II (Tsuda et al. 2004). An earlier study confirmed that MBF1a and MBF1b are involved in the regulation of developmental processes (Tsuda and Yamazaki 2004). In contrast, the expression of MBF1c is induced in Arabidopsis thaliana in response to heat, drought, salinity, hydrogen peroxide, and exogenously applied plant hormones (Suzuki et al. 2005). Additionally, MBF1 proteins reportedly function as non-DNA-binding transcriptional co-activators in various organisms, from yeast to humans (Liu et al. 2003; Suzuki et al. 2005). However, the identification of CTAGA as a putative binding sequence element for MBF1c suggests that Arabidopsis thaliana MBF1c functions as a transcriptional regulator that binds to DNA (Suzuki et al. 2011).

In this study, we confirm that Arabidopsis thaliana TPS5, which encodes a trehalose-synthesizing enzyme with TPS-like and TPP-like domains, is required for defense responses to Botrytis cinerea, but it enhances the susceptibility of plants to P. syringae. The encoded TPS5 regulates defenses against pathogens by interacting with MBF1c, which induces TPS5 expression during pathogen infection. Although TPS5 is required for basal defenses against Botrytis cinerea, and functions upstream of PDF1.2, elevated trehalose levels promote
P. syringae growth and disease development, via a mechanism involving the suppression of the plant defense gene, PR1. Our results reveal a previously unrecognized regulatory function for TPS5-dependent trehalose synthesis in plant defenses against pathogens.

RESULTS
Expression of TPS5 during plant defense responses
The TPS5 gene encodes an enzyme putatively involved in trehalose biosynthesis. To functionally characterize TPS5, we first examined its expression in A. thaliana. The basal TPS5 expression levels in different organs were assessed using quantitative real-time polymerase chain reaction (qRT-PCR) assays. The TPS5 expression level was higher in senescent leaves than in the other tested organs (Figure S1C).

An analysis of the effects of defense-related hormones on TPS5 expression indicated that treatments with SA or JA up-regulated expression (Figure 1A). To confirm the TPS5 expression levels due to biotic stress, we analyzed TPS5 transcript levels in plants infected by B. cinerea; within 24 h post-inoculation, transcript abundance was elevated in inoculated plants relative to the control plants (Figure 1B).

The TPS5 expression was next assessed in response to P. syringae; transcript levels increased within 8 h of infiltration with P. syringae pv. tomato strain DC3000 (Figure 1B). Moreover, the qRT-PCR data confirmed that the TPS5 expression level was higher than that of the other 10 TPS genes in response to pathogen stress in A. thaliana (Figure 1C). These results indicated that TPS5 may be involved in basal defenses against pathogens.

TPS5 enhanced resistance to B. cinerea but promoted susceptibility to P. syringae
To elucidate the regulatory functions of TPS5 related to plant defense, we first identified two TPS5 knockout mutants, tps5-1 and tps5-2, which have T-DNA
fragments inserted into the first exon and the promoter of AT4G17770, respectively (Figure S1A). We then generated and analyzed transgenic A. thaliana plants expressing TPS5 under the control of the Cauliflower mosaic virus 35S promoter. Experiments confirmed that TPS5 transcript levels were lower and higher in the tps5 mutants and independent 35S:TPS5 transgenic plants, respectively, than in the wild-type plants (Figure S1B).

The performance of wild-type, tps5, and 35S:TPS5 plants was assessed in response to B. cinerea and P. syringae. Wild-type plants infected by B. cinerea exhibited a certain level of chlorosis, with visible necrotic lesions. The tps5 mutant leaves were severely necrotic or macerated at 3 d-post-inoculation (dpi; Figure 2A; Figure S2A). By contrast, plants over-expressing TPS5 had less extensive disease symptoms compared with wild-type plants, suggesting that the over-expression of TPS5 enhanced the resistance to B. cinerea (Figure 2A; Figure S2A). Additionally, the average lesion areas on the tps5 mutant leaves and transgenic seedlings were significantly higher and lower, respectively, than the average lesion areas on the wild-type control plants (Figure 2B). These results indicated that TPS5 positively regulates plant resistance to B. cinerea.

Three-week-old soil-grown plants were inoculated with P. syringae. Bacterial growth and disease symptoms were less extensive in the tps5 mutant plants than in the wild-type controls (Figure 2D; Figure S2B). By contrast, lines that over-expressed TPS5 had significantly higher levels of bacterial growth than wild-type and tps5 mutant plants at 3 dpi with P. syringae (Figure 2E). Additionally, chlorosis and disease symptoms were more extensive in the 35S:TPS5 plants than in the wild-type plants (Figure 2D; Figure S2B).

To determine whether the altered responses of the tps5 mutants and 35S:TPS5 transgenic plants to B. cinerea and P. syringae were related to modified JA- or SA-mediated defense mechanisms, two reliable molecular markers, PDF1.2 and PR1, were analyzed by qRT-PCR. The PDF1.2 transcript level was lower and the PR1 transcript level was higher in the tps5 mutant than in the wild-type controls, whereas the opposite pattern was
observed for the 35S:TPS5 plants at 1, 2, and 3 dpi (Figure 2C, F). These results indicated that TPS5 may positively regulate defense responses against necrotrophs like B. cinerea, but negatively regulate defense responses against biotrophs like P. syringae.

Effect of trehalose on pathogen infection

To study the effect of trehalose on pathogen infection, A. thaliana leaves were sprayed with a 10 µM trehalose solution before inoculations with B. cinerea or P. syringae. Noticeable disease symptoms were induced by B. cinerea in wild-type plants treated with water. By contrast, only a few necrotic spots were observed in plants treated with trehalose, and the leaves remained green, with relatively little tissue damage (Figure 3A). Trypan blue staining revealed greater cell mortality in leaves pre-treated with water than in leaves pre-treated with trehalose (Figure 3B).

Experiments performed on detached leaves treated with trehalose prior to the inoculation with B. cinerea were consistent with those using whole plants (Figure S3). At 2 dpi with P. syringae, bacterial growth was significantly higher in leaves pre-treated with trehalose than in leaves pre-treated with water (Figure 3C, D); a similar result was obtained for disease symptoms (Figure 3C).

