The de novo Biosynthesis of Vitamin B6 Is Required for Disease Resistance Against *Botrytis cinerea* in Tomato

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Vitamin B6 (VB6), an essential cofactor for numerous metabolic enzymes, has recently been shown to act as a potent antioxidant and play important roles in developmental processes and stress responses. However, little is known about the possible function of VB6 in plant disease resistance response against pathogen infection. In the present study, we explored the possible involvement of VB6 in defense response against *Botrytis cinerea* through functional analysis of tomato VB6 biosynthetic genes. Three de novo VB6 biosynthetic genes (*SIPDX1.2*, *SIPDX1.3*, and *SIPDX2*) and one salvage pathway gene (*SISOS4*) were identified and the *SIPDX1.2*, *SIPDX1.3*, and *SIPDX2* genes were shown to encode functional enzymes involved in de novo biosynthesis of VB6, as revealed by complementation of the VB6 prototrophy in yeast *snz1* and *sno1* mutants. Expression of *SIPDX1.2*, *SIPDX1.3*, and *SISOS4* genes was induced by infection with *B. cinerea*. Virus-induced gene silencing-mediated knockdown of *SIPDX1.2* or *SIPDX1.3* but not *SIPDX2* and *SISOS4* led to increased severity of disease caused by *B. cinerea*, indicating that the VB6 de novo biosynthetic pathway but not the salvage pathway is involved in tomato defense response against *B. cinerea*. Furthermore, the *SIPDX1.2*- and *SIPDX1.3*-silenced tomato plants exhibited reduced levels of VB6 contents and reactive oxygen species scavenging capability, increased levels of superoxide anion and H₂O₂ generation, and increased activity of superoxide dismutase after infection by *B. cinerea*. Our results suggest that VB6 and its de novo biosynthetic pathway play important roles in regulation of defense response against *B. cinerea* through modulating cellular antioxidant capacity.

Vitamin B6 (VB6) refers to a group of six water-soluble vitamers, including pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their phosphorylated derivatives (pyridoxal 5'-phosphate [PLP], pyridoxamine 5'-phosphate [PMP], and pyridoxine 5'-phosphate [PNP]), among which PLP is the active form of VB6. The VB6 vitamers are important compounds for all living organisms and function as cofactors in many enzymatic reactions involved in amino acid and fatty acid metabolisms (Drewke and Leistner 2001; Mittenhuber

2001; Mooney et al. 2009). The de novo biosynthetic pathway requires two protein families, PDX1s and PDX2, which form a PLP synthase complex to synthesize PLP directly. In this pathway, PDX1 catalyzes the final pyridine ring closure whereas PDX2 acts as a glutaminase to supply nitrogen for the PLP heterocycle by hydrolyzing glutamine to glutamate (Dong et al. 2004; Hill and Spenser 1996; Tambasco-Studart et al. 2005, 2007; Wetzel et al. 2004). Alternatively, biosynthesis of the VB6 vitamers can also occur through the salvage pathway, which functions to convert different VB6 vitamers between each other by specific enzymes (McCormick and Chen 1999). Recent studies have demonstrated that both the de novo pathway and the salvage pathway operate in higher plants, as revealed by the identification of a number of genes or enzymes that are involved in VB6 biosynthesis in *Arabidopsis* at molecular and biochemical levels. There are three *PDX1* genes (*AtPDX1.1*, *AtPDX1.2*, and *AtPDX1.3*) and one *PDX2* gene (*AtPDX2*) which are involved in the de novo biosynthetic pathway (Tambasco-Studart et al. 2005, 2007); while a pyridoxal kinase (*AtSOS4*), a PNP/PMP oxidase (*AtPDX3*), and a pyridoxal reductase (*AtPLR1*) were found to be involved in the salvage pathway (González et al. 2007; Herrero et al. 2011; Lum et al. 2002; Sang et al. 2007, 2011; Shi et al. 2002). Several studies have shown that the de novo pathway is essential in VB6 biosynthesis and is critical in maintaining the balance of VB6 content in plants, because the salvage pathway cannot complement the defect of the de novo pathway deficient plants (Shi and Zhu 2002; Shi et al. 2002; Titiz et al. 2006).

In addition to the importance as enzyme cofactors, VB6 has also been demonstrated to possess antioxidant activity and play important roles in regulating cellular antioxidant defense (Vanderschuren et al. 2013), which may be greater in the antioxidant activity than vitamins C and E (Bilski et al. 2000; Denslow et al. 2005, 2007; Stocker et al. 2003). It was observed that the genes involved in the de novo biosynthetic pathway are upregulated in plant response to various abiotic stresses, including high light, chilling, drought, ozone, and UV-B radiation (Denslow et al. 2005, 2007; Shi et al. 2002; Titiz et al. 2006). *Arabidopsis* mutants deficient in the *PDX1* genes have decreased levels of VB6 and show abnormal growth phenotypes and increased sensitivity to salt and osmotic stress and high light intensity (Chen and Xiong 2005; Havaux et al. 2009; Titiz et al. 2006). In contrast, overexpression of the *PDX* gene resulted in increased tolerance to oxidative stress (Raschke et al. 2011). During these abiotic stress responses, it was shown recently that VB6 vitamers are efficient quenchers of both singlet oxygen and superoxide anions (Bilski et al. 2000; Denslow et al. 2005). This is evident in the conditional *Arabidopsis flu* mutant plants, in which VB6 was able to protect *flu* protoplasts from cell death induced by the release of the singlet oxygen

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under light condition (Danon et al. 2005). On the other hand, the pyridoxal kinase SOS4 and the pyridoxal reductase PLR1 in the salvage pathway were shown to play important roles in response to various abiotic stresses, including salt, cold, and osmotic stress (González et al. 2007; Herrero et al. 2011; Rueschhoff et al. 2013; Shi and Zhu 2002).

Recent evidence also suggests the involvement of VB6 in plant defense response against different types of pathogens. Expression of the *PDX* genes was induced in tobacco plants after treatment with signaling molecules such as salicylic acid, methyl jasmonate, and ethylene but was decreased after inoculation with an incompatible pathogen, *Pseudomonas syringae* pv. *phaseolicola* (Denslow et al. 2005). Excess VB6 in tobacco leaves affected the development of a hypersensitive response (HR) caused by *P. syringae* pv. *phaseolicola* but increased disease severity caused by a compatible bacterium, *P. syringae* pv. *tabaci* (Denslow et al. 2005). The *Arabidopsis pdx1.3* mutant plants showed an elevated expression level for the *PR-5* gene (Ristilä et al. 2011). These findings imply that the VB6 vitamers might play important roles in regulating plant defense responses against different types of pathogens. However, direct molecular evidence supporting the hypothesis that VB6 as an antioxidant functions in plant defense is still lacking. The objective of the present study was to explore the possible involvement of VB6 in defense response against *Botrytis cinerea* by functional analysis of tomato VB6 biosynthetic genes. We found that silencing of *SIPDX1.2* or *SIPDX1.3* but not *SIPDX2* and *SISOS4* led to enhanced susceptibility to *B. cinerea*, reduced levels of VB6 content and total antioxidant capability, increased levels of superoxide anion and H₂O₂ generation, and increased activity of superoxide dismutase (SOD) activity after *Botrytis* infection. Our results suggest that VB6 and its de novo biosynthetic pathway play important roles in regulation of defense response against *B. cinerea* through modulating cellular antioxidant capacity.

RESULTS

Identification of the VB6 biosynthetic genes in tomato.