To determine whether the tested pathogens were affected by exogenous trehalose, we next conducted in vitro pathogen growth assays. The growth rate of B. cinerea isolates (Figure S4A) and the germination of conidia (Figure S4B) were tested on potato dextrose agar medium, with or without trehalose. The presence of trehalose had no significant effect on B. cinerea growth. Regarding P. syringae growth, the bacterium was pre-cultured for 24 h in King’s liquid medium, with or without trehalose, before we counted colonies. There was no significant difference in P. syringae growth in the medium with or without trehalose (Figure S4C). These assays established that exogenous trehalose did not affect the growth of B. cinerea and P. syringae. Taken
together, these findings suggested that trehalose metabolism negatively regulates *B. cinerea* infection, while positively regulating *P. syringae* infection.

**TPS5 is necessary for trehalose synthesis after pathogen infection**

Trehalose levels were similar in uninfected wild-type and *tps5* mutant plants, indicating that *TPS5* does not contribute to basal trehalose content in *A. thaliana* leaves (Figure 4A). Because *B. cinerea* and *P. syringae* infections induce *TPS5* expression, trehalose levels were measured in leaves infected by *B. cinerea* or *P. syringae* to assess the impact on trehalose content. Here, infection by either *B. cinerea* or *P. syringae* increased trehalose content in wild-type but not *tps5-1* leaves (Figure 4A). Moreover, the basal trehalose content was higher in 35S:*TPS5* plants than in wild-type plants (Figure 4D). Meanwhile, trehalose treatment enhanced the resistance of the *tps5-1* mutant plants to *B. cinerea* (Figure 4B).

The expression level of *B. cinerea ActinA* was measured to assess the biomass of the fungal pathogen on infected plants. The trehalose treatment significantly down-regulated the expression of *ActinA* (Figure 4B), but increased *P. syringae* levels (Figure 4C). The impact of trehalose on plant defense responses was further tested with *tre1* mutants, which accumulate more trehalose than wild-type plants because of a lack of trehalase (Figure 5A). The expression levels of *B. cinerea ActinA* were lower in the *tre1* mutants than in the wild-type controls (Figure 5B). However, *P. syringae* levels were significantly higher in the *tre1* mutants than in the wild-type plants (Figure 5C). These results suggested a role for trehalose in *A. thaliana* defense responses.

**TPS5 interacts with MBF1c and is regulated by MBF1c**

To study the molecular mechanism underlying *TPS5* functions, during pathogen infection, a yeast two-hybrid assay was conducted using the full-length *TPS5* as bait to detect potential interacting proteins. We observed that MBF1c was frequently represented among 28 colonies with potential
interacting proteins. To verify the interaction in yeast cells, the *MBF1c* open reading frame was introduced into the pGBK-T7 vector. As shown in Figure 6A, TPS5 strongly interacted with MBF1c.

The TPS5 C-terminus contains three conserved domains. To ascertain which TPS5 region is required for the interaction with MBF1c, five truncated TPS5 variants were inserted into the pGAD-T7 vector. Deletion of the N-terminus (AD-TPS5, D276–862) did not affect the interaction with MBF1c. By contrast, deletion of the C-terminal residues (AD-TPS5, D1–568) eliminated the interaction between TPS5 and MBF1c (Figure 6A), suggesting the importance of the C-terminal domain.

This interaction was also confirmed, in planta, by bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (CoIP) assays. For the BiFC assay, MBF1c and TPS5 were fused to the C-terminal yellow fluorescent protein (YFP) fragment and the N-terminal YFP fragment, respectively, to generate the MBF1c-cYFP and TPS5-nYFP fusion proteins. The YFP signal was observed in *Nicotiana benthamiana* leaf cells co-expressing the sequences encoding MBF1c-cYFP and TPS5-nYFP (Figure 6B). Fluorescence was undetectable in the negative controls (Figure 6B). Additionally, the TPS5–MBF1c interaction was verified by a CoIP assay involving plant total protein extracts (Figure 6C). These results demonstrated that TPS5 interacts with MBF1c in plant cells, a finding consistent with the hypothesis that their function requires the formation of a protein complex.

Our qRT-PCR analysis indicated that *TPS5* expression was down-regulated in *mbf1c* plants, but up-regulated in transgenic plants in which *MBF1c* was ectopically expressed (*OE4*; Figure 6D). Moreover, trehalose content was increased in wild-type plants infected by a pathogen and in plants ectopically expressing *MBF1c*, but not in the *mbf1c* mutant (Figure 6E). These results suggested that MBF1c regulates trehalose accumulation during pathogen infection.

To further clarify how MBF1c impacts trehalose synthesis, we performed
chromatin immunoprecipitation (ChIP) experiments using a transgenic line producing a MYC-MBF1c fusion protein. Because MBF1c reportedly specifically binds to a core DNA motif (CTAGA), we analyzed this motif sequence in the promoter regions of the 11 *A. thaliana TPS* family genes. Motifs were identified not only in the promoter of *TPS5*, whose expression was notably induced by pathogen infection, but also in the promoters of *TPS4*, *TPS7*, and *TPS10*, whose expression was not induced or only slightly induced by pathogen infection (Figure 1C; Figure S7A).

PCR amplification of the promoter regions of these four *TPS* genes suggested that MYC-MBF1c specifically bound to the motif-containing promoter region of *TPS5* (Figure S7B). The qRT-PCR data from the ChIP assay, using an antibody against MYC, further supported this notion. Moreover, obvious *B. cinerea*-dependent binding to the promoter regions of *TPS5* was detected, but not binding to the promoter regions of *TPS4*, *TPS7*, and *TPS10* (Figure S7C). Yeast one-hybrid assays also indicated that MBF1c can bind to the *TPS5* promoter (Figure S8A, B).