To identify the VB6 biosynthetic genes in tomato, the genomic database (tomato gene mode CDS ITAG release 2.31) was searched by the BlastP program using amino acid sequences of the *Arabidopsis* PDX1, PDX2, and SOS4 proteins as queries (Shi et al. 2002; Tambasco-Studart et al. 2005, 2007). These searches identified two putative *PDX1* genes (Soly03g120090 and Soly06g081980), one putative *PDX2* gene (Soly11g069290), and one putative *SOS4* gene (Soly02g091340) in the whole tomato genome. Further searches using the predicted cDNA sequences of these VB6 biosynthetic genes against the Combined Tomato UniGene database were able to retrieve corresponding full-length cDNAs for Soly03g120090 (SGN-U582020), Soly06g081980 (SGN-U577360 and SGN-U590632), and Soly11g069290 (SGN-U567386) and Soly02g091340 (SGN-U580571), indicating that these putative VB6 biosynthetic genes are expressed in tomato plants. The sequences of these putative *SIPDX1*, *SIPDX2*, and *SISOS4* genes were further confirmed by our reverse-transcriptase polymerase chain reaction (RT-PCR)-based cloning and sequencing. Surprisingly, we only identified two *SIPDX1* genes, Soly03g120090 and Soly06g081980. This is in agreement with the observation that only two *StPDX1* genes were identified but is in contrast to *Arabidopsis* that has three *AtPDX1* members (Mooney et al. 2013; Tambasco-Studart et al. 2005). Alignment of protein sequences with *AtPDX1*s revealed that Soly03g120090 has 67.5% identity to *AtPDX1.2* (61.3 and 65.2% identity to *AtPDX1.1* and *AtPDX1.3*, respectively) and Soly06g081980 has 90.6% identity to *AtPDX1.3* (87.4 and

60.2% identity to *AtPDX1.1* and *AtPDX1.2*, respectively); thus, we named Soly03g120090 and Soly06g081980 for *SIPDX1.2* and *SIPDX1.3*, respectively. The *SIPDX1.3* protein contains a characteristic sequence of LPVVNFAAGGVATPA DAAL (UPF0019), while the *SIPDX1.2* protein lacks this characteristic sequence, which is consistent with those for *AtPDX1.2* and *AtPDX1.3* (Tambasco-Studart et al. 2005). The putative *PDX2* gene Soly11g069290, named *SIPDX2*, encodes a protein showing 70.9% identity to *AtPDX2*, and containing almost the entire signature sequence of this protein family ([GA]LI[LIV]PGGEST[STA], UPF0030) (Tambasco-Studart et al. 2005). The putative *SOS4* gene Soly02g091340, named *SISOS4*, encodes a protein showing 82.5% identity to *AtSOS4*, and containing conserved motifs that may be involved in substrate binding or catalysis (Shi et al. 2002).

Biochemical characterization of *SIPDX1*s and *SIPDX2*.

To determine whether the cloned *SIPDX1.2*, *SIPDX1.3*, and *SIPDX2* encode functional enzymes, the coding sequence of these genes were cloned into pYES2, an *S. cerevisiae* expression vector under the control of a GAL1 promoter for high-level inducible expression in yeast by galactose, and were tested for their ability to complement the *SNZ1* (functional *PDX1* homolog) and *SNO1* (functional *PDX2* homolog) disruption mutants, BQS1037 and MML21, respectively (Rodríguez-Navarro et al. 2002; Tambasco-Studart et al. 2005). When grown on synthetic complete (SC) medium supplemented with VB6, the BQS1037 strains transformed with the *SIPDX1.2* or *SIPDX1.3* genes and the MML21 strain transformed with gene *SIPDX2* showed growth rates similar to those of strains transformed with the *S. cerevisiae* *SNZ1* or *SNO1* genes and with the corresponding empty vectors, respectively (Fig. 1). In the absence of VB6, growth defects were observed in the *SIPDX1.2*- or *SIPDX1.3*-transformed BQS1037 strains, which grew faster than the empty-vector-transformed strain but slower than the *S. cerevisiae* *SNZ1*-transformed strain (Fig. 1A). Meanwhile, the *SIPDX1.3*-transformed BQS1037 strain showed better growth than the *SIPDX1.2*-transformed BQS1037 strain (Fig. 1A). These data suggest that the cloned *SIPDX1.2* and *SIPDX1.3* restored the VB6 prototrophy in yeast. Likewise, when grown on SC medium without VB6, the *SIPDX2*-transformed MML21 strain showed similar growth to the strain transformed with the *S. cerevisiae* *SNO1* gene but enhanced growth to the strain transformed with the empty vector (Fig. 1B), indicating that the cloned *SIPDX2* has a homological biochemical function as the *SNO1* gene in the de novo VB6 biosynthetic pathway.

Silencing of *SIPDX1.2* and *SIPDX1.3* led to enhanced susceptibility to *B. cinerea*.