If the inability to induce *TPS5* expression is the primary cause of the sensitivity of *mbf1c* mutant plants to *B. cinerea* infection, the over-expression of *TPS5* should rescue these mutants. We observed that the *mbf1c* mutant plants, expressing *TPS5* under the control of the 35S promoter, were indeed better protected against *B. cinerea* than the *mbf1c* mutants (Figure S7D). These results demonstrated that the MBF1c transcription factor regulates *TPS5*-dependent trehalose synthesis by interacting with TPS5 and binding to the *TPS5* promoter region.

**MBF1c can regulate the defense response against *B. cinerea* and *P. syringae***

To elucidate the contribution of *MBF1c* to disease resistance, we characterized two *MBF1c* T-DNA-insertion alleles, designated as *mbf1c-1* (SALK_083813) and *mbf1c-2* (CS873275) that were confirmed by primer-specific PCR (Figure
S5A, B). To measure $MBF1c$ transcript levels in these mutant plants, we conducted a qRT-PCR analysis using RNA samples from plants infected with $B. \textit{cinerea}$ or $P. \textit{syringae}$. In response to pathogen infection, $MBF1c$ transcript levels increased in wild-type plants, but not in $mbf1c$ mutant plants (Figure S5C). Moreover, $MBF1c$ expression was induced by SA and MeJA treatments (Figure S5E). Thus, $MBF1c$ expression was responsive to pathogen infections.

Disease symptoms were detected on wild-type leaves spray-inoculated with $B. \textit{cinerea}$ (Figure 7A), whereas leaves of mutant plants were decayed and macerated at 3 dpi, with the average lesion size being larger in mutant compared with wild-type leaves (Figure 7A). To determine whether the altered response of $mbf1c$ mutants, to $B. \textit{cinerea}$, was related to changes in JA-mediated defense pathways, we examined the expression of $PDF1.2$. Following infection by $B. \textit{cinerea}$, the $PDF1.2$ transcript levels were much higher in wild-type than $mbf1c$ plants (Figure 7B).

To further examine the effects of MBF1c on $B. \textit{cinerea}$ and $P. \textit{syringae}$, wild-type plants as well as $MBF1c$ over-expression and mutant lines were challenged with these two pathogens. Our qRT-PCR data confirmed the elevated $MBF1c$ transcript level in independent $35S:MBF1c$ transformants ($OE4$ and $OE8$; Figure S5D). The plants over-expressing $MBF1c$ developed fewer disease symptoms than wild-type plants, at 3 dpi, indicating that ectopically expressed $MBF1c$ could enhance resistance to $B. \textit{cinerea}$ (Figure S6B).

The $PDF1.2$ transcript levels were correspondingly higher in plants that over-expressed $MBF1c$ than in wild-type plants (Figure S6B). Moreover, $P. \textit{syringae}$ growth was significantly greater in $35S:MBF1c$ line $OE8$ than in the wild-type controls (Figure 7C). However, at 4 dpi, chlorosis was more extensive in the inoculated leaves of $35S:MBF1c$ transgenic plants than in leaves of wild-type and mutant plants (Figure 7C). Infection by $P. \textit{syringae}$ resulted in $PR1$ expression levels that were considerably higher in wild-type than in the $35S:MBF1c$ plants (Figure 7D), suggesting that transgenic plants
over-expressing MBF1c are more susceptible to P. syringae than the wild-type controls. These results provide support for the hypothesis that MBF1c positively regulates defense responses against B. cinerea, while negatively regulating defenses against P. syringae.

Trehalose accumulated in wild-type plants during B. cinerea infection under control conditions. By contrast, trehalose did not accumulate in mbf1c mutant plants during B. cinerea infection (Figure 6E). Additionally, the TPS5 expression levels were lower in mbf1c than in wild-type plants (Figure 6D). Thus, mbf1c plants may have been more susceptible to B. cinerea due to the decreased trehalose accumulation during the B. cinerea infection. These data indicated that MBF1c can affect basal plant defenses, possibly by inducing the accumulation of trehalose.

TPS5 coordinates with MBF1c in basal defense responses
To analyze the functions of TPS5 and MBF1c in the tps5 mbf1c-null background, a double mutant was obtained by crossing the tps5-1 and mbf1c-1 mutants. We then examined the responses of wild-type plants as well as the mbf1c and tps5-1 mbf1c-1 double mutant plants to P. syringae infection. Here, the fewest disease symptoms were observed in these double mutant plants (Figure 8A). Bacterial growth increased in the mbf1c mutant plants, which was in contrast to the results for the double mutant lines. An even greater increase in bacterial growth and the most severe disease symptoms were observed in wild-type plants. The double mutants exhibited up-regulated PR1 expression at 5 dpi, whereas wild-type plants had the lowest PR1 transcript levels at this time point. These results were consistent with the fact that wild-type plants exhibited the most severe disease symptoms (Figure 8B). Thus, the disruption of both TPS5 and MBF1c decreased the susceptibility of plants to P. syringae.

For B. cinerea infection, most leaves of the double mutant plants showed the appearance of chlorotic or macerated symptoms at 2 dpi. These symptoms
worsened at 4 dpi, resulting in severely damaged plants, whereas most of the wild-type leaves remained green (Figure 8C). Correspondingly, the PDF1.2 transcript levels were significantly down-regulated in double mutant leaves treated with B. cinerea (Figure 8D). These results indicated that TPS5, in combination with MBF1c, can positively regulate defenses against B. cinerea, while negatively regulating defenses against P. syringae.

**Over-expression of TPS5 enhances the phenotype of sid2 mutants upon a P. syringae infection**

We analyzed the expression of ICS1 and PBS3, which encode the enzymes responsible for most of the SA synthesis during infections, to investigate whether plants over-expressing TPS5 were more susceptible to P. syringae due to a decrease in SA biosynthesis. The ICS1 and PBS3 expression levels were up-regulated at 48 h post-inoculation in wild-type and 35S:TPS5 plants (Figure 9A, B). The ICS1 expression levels in 35S:TPS5 plants and tps5 mutants were lower and higher, respectively, than in the wild-type controls (Figure 9A). Although these results indicated that the expression of ICS1 was somewhat repressed by high TPS5 expression levels, this difference was not significant.