To explore the involvement of VB6 in tomato defense response, we examined whether blocking of VB6 biosynthesis by virus-induced gene silencing (VIGS)-mediated silencing of the genes involved in the de novo and salvage pathways affected the defense response of tomato plants to fungal infection. For this purpose, we made *Tobacco rattle virus* (TRV)-mediated VIGS constructs for *SIPDX1.2*, *SIPDX1.3*, *SIPDX2*, and *SISOS4* genes. Standard VIGS protocol was performed and the VIGS-infiltrated plants were used for various assays 3 weeks later, when the bleaching phenotype in the pTRV-phytoene desaturase (PDS)-infiltrated plants appeared. Silencing efficiency was examined by quantitative (q)RT-PCR analysis of the transcript abundance of the target genes in the pTRV-*SIPDX1.2*-, pTRV-*SIPDX1.3*-, pTRV-*SIPDX2*-, or pTRV-*SISOS4*-infiltrated plants and compared with those in the pTRV- β -glucuronidase (GUS)-infiltrated negative control plants. Results revealed that the transcript levels of the *SIPDX1.2*, *SIPDX1.3*, *SIPDX2*, and *SISOS4* genes were significantly re-

duced in the VIGS-infiltrated plants compared with those in the pTRV-GUS-infiltrated control plants and the silencing efficiencies were >75% (Fig. 2A). In our study, we observed that the *SIPDX1.3*-silenced plant showed an abnormal leaf developmental phenotype (e.g., yellowish and small leaves), indicating a possible function for *SIPDX1.3* in development. This is similar to the previous observations that the *Arabidopsis pdx1.3* mutant plants had reduced leaf size and developed yellowish leaves due to a reduction of chlorophyll content (Titiz et al. 2006; Wagner et al. 2006). No significant morphological phenotypes were observed in the *SIPDX1.2*-, *SIPDX2*-, or *SISOS4*-silenced plants compared with the control plants.

A detached leaf assay was first used to examine the disease phenotype of the VIGS-infiltrated plants after inoculation with *B. cinerea*. Detached leaves from 5-week-old VIGS-infiltrated plants were inoculated by dropping 2.5 μ l of spore suspension (1×10^5 spores/ml) onto the leaf surface and disease development was evaluated at 3 days postinoculation (dpi). Under our disease assay conditions, a typical disease symptom (e.g., necrotic lesions) was observed in the leaves from all VIGS-infiltrated plants but the lesions in the leaves from the *SIPDX1.2*- or *SIPDX1.3*-silenced plants expanded much more rapidly compared with others (Fig. 3A). At 3 dpi, the lesion sizes in leaves from the pTRV-SIPDX1.2- or pTRV-SIPDX1.3-infiltrated plants were approximately 7.5 and 7.1 mm, respectively; 30 to 37% larger than those on the controls (approximately 5.5 mm) (Fig. 2B). However, the lesions on the leaves from the pTRV-SIPDX2- or pTRV-SISOS4-infiltrated plants, measuring approximately 5.9 and 5.8 mm, respectively, were similar in size to the controls (Fig. 2B). A whole-plant inoculation method was further used to confirm the disease phenotypes observed above in the VIGS-infiltrated plants after inoculation by foliar spraying with spore suspension of *B. cinerea*. The pTRV-SIPDX1.2- or pTRV-SIPDX1.3-infiltrated plants showed more significant diseases at 3 dpi compared with the control plants (Fig. 3B) and most of the plants (>90%) died and were rotten

at 7 dpi; however, no significant difference in disease severity and progress was observed among the pTRV-SIPDX2-, pTRV-SISOS4-, and pTRV-GUS-infiltrated plants during our experimental period. Analysis of the transcript for the *B. cinerea BcActinA* gene as indicative of the rate of fungal growth in planta showed similar results compared with analysis of the disease phenotypes. At 3 dpi, growth of *B. cinerea* in leaf tissues of the pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants was approximately 28.6- and 25.4-fold higher compared with the controls (Fig. 2C). No significant difference in fungal growth was observed in the pTRV-SIPDX2-, pTRV-SISOS4-, and pTRV-GUS-infiltrated plants (Fig. 2C). These results indicate that silencing of *SIPDX1.2* and *SIPDX1.3* resulted in increased susceptibility against *B. cinerea* and that *SIPDX2* and *SISOS4* may have no contribution to disease resistance against *B. cinerea* in tomato.