Our data, described herein, suggested that the trehalose-mediated increased susceptibility of plants to P. syringae may not be due to decreased SA production. To test this possibility, we incorporated 35S:TPS5 into the sid2-2 mutant background to obtain sid2-2 35S:TPS5 plants, which were then infected with P. syringae. Bacterial growth was greater in the sid2-2 35S:TPS5 line than in either 35S:TPS5 or sid2-2 lines (Figure 9C, D). We confirmed that TPS5 over-expression in the sid2-2 background enhanced the susceptibility of plants to P. syringae because of the additive effects of elevated trehalose levels and impaired SA synthesis. Pathogen growth was greater in sid2-2 35S:TPS5 plants than in plants with either 35S:TPS5 or sid2-2 alone. Thus, TPS5 over-expression apparently can promote bacterial growth, possibly in an
DISCUSSION

Trehalose is a natural sugar that is important for plant development and abiotic stress tolerance (Wahl et al. 2013; Henry et al. 2015). Many research groups are interested in trehalose metabolism and signaling (Satoh-Nagasawa et al. 2006). However, it remained unclear as to whether trehalose metabolism regulates responses to pathogens in A. thaliana. In the present study, we demonstrate that trehalose affects A. thaliana basal defense responses.

A decrease in disease symptoms, including the number of lesions, was observed following a pre-treatment with trehalose prior to infection by B. cinerea (Figure 3A). This indicates that trehalose can interfere with fungal infection and, thereby, restrict fungal growth. The insertion of T-DNA fragments into TPS5 resulted in decreased resistance to B. cinerea (Figure 2A). We also observed a transient increase in trehalose levels in wild-type plants, but not in tps5 mutant plants (Figure 4A). These results suggest that TPS5 is required for A. thaliana defenses against B. cinerea.

There is evidence that trehalose may function as a general toxin that can disrupt fungal infection. In addition, several studies have confirmed that exogenously applied trehalose can negatively affect seedling growth and development (Schluepmann et al. 2004; Bae et al. 2005; Gravot et al. 2011). However, another study concluded that trehalose does not have a toxic effect, as evidenced by the lack of any visible symptoms following the treatment of mature plants with trehalose (Djonović et al. 2013). Our data also indicate that exogenously applied trehalose does not visibly influence plant development.

It was earlier shown that trehalose production, by PA14, is required for virulence when the pathogen infects A. thaliana (Djonović et al. 2013), but the precise effects of trehalose on plant defenses remained unclear. Trehalose may directly affect pathogen biology, for example, by enabling the pathogen to sense the plant host and up-regulate the expression of virulence genes, or by
altering the metabolism of the pathogen in ways that allow it to better grow within the host and, thereby, cause disease (Wilson et al. 2007; Djonović et al. 2013). Our results indicate that exogenously-applied trehalose does not affect the growth of \textit{B. cinerea} and \textit{P. syringae} (Figure S4A–C).

Earlier investigations concluded that JA signaling is required for disease resistance against necrotrophic pathogens (Seo et al. 2001; Zheng et al. 2006). In our study, \textit{TPS5} expression was induced by JA (Figure 1A) and \textit{B. cinerea} (Figure 1B). The knockout of \textit{TPS5} increased the susceptibility of plants to \textit{B. cinerea} because of the suppression of JA-mediated expression of defense genes (Figure 2C). By contrast, over-expression of \textit{TPS5} increased the plant resistance to \textit{B. cinerea}, via activation of the JA-mediated defense gene, \textit{PDF1.2} (Figure 2A, C). These findings indicate that \textit{TPS5} encodes a positive regulator of JA-mediated defense responses.

The \textit{TPS5} knockout plants examined in this study exhibited enhanced resistance towards \textit{P. syringae}, whereas plants over-expressing \textit{TPS5} were less resistant to \textit{P. syringae} than the wild-type plants (Figure 2D; Figure S2B). Suppression of JA-mediated defense responses is compensated by the activation of the SA-dependent defense responses (Seo et al. 2001). The enhanced disease resistance established in \textit{tps5} mutant plants was accompanied by an up-regulated expression of the SA-dependent \textit{PR1} gene (Figure 2F). These results demonstrate that \textit{TPS5} negatively regulates SA-mediated defense responses.

There are three \textit{MBF1} genes in \textit{A. thaliana} and earlier studies showed that \textit{MBF1c} functions upstream of SA, ET, and trehalose signaling in response to heat stress (Suzuki et al. 2005; Suzuki et al. 2008). Moreover, \textit{MBF1c} is required for the production of many heat shock proteins and ascorbate peroxidase 1 during heat stress responses (Suzuki et al. 2005; Suzuki et al. 2008). However, the mode of action of \textit{A. thaliana} \textit{MBF1c} during exposures to biotic stress remains unclear.

Our studies establish that \textit{MBF1c} positively regulates defense responses
against *B. cinerea*, but negatively regulates defense responses against *P. syringae* (Figure 7A–D; Figure S6B). In this regard, we confirmed that TPS5 can interact with MBF1c, *in vivo*, and that they function cooperatively during basal pathogen defense responses. The transcriptional co-activator MBF1c was shown to interact with TPS5 in yeast cells during heat stress conditions (Suzuki et al. 2008). Such an interaction between TPS5 and MBF1c, during pathogen infection, was confirmed by our findings.

The knockout of *MBF1c* resulted in plants with lower trehalose levels than in wild-type plants following *B. cinerea* infection (Figure 6E), as well as increased susceptibility to *B. cinerea* (Figure 7A). These findings provide support for the notion that the MBF1c transcription factor regulates trehalose synthesis by interacting with TPS5 and binding to the TPS5 promoter region (Figure S7A–C; Figure S8A, B). Additionally, the over-expression of TPS5 rescued the sensitivity of *mbf1c* mutants to *B. cinerea* (Figure 7D).

Based on our collective findings, we developed a working model for the roles of trehalose during pathogen infection (Figure S9). Here, an enhanced susceptibility to *P. syringae*, due to increased trehalose levels, occurs through a mechanism involving suppression of the plant defense gene, *PR1*. Plants over-expressing TPS5 restrict *B. cinerea* growth and disease development, via a mechanism that involves the up-regulated expression of the plant defense gene, *PDF1.2*.

Characterizing the roles of plant defense (SA and JA) pathways is important for clarifying plant–pathogen interactions. Additional signaling pathways remain to be investigated regarding how they influence the ability of pathogens to infect host plants and cause disease (Gimenez-Ibanez and Solano 2013). Our findings suggest that diverse trehalose-regulated pathogenic mechanisms should be considered. Elucidating the effects of trehalose on pathogen infections may have direct applicability to enhance agricultural production.

In summary, we analyzed the function of a class II TPS and the role of
trehalose metabolism in plant defense responses. We demonstrated that exogenously-applied trehalose can increase *A. thaliana* resistance to *B. cinerea*, but promote the growth of *P. syringae*. Furthermore, TPS5-dependent trehalose metabolism can positively regulate *A. thaliana* resistance to *B. cinerea*, whereas it negatively regulates defense responses to *P. syringae*. However, the molecular mechanisms underlying these observations will need to be thoroughly investigated.

**MATERIALS AND METHODS**

**Materials**

The *A. thaliana* wild-type and mutant lines used in this study were in the Columbia genetic background. We obtained *mbf1c-1* (SALK_083813), *mbf1c-2* (CS873275), *tre1-1* (SALK_147073), and *tre1-2* (CS871511) mutants from the Arabidopsis Biological Resource Center. The *tps5-1* (SALK_144791) mutant was a gift from Dongping Li (Hunan Normal University). Seeds were sown on half-strength Murashige and Skoog medium and incubated at 4°C for 3 d to break the residual dormancy. *Arabidopsis thaliana* plants were then grown in a growth cabinet set at 22°C with a photoperiod of 8 h light (100 µE m⁻² s⁻¹) and 16 h darkness.

**Constructs**

To prepare the 35S:TPS5 construct, the TPS5 open reading frame was amplified by PCR from *A. thaliana* wild-type leaf cDNA and inserted into the pOCA30 vector. The 35S:TPS5 plasmid was inserted into wild-type plants according to the floral dip method (Clough and Bent 1998). For the production of TPS5-nYFP and MBF1c-cYFP fusion proteins, full-length TPS5 and MBF1c open reading frames were amplified without their stop codons. The PCR products were cloned into the pFGC-YN/YC vectors. To prepare the Pro<sub>MBF1c</sub>:Myc-MBF1c fusion construct, the approximately 1.2-kb MBF1c
promoter was amplified by PCR from *A. thaliana* wild-type genomic DNA, inserted into the pOCA28-polyA vector, and fused to the MBF1c open reading frame.

**Generation of the sid2-2 35S:TPS5 and mbf1c-1 35S:TPS5 lines**

To generate the sid2-2 35S:TPS5 and mbf1c-1 35S:TPS5 lines, homozygous sid2-2 and mbf1c-1 lines were crossed with 35S:TPS5 transgenic plants. We used PCR to identify homozygous plants for the sid2-2 and mbf1c-1 mutations.

**Fungal culture and disease assays**

A *B. cinerea* isolate from Yunnan University was cultured on potato dextrose agar medium and incubated at 26°C in darkness. *B. cinerea* spores were collected and inoculated onto *A. thaliana* plants, as previously described (Mengiste et al. 2003). *A. thaliana* plants were infected with *B. cinerea* by spraying with a conidial spore suspension (1 × 10⁶ spores mL⁻¹ in Sabouraud maltose broth). Infected leaves were harvested for subsequent analyses.

**Treatments**

A JA stock solution was diluted to 100 μM with water and sprayed onto plants. Meanwhile, SA was dissolved in water as a 100 mM stock solution and the pH was adjusted to 6.5 with KOH. Plants were sprayed with a 10 mM SA solution. Trehalose was dissolved in water as a 10 μM solution and was freshly prepared before use. Both sides of leaves were sprayed with the trehalose solution (six or eight sprays) before plants were infected with the pathogens. Control plants were sprayed with sterile water.

For the *P. syringae* treatment, inoculations involved the infiltration of leaves with *P. syringae* pv. tomato strain DC3000 (OD₆₀₀ = 0.0001 in 10 mM MgCl₂). Inoculated leaves were harvested at specific post-infection time points. Diluted leaf extracts were plated on King’s B medium supplemented with rifampicin (100 μg mL⁻¹) and kanamycin (25 μg mL⁻¹) and incubated at 28°C before
counting the colony-forming units.

**RT-PCR experiments**
Total RNA (2 μg) was reverse transcribed in a 20 μL reaction mixture using Superscript II reverse transcriptase. After the reaction was completed, 1 μL aliquots were used as the template for PCR amplifications with specific primers (Table S1). Data presented were derived from at least three independent biological replicates comprising at least three technical replicates. Expression levels were normalized against ACTIN2 expression levels. Values are presented as the mean ± standard error. Means were analyzed using Student’s *t*-test.

**Yeast two-hybrid assays**
To confirm protein–protein interactions in yeast two-hybrid assays, the full-length MBF1c CDS was cloned into the bait vector pGBK T7, while the full-length TPS5 CDS and truncated TPS5 CDS were cloned into the pGADT7 vector. The primers used for the yeast two-hybrid are listed in Table S1.

**Yeast one-hybrid assays**
Yeast one-hybrid assays were conducted according to the manufacturer’s instructions (Clontech). Normal or mutated sequences (60 bp) were cloned into the pHis2 vector, which was then linearized and integrated into the yeast genome (Y1H Gold). Yeast cells were then transformed with the pGADT7-AD plasmid containing MBF1c. The ability of the transformed yeast cells to grow on −Leu/−Trp/−Ura medium in the presence of 60 mM 3-amino-1,2,4-triazole (Sigma) was assessed to identify positive interactions.