Expression of VB6 biosynthetic genes and changes of VB6 content in response to *B. cinerea*.

The enhanced susceptibility against *B. cinerea* in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants led us to hypothesize that *B. cinerea* may interfere with the VB6 biosynthesis in tomato plants during its infection process. Therefore, we first examined the expression patterns of the *SIPDX1.2*, *SIPDX1.3*, and *SISOS4* genes in wild-type tomato plants after infection with *B. cinerea*. Expression levels of *SIPDX1.2*, *SIPDX1.3*, and *SISOS4* were comparable at different time points in mock-inoculated plants but were significantly induced with different patterns in *B. cinerea*-inoculated plants (Fig. 4A). After inoculation with *B. cinerea*, expression levels of *SIPDX1.2* increased significantly at 24 and 48 h postinoculation (hpi); more than three times higher than those in the mock-inoculated plants (Fig. 4A). Expression levels of the *SIPDX1.3* and *SISOS4* also increased approximately 0.6 to 1.8 times higher than those in the mock-inoculated plants (Fig. 4A). These results indicate that expression of the genes involved in the de novo pathway

Fig. 1. Functional complementation of tomato *SIPDX1s* and *SIPDX2* genes in yeast with defects in *SNZ1* (functional *PDX1* homolog) and *SNO1* (functional *PDX2* homolog). Left: Growth of **A**, 6-day-old BQS1037 and **B**, 4-day-old MML21 in the absence of VB6. Right: Growth of **A**, 3-day-old BQS1037 and **B**, MML21 in the presence of VB6. Each row represents a serial 10-fold dilution from a starter culture with an optical density at 600 nm of 0.5.

as well as in the salvage pathway can be induced by infection of *B. cinerea*.

To examine whether the *B. cinerea*-induced expression of the VB6 biosynthetic genes affects the biosynthesis of VB6 in tomato plants, we analyzed the changes in VB6 contents in the pTRV-SIPDX1.2- and pTRV-SIPX1.3-infiltrated plants before and after infection with *B. cinerea*. Before inoculation with *B. cinerea*, the pTRV-SIPDX1.2-infiltrated plants contained VB6 content in leaf tissues similar to that in the control plants; however, the VB6 content in leaf tissues of the pTRV-SIPDX1.3-infiltrated plants was significantly reduced, showing only approximately 50% of the VB6 content in the control plants (Fig. 4B). At 48 hpi with *B. cinerea*, VB6 content was increased markedly in the control plants, resulting in an increase of 53% compared with the content before inoculation (Fig. 4B). Although the VB6 contents in the pTRV-SIPDX1.2- and pTRV-SIPX1.3-infiltrated plants were also increased after infection with *B. cinerea* (Fig. 4B), the increase was <23% compared with the contents before inoculation and was less evident than that observed in the control plants. These data indicate that silencing of the *SIPDX1.3* gene decreased the capability of the de novo VB6 biosynthesis but silencing of either the *SIPDX1.2* or *SIPDX1.3* gene can reduce the *B. cinerea*-triggered VB6 biosynthesis in tomato plants.

Increased accumulation of reactive oxygen species in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants after *Botrytis* sp. infection.

Considering that reactive oxygen species (ROS) has been implicated in susceptible responses of plants against necrotrophic fungal pathogens such as *B. cinerea* (Veronese et al. 2006) and that VB6 plays important roles in regulating cellular antioxidant defense (Vanderschuren et al. 2013), we asked whether decreased VB6 content in the *SIPDX1.2*- or *SIPDX1.3*-silenced plants affected the accumulation of ROS in response to *B. cinerea*. We determined ROS content by in situ detection of superoxide anion and quantitative measurement of H_2O_2 and compared the accumulation of ROS in the pTRV-SIPDX1.2-, pTRV-SIPDX1.3-, and pTRV-GUS-infiltrated plants before and after infection with *B. cinerea* (Fig. 5). Without infection, no significant accumulation of superoxide anion and H_2O_2 in the leaves was observed among the pTRV-SIPDX1.2-, pTRV-SIPDX1.3-, and pTRV-GUS-infiltrated plants, indicating that silencing of *SIPDX1.2* or *SIPDX1.3* did not affect the accumulation of superoxide anion and H_2O_2 in tomato plants. In con-

trast, infection with *B. cinerea* accelerated markedly the accumulation of superoxide anion and H_2O_2 in the leaves but the accumulation levels in the pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants were more evident and higher than that in pTRV-GUS-infiltrated plants (Fig. 5). At 24 hpi, accumulation of H_2O_2 in pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants showed an increase of 25 to 30% over that in the pTRV-GUS-infiltrated plants (Fig. 5B). No difference in the ROS accumulation was observed between the pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants with or without inoculation. These data indicate that silencing of *SIPDX1.2* and *SIPDX1.3* can promote the accumulation of superoxide and H_2O_2 in tomato plants when infected with *B.*

Fig. 2. Silencing of *SIPDX1.2* and *SIPDX1.3* resulted in increased severity of disease caused by *Botrytis cinerea*. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, pTRV-SIPDX2, pTRV-SISOS4, or pTRV-GUS (as a negative nonsilenced control [NS]) constructs, and inoculation was carried out at 3 weeks after virus-induced gene silencing infiltration by **B**, dropping spore suspension on detached leaves or **C**, foliar spraying with spore suspension. **A**, Silencing efficiency for target genes in silenced plants. The silencing efficiency was calculated by comparing the transcript levels of *SIPDX1.2*, *SIPDX1.3*, *SIPDX2*, and *SISOS4* in pTRV-SIPDX1.2-, pTRV-SIPDX1.3-, pTRV-SIPDX2-, or pTRV-SISOS4-infiltrated plants to those in the NS plants, which were set as 1. **B**, Lesion sizes on selected leaves in detached leaf inoculation assays at 3 days postinoculation (dpi). Lesion sizes were measured on a minimum of 30 leaves in each experiment. **C**, Growth of *B. cinerea* in inoculated plants from whole-plant inoculation experiments. Fungal growth in planta was assumed at 3 dpi by analyzing the transcript level of *B. cinerea* *BcActinA* gene by quantitative reverse-transcriptase polymerase chain reaction using the *SActin* gene as an internal control. Relative fungal growth was shown as folds of transcript levels of *BcActin* compared with *SActin*. Data presented are the means $\pm$ standard deviation from three independent experiments, and different letters above the columns indicate significant differences at $P < 0.05$.

cinerea, implying a relationship between decreased VB6 content and increased accumulation of ROS in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants.

Changes of ROS scavenging capability in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants.

Increased ROS accumulation in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants after infection with *B. cinerea* led us to examine whether silencing of *SIPDX1.2* or *SIPDX1.3* affects

the capability of ROS scavenging system in tomato plants. We first analyzed the changes of ROS scavenging capability (RSC), representing the ability to scavenge superoxide anion, in the pTRV-*SIPDX1.2*- and pTRV-*SIPDX1.3*-infiltrated plants and compared them with those in the pTRV-GUS-infiltrated plants. Without inoculation with *B. cinerea*, silencing of either the *SIPDX1.2* or *SIPDX1.3* gene decreased the RSC in tomato plants (the mock-inoculation control and at 0 hpi the inoculation treatment) compared with that in the pTRV-GUS-

Fig. 3. *Botrytis cinerea*-caused disease phenotype in *SIPDX1.2*-, *SIPDX1.3*-, *SIPDX2*-, and *SISOS4*-silenced tomato plants. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-*SIPDX1.2*, pTRV-*SIPDX1.3*, pTRV-*SIPDX2*, pTRV-*SISOS4*, or pTRV-GUS (as a negative nonsilenced control [NS]) constructs, and inoculation was carried out at 3 weeks after virus-induced gene silencing infiltration by **A**, dropping spore suspension on detached leaves or **B**, foliar spraying with spore suspension. **A**, Disease symptoms on detached leaves at 3 days postinoculation (dpi). **B**, Disease phenotype on representative leaves and whole plants at 3 and 7 dpi, respectively. Red arrows indicate the representative diseased leaves in the inoculated whole plants.