**BiFC and CoIP assays**
For the BiFC assays, the full-length MBF1c CDS was inserted into the pFGC-cYFP vector to generate a C-terminal in-frame fusion with cYFP, while
the TPS5 CDS was incorporated into the pFGC-nYFP vector to form an N-terminal in-frame fusion with nYFP. The resulting plasmids were inserted into Agrobacterium tumefaciens strain EHA105 cells for an infiltration of N. benthamiana plants. Infected tissues were analyzed 48 h after the infiltration (Hu et al. 2013b). The DAPI fluorescence and YFP signals were observed with a confocal laser scanning microscope (Olympus, Tokyo, Japan). Primers used to prepare the BiFC constructs are listed in Table S1.

For the Co-IP assays, the full-length MBF1c, JAZ1, and TPS5 CDSs were individually cloned in the sense orientation into tagging plasmids downstream of the MYC or FLAG tag sequence so that expression was under the control of the Cauliflower mosaic virus 35S promoter. The constructs were inserted into A. tumefaciens strain EHA105 cells. The constructs encoding MYC-MBF1c or MYC-JAZ1 were transiently co-expressed with the construct encoding FLAG-TPS5 in Nicotiana benthamiana. Infected leaves were harvested 48 h after the infiltration for a subsequent protein extraction, as previously described (Shang et al. 2010). Briefly, FLAG-TPS5 was immunoprecipitated with an anti-FLAG antibody and the co-immunoprecipitated protein was then detected using an anti-MYC rabbit antibody (Sigma-Aldrich).

**Chromatin immunoprecipitation**

Two-week-old ProMBF1c:Myc-MBF1c/mbf1c-1 (MYC-MBF1c) transgenic plants were analyzed by ChIP assays, conducted as previously described (Mukhopadhyay et al. 2008; Saleh et al. 2008; Jiang et al. 2014). Fragmented chromatin was immunoprecipitated using an anti-MYC antibody. The DNA extracts separated from the DNA–protein complexes were analyzed by a qRT-PCR. The primer sets used in this analysis amplified various regions of the JAZ and TPS loci. The ChIP assays were conducted three times.

**Other methods**

Trehalose content was measured, as previously described (Suzuki et al. 2008).
To visualize dying plant cells, leaves were stained with trypan blue using an established procedure (Koch and Slusarenko, 1990).

**Accession numbers**

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative databases under the following accession numbers: TPS1 (AT1G78580), TPS2(AT1G16980), TPS3(AT1G17000), TPS4(AT4G27550), TPS5(AT4G17770), TPS6(AT1G68020), TPS7(AT1G06410), TPS8(AT1G70290), TPS9(AT1G23870), TPS10(AT1G60140), TPS11(AT2G18700), TRE1(AT4G24040), MBF1C(AT3G24500), PR1(AT2G14610), PDF1.2(AT5G44420), SID2/ICS1(AT1G74710), PBS3(AT5G13320).

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**AUTHOR CONTRIBUTIONS**

X.W. carried out experiments and wrote the manuscript. Y.D performed expressions analyses. D.Y. designed the experiment, supervised the study and revised the manuscript.

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Figure Legends

Figure 1. Analysis of TPS5 expression

(A) Induced expression of TPS5 in response to defense-related hormones
MeJA and SA. RNA samples were prepared from 4-week-old wild-type plants, at the indicated times, after spraying with MeJA (0.1 mM) and SA (2 mM). (B) Expression of TPS5 after pathogen infection. For P. syringae treatment, 4-week-old wild-type plants were infiltrated with a suspension of P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl2), and inoculated leaves were collected at the indicated times. For B. cinerea treatment, 4-week-old wild-type plants were inoculated by spraying with a spore suspension. Plants were maintained under high humidity and whole seedlings were collected for isolation of RNA, at the indicated times. (C) Relative expression of all TPS transcripts at 48 h after P. syringae and B. cinerea infection. Sterile water was used as control (Student’s t-test indicated that the values were significantly different from the wild-type treated with water, *P < 0.05, **P < 0.01, ***P<0.001).

Figure 2. Responses of the tps5 mutant and 35S:TPS5 lines to B. cinerea and P. syringae

(A, B) Disease response of B. cinerea drop-inoculated plants at 3 dpi. (C) Expression of JA-regulated defense gene, PDF1.2, during B. cinerea infection. Treatment times are indicated. Mean ± SE are shown (n = 5 whole plants per treatment). (D, E) Disease symptoms and bacterial growth on leaves inoculated with a virulent strain of P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl2) 3 dpi. Bacterial disease assays were performed at least three times with similar results. (F) Expression of SA-regulated defense gene PR1 during P. syringae infection. Treatment times are indicated. Mean ± SE are shown (n = 10 leaves per treatment). In (B), values are mean ± SE, student’s t-test indicated by two asterisks were significantly different from the wild-type (P < 0.01; n = 5 experiments). In (C) and (F), error bars represent SE. Asterisks indicate significant differences (*P < 0.05, **P<0.01) from the wild type (WT) samples for that time point.
Figure 3. Influence of exogenous trehalose on pathogens growth

(A) Disease response of B. cinerea spray-inoculated plants. Before B. cinerea was inoculated, plants were treated with trehalose solution (0.01 mM) or sterile water without trehalose. (B) Leaves from 4-week-old soil-grown wild-type plants were spray-inoculated with B. cinerea and analyzed for development of cell death by staining with trypan blue at 4 dpi. (C) Disease symptoms on plants inoculated with a virulent strain of P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl₂) 2 dpi. (D) P. syringae growth on plants showing increased susceptibility of the trehalose solution-treated plants. Values are mean ± SE (n = 5 experiments). Asterisks indicate significant differences (**P<0.01) from the sample without trehalose treatment for that time point.

Figure 4. TPS5 is required for the transient accumulation of trehalose in B. cinerea- and P. syringae-infected plants

(A) Trehalose content in uninfected and P. syringae- or B. cinerea-infected wild-type and tps5-1 plants. Sterile water was used as control. Asterisk indicates a significant difference (*P<0.05). (B) Accumulation of ActinA mRNA of B. cinerea in spray-inoculate wild-type and tps5-1 plants treated with trehalose (0.01mM). (C) Growth of P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl₂) assessed at the indicated times. Wild-type and tps5-1 plants were treated with trehalose (0.01 mM). (D) Basal trehalose content in wild-type and three independently derived 35S:TPS5 lines in the tps5-1 genetic background. Student’s t-test indicated that the values were significantly different from the wild-type for that time point, *P<0.05, **P<0.01).

Figure 5. Trehalose hyper-accumulating tre1 plants exhibit heightened resistance to B. cinerea, but enhanced susceptibility to P. syringae

(A) Measurements of trehalose in leaves of wild-type, tps5-1 and tre1 mutant plants. Values are mean ± SE (n = 5 replicates). Asterisk indicates a
significant difference (*P < 0.05, **P < 0.01) from the wild type. (B) Accumulation of *B. cinerea* ActinA mRNA in spray-inoculated wild-type and *tre1* mutants plants. Error bars represent SE. Asterisks indicate significant differences (*P < 0.05, **P < 0.01) from the wild-type sample for that time point. (C) Growth of *P. syringae* in wild-type and *tre1* mutants plants following syringe infiltration (optical density at 600 nm = 0.0001 in 10 mM MgCl₂). Values are mean ± SE (n = 3 biological replicates).

**Figure 6. TPS5 physically interacts with MBF1c**

(A) Analysis of yeast two-hybrid assays. The C-terminal fragment of TPS5 is involved in the interaction of TPS5 with MBF1c. Left: Diagram of full-length and truncated TPS5 constructs with specific deletions. Right: Interactions were indicated by the ability of yeast cells to grow on synthetic dropout medium lacking Leu, Trp, His, and Ade. Empty pGBK7 vector was used as a negative control. (B) BiFC analysis. Fluorescence was observed in nuclear compartments of *N. benthamiana* leaf epidermal cells; the fluorescence resulted from complementation of TPS5-nYFP with MBF1c-cYFP. No signal was observed from negative controls. DAPI, 4,6-diamidino-2-phenylindole. (C) Co-IP analysis. Flag-fused TPS5 protein was immunoprecipitated using an anti-Flag antibody, and coimmunoprecipitated MBF1c and JAZ1 factors were then detected using an anti-MYC rabbit antibody. Protein input for Flag-TPS5 protein in immunoprecipitated complexes was also detected and shown. MYC-JAZ1 was used as a negative control. (D) Expression of *TPS5* in 35S:MBF1c (OE4) and *mbf1c* plants compared with the wild type during *B. cinerea* infection. (E) Measurements of trehalose in wild-type, *mbf1c* and 35S:MBF1c (OE4) plants at different times during *B. cinerea* infection. In (D) and (E), error bars represent SE. Asterisks indicate significant differences (*P < 0.05, **P < 0.01) from the wild-type samples for that time point.

**Figure 7. Phenotypic analyses of the *mbf1c* mutants and MBF1c**
overexpression plants

(A) Disease symptoms in wild-type, mbf1c-1 and mbf1c-2 plants at 3 dpi with B. cinerea. (B) Expression of JA-regulated defense gene PDF1.2 during B. cinerea infection. Treatment times are indicated. (C) Altered disease symptom development. Pathogen inoculation of MBF1c over-expression lines, mbf1c mutant and wild-type plants were infiltrated with a suspension of P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl₂). Images of representative inoculated leaves taken at 4 dpi. Growth of the bacterial pathogen was assessed at 0 and 4 dpi. Mean ± SE. (D) Expression of SA-regulated defense gene PR1 during P. syringae infection. In (A), values are mean ± SE, student’s t-test indicated by one asterisks were significantly different from the wild-type (P < 0.05; n = 4 experiments). In (B) and (D), error bars represent SE. Asterisks indicate significant differences (**P<0.01, ***P<0.001,) from the WT samples for that time point.

Figure 8. Phenotype analysis of tps5 mbf1c-1 double mutants upon B. cinerea and P. syringae infection

(A, B) Double mutant plants have elevated PR1 expression levels and reduced susceptibility to P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl₂) compared with wild-type plants. Growth of P. syringae in plants following syringe infiltration.(C, D) Double mutants plants have reduced PDF1.2 expression levels and reduced resistance to B. cinerea compared with wild-type plants. In (B) and (D), error bars represent SE. Asterisks indicate significant differences (*P < 0.05, **P<0.01, ***P<0.001) from the wild-type sample for that time point.

Figure 9. Overexpression of TPS5 enhances the phenotype of sid2 mutants upon P. syringae infection

(A, B) Expression of ICS1 and PBS3 in plants infected by P. syringae (optical density at 600 nm = 0.0001 in 10 mM MgCl₂). Values are mean ± SE (n = 3
biological replicates). (C, D) Growth of *P. syringae* in wild-type, *sid2*, 35S:TPS5, and *sid2-2 35S:TPS5* plants following syringe infiltration (optical density at 600 nm = 0.0001 in 10 mM MgCl₂). Values are mean ± SE (n = 3 biological replicates).

**Supplemental Data**

**Figure S1. Molecular characterization of TPS5**

(A) Genomic organization of the *tps5* T-DNA insertion alleles. Untranslated regions (UTRs) are represented as open boxes and exons as closed boxes; triangles, T-DNA insertion sites; ATG, initiation codon; TAA termination codon. (B) Downregulation of TPS5 transcript abundance in *tps5* lines and elevated TPS5 expression in 35S:TPS5 transgenic lines compared with wild-type plants is observed, as confirmed by qRT-PCR. Experiments were repeated three times with similar results. Student’s *t*-test indicated that the values were significantly different from the wild-type (*P < 0.05, **P < 0.01, ***P<0.001). (C) TPS5 expression levels in plants tissues. FL, flowers; RT, roots; ST, stems; NL: Young leaves; SL, senescent leaves; YS, young siliques; MS, mature siliques. Student’s *t*-test indicated that the values were significantly different from the wild-type (*P < 0.05).