infiltrated plants (Fig. 6). The RSC increased significantly at 24 hpi with *B. cinerea* in all plants, indicating that infection of *B. cinerea* can trigger the response of RSC in plants (Fig. 6). However, the increase patterns of the RSC differed among the pTRV-SIPDX1.2-, pTRV-SIPDX1.3-, and pTRV-GUS-infiltrated plants when compared with their corresponding mock-inoculated control plants. The RSC in the *B. cinerea*-infected pTRV-GUS-infiltrated plants increased almost onefold (98.86%) over that in the mock-inoculated plants; however, the RSC in the *B. cinerea*-infected pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants showed approximately 65.71 to 68.32% increase compared with their corresponding mock-inoculated plants (Fig. 6). These data reflect a reduction of the RSC increase in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants compared with the increase in the pTRV-GUS-infiltrated plants after infection, suggesting that silencing of either the *SIPDX1.2* or

SIPDX1.3 gene in tomato suppresses the *B. cinerea*-triggered RSC.

We next analyzed the changes in activity of SOD and catalase (CAT) and contents of reduced or oxidized glutathione (GSH or GSSG, respectively), key components of the ROS scavenging system, in the pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants and compared them with those in the pTRV-GUS-infiltrated plants. Without inoculation with *B. cinerea*, similar activity of SOD and CAT and contents of GSH and GSSG were observed, indicating that silencing of *SIPDX1.2* or *SIPDX1.3* itself had no effect on these components of the ROS scavenging system in tomato plants (Figs. 7 and 8). The activity of SOD and CAT was increased but the GSH content was reduced after inoculation with *B. cinerea* (Figs. 7 and 8), similar to previous observations in tomato plants after infection with *B. cinerea* (Kuźniak and Skłodowska

Fig. 4. Expression of *SIPDX1.2* and *SIPDX1.3* and changes in VB6 contents after *Botrytis* infection. **A**, Expression patterns of *SIPDX1.2* and *SIPDX1.3* in tomato plants after infection with *Botrytis cinerea*. Four-week-old tomato plants were inoculated by foliar spraying with spore suspension of *B. cinerea* and leaf samples were collected at indicated time points after inoculation. Gene expression was analyzed by quantitative reverse-transcriptase polymerase chain reaction with actin gene *SlActin* as an internal control. **B**, Changes of VB6 contents in *SIPDX1.2*- and *SIPDX1.3*-silenced plants after *Botrytis* infection. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, or pTRV-GUS (as a negative nonsilenced control) constructs and inoculated with spore suspension of *B. cinerea* at 3 weeks after virus-induced gene silencing infiltration. Leaf samples were collected at 0 and 48 h after inoculation and were subject to analyze total VB6 content by yeast bioassay. Data presented in B are the means $\pm$ standard deviation from three independent experiments and different letters above the columns indicate significant differences at $P < 0.05$.

Fig. 5. Increased accumulation of reactive oxygen species (ROS) in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants after *Botrytis* infection. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, or pTRV-GUS (as a negative non-silenced control) constructs and foliar spraying inoculated with spore suspension of *Botrytis cinerea* at 3 weeks after virus-induced gene silencing infiltration. Leaf samples were collected 0 and 24 h after inoculation and were subjected to **A**, in situ detection of superoxide anion or **B**, quantitative measurement of H₂O₂ accumulation. Data presented in B are the means $\pm$ standard deviation from three independent experiments and different letters above the columns indicate significant differences at $P < 0.05$.

2004, 2005a and b). At 24 hpi, no significant difference in the CAT activity and the GSH and GSSG contents was observed between the pTRV-SIPDX1.2- or pTRV-SIPDX1.3-infiltrated plants and the pTRV-GUS-infiltrated plants (Figs. 7B and 8A). However, the SOD activity was significantly higher in the pTRV-SIPDX1.2- or pTRV-SIPDX1.3-infiltrated plants, showing an increase of 30% compared with that in the pTRV-GUS-infiltrated plants (Fig. 7A). Notably, no difference in RSC, activity of SOD and CAT and contents of GSH or GSSG was observed between the pTRV-SIPDX1.2- and pTRV-SIPDX1.3-infiltrated plants with or without inoculation.