**Figure S2. Phenotype analysis of *tps5* mutant and TPS5 overexpression plants**

(A) Disease responses of *B. cinerea* drop-inoculated plants at 3 dpi. (B) Disease symptoms of the *tps5-2* mutant and 35S:TPS5 lines to *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM MgCl₂) compared with wild-type plants, respectively. Images of wild-type and *tps5-2* mutant plants taken at 3 d (Upper panel), whereas wild-type and 35S:TPS5 lines were taken at 2 d (Lower panel) after *P. syringae* infection.
**Figure S3. Influence of exogenous trehalose on** B. cinerea **growth**

(A, B) Disease responses of spray-inoculated leaves after treatment with trehalose solution (0.01mM) or sterile water. Asterisk indicates a significant difference from leaves treated with trehalose for that time point (**P<0.05). (C) Accumulation of B. cinerea ActinA mRNA in spray-inoculated plants treated with trehalose (0.01 mM) or sterile water. Asterisk indicates a significant difference from leaves treated with trehalose for that time point (*P<0.05).

**Figure S4. Exogenous trehalose does not affect pathogen physiology as tested by in vitro growth assays**

(A) Growth rate of B. cinerea isolates on PDA or PDA medium supplemented with trehalose (0.01mM). Abscissa numbers represent the growth of days. (B) Effect of trehalose on conidia germination of B. cinerea. Distribution of germ tubes (short, long, middle) produced from conidia incubated for 24 h in Petri dishes without or with trehalose (0.01mM). Percentage of each type of conidia expressed from assessment on 100 conidia for each condition and replicate of the experiment gave similar results. (C) Growth of P. syringae pre-cultivated for 24 h in King's liquid medium containing nonsupplemented or supplemented with trehalose (0.01mM). Data represent mean ± SE (n = 10 experiment replicates).

**Figure S5. Molecular characterization of the mbf1c mutants and MBF1c expression levels**

(A—C) Screening of MBF1c mutant lines. (A) schematic representation of the AtMBF1c gene structure. Untranslated regions (UTRs) are represented as open boxes and exons as closed boxes; triangles, T-DNA insertion sites; ATG, initiation codon; TGA, termination codon. (B) Expression analysis of MBF1c performed using semi-quantitative PCR of RNAs from wild-type, mbf1c-1 and mbf1c-2 plants. (C) Relative expression of MBF1c at 48 h after B. cinerea
infection in wild-type and *mbf1c* mutant plants, confirmed by qRT-PCR. Student's t-test indicated that the values were significantly different from the wild-type (**P<0.001**). (D) Increased *MBF1c* expression in *35S:MBF1c* overexpression lines 4 and 8, as confirmed by qRT-PCR, compared with wild-type plants. (Student's *t*-test ***P<0.001**).(E) Increased *MBF1c* expression in leaves treated with MeJA and SA, as confirmed by qRT-PCR.

**Figure S6. Expression analysis of *MBF1c* and phenotype analysis of *MBF1c* overexpression plants**

(A) *MBF1c* expression was induced in leaves of wild-type seedlings after treatment with *B. cinerea* or *P. syringae* (optical density at 600 nm = 0.0001 in 10 mM MgCl₂). Treatment times are indicated. (B) Comparison of *MBF1c* overexpression plants with wild-type plants at 3 dpi after treatment with *B. cinerea* and expression of JA-regulated defense gene *PDF1.2* during *B.cinerea* infection. In (A) and (B), error bars represent SE. Asterisks indicate significant differences (**P < 0.05**) from the wild type.

**Figure S7. MBF1c binds to the TPS5 promoter region**

(A) Illustration of the *TPS4, TPS5, TPS7* and *TPS10* promoter regions showing the presence of MBF1c binding motifs. Arrows indicate positions of primers used for ChIP-PCR experiments. Shown are 2-kb upstream sequences of the *TPS* genes. Translational start site (ATG) is shown at position +1. (B) Gel images showing the amplified products from the ChIP assay. ChIP assays were performed using three-week-old seedlings expressing the MYC-MBF1c fusion protein treated with *B. cinerea* for 48 h. Antibody to the MYC tag was used to immunoprecipitate MYC-MBF1c and associated DNA fragments. DNA was amplified by using primers specific to the region containing the MBF1c binding element. Shown are representative data from one biological replicate; this experiment was repeated three times, yielding similar results. (C) Real-time RT-PCR data from ChIP assay with antibody against MYC with
ACTIN2 promoter as a negative control. Values are the mean ± SE (n = 3 replicates) Asterisks indicate significant differences (*P <0.05, **P<0.01) from pACTIN2 for that time point. (D) Overexpression of TPS5 rescues mbf1c mutants sensitivity to B. cinerea. Plants were grown for three weeks for treatment with B. cinerea. Asterisk indicates a significant difference from mbf1c-1 samples for that time point (**P<0.001).

Figure S8. Yeast one-hybrid assays detect MBF1c binding to the TPS5 promoter
(A) A 60bp fragments of the TPS5 promoter containing normal MBF1c binding box or mutated MBF1c binding box. (B) Yeast one-hybrid assay showing specific binding (CTAGA) by MBF1c. Experiments were repeated four times and similar results were obtained. Positive transformants that grew on medium (lacking Trp, Leu and Ura) were incubated on plates supplemented with 60mmol/L 3-AT(3-Amino-1,2,4-triazole).

Figure S9. Working model for the roles of trehalose during pathogen attack
Expression of TPS5 and MBF1c are induced by Pseudomonas syringae and Botrytis cinerea infection. Upon P. syringae attack, expression of the salicylic acid (SA) biosynthesis gene, ICS1, is upregulated, SA levels increase, and plant defenses (e.g. PR1 expression) are induced to restrict P. syringae growth and disease in the host. In plants overexpressing the TPS5 gene, trehalose levels are elevated, which promotes P. syringae growth and disease development, via a mechanism that involves suppression of the plant defense gene (PR1). Upon B. cinerea attack, JA levels increase, and plant defenses (e.g. PDF1.2 expression) are induced to restrict B. cinerea growth and disease in the host. In plants overexpressing the TPS5 gene, B. cinerea growth and disease development is restricted via a mechanism that involves the activation of plant defense gene, PDF1.2.
Table S1. Primers used in this study

Figure 1
Figure 4

Figure 5
Figure 6

Figure 7
Figure 8

Figure 9