DISCUSSION

Functions of VB6 as antioxidant compounds in plant abiotic stress responses has been well documented recently (Bilski et al. 2000; Danon et al. 2005; Denslow et al. 2005; González et al. 2007; Havaux et al. 2009; Herrero et al. 2011; Raschke et al. 2011; Rueschhoff et al. 2013; Shi and Zhu 2002; Titiz et al. 2006). Although a previous study has implicated the involvement of VB6 in HR and disease development (Denslow et al. 2005), direct molecular evidence of the function of VB6 in plant defense response against pathogen attack remains elusive. In this study, we presented molecular evidence that the de novo VB6 biosynthetic pathway but not the salvage pathway is involved in tomato defense response against *B. cinerea* because silencing of tomato *SIPDX1.2* and *SIPDX1.3* but not *SISOS4* led to increased disease severity. Combination of the data from our molecular and biochemical analyses led to a conclusion that VB6 and its de novo biosynthetic pathway have a positive function in regulation of defense response through modulating cellular antioxidant capacity. These findings extend our knowledge of the biological function of VB6 and its biosynthesis, which integrate into the plant defense response against pathogen attack.

The de novo pathway contributes predominantly to the biosynthesis of VB6 and, hence, affects the total VB6 content, whereas the salvage pathway involves the interconversion

among the different VB6 vitamers (Dong et al. 2004; Hill and Spenser 1996; McCormick and Chen 1999; Wetzel et al. 2004). In the present study, blocking of the de novo VB6 biosynthetic pathway by silencing of *SIPDX1.2* or *SIPDX1.3* resulted in an increased level of disease caused by *B. cinerea* but blocking of the salvage pathway by silencing of the *SISOS4* gene did not alter the disease phenotype (Figs. 2 and 3). Similar results were also found in *Arabidopsis*: mutations in *AtPDX1.2* or *AtPDX1.3* led to increased levels of diseases caused by *B. cinerea* and *P. syringae* pv. *tomato* DC3000 (Y. Zhang and F. Song, unpublished data). Furthermore, silencing of *SIPDX1.3* but not *SIPDX1.2* resulted in a decreased level of VB6 in leaf tissues of tomato plants (Fig. 4). This is in agreement with previous findings that the *Arabidopsis* AtPDX1.3, together with AtPDX1.1, interacts with AtPDX2 to form a complex that directly synthesizes the cofactor form of VB6 (Tambasco-Studart et al. 2005; Wagner et al. 2006). Surprisingly, silencing of the *SIPDX2* gene, a key gene in the de novo pathway, did not alter the phenotype of disease caused by *B. cinerea* in our repeated experiments (Figs. 2 and 3). Taken together, it is likely that the de novo pathway of VB6 biosynthesis is essential for plant disease resistance against different types of patho-

Fig. 6. Reduced capacity of reactive oxygen species (ROS) scavenger in *SIPDX1.2*- and *SIPDX1.3*-silenced plants. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, or pTRV-GUS (as a negative nonsilenced control) constructs and foliar spraying inoculated with spore suspension of *Botrytis cinerea* at 3 weeks after virus-induced gene silencing infiltration. ROS scavenger capacity represents the overall ability of the samples to scavenge superoxide anion generated from the xanthine and xanthine oxidase system in the reactions. Data presented are the means $\pm$ standard deviation from three independent experiments and different letters above the columns indicate significant differences at $P < 0.05$.

Fig. 7. Changes in superoxide dismutase (SOD) and catalase (CAT) activity in *SIPDX1.2*- and *SIPDX1.3*-silenced plants. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, or pTRV-GUS (as a negative nonsilenced control) constructs and foliar spraying inoculated with spore suspension of *Botrytis cinerea* at 3 weeks after virus-induced gene silencing infiltration. Activities of **A**, SOD and **B**, CAT were analyzed using commercial kits. Data presented are the means $\pm$ standard deviation from three independent experiments and different letters above the columns indicate significant differences at $P < 0.05$.

gens. Previous studies with *Arabidopsis* mutants have shown that the de novo pathway is essential and critical in biosynthesis and maintaining the balance of VB6 content in plants, and that the salvage pathway cannot compensate for the defect of the de novo pathway-deficient plants (Tambasco-Studart et al. 2005; Titiz et al. 2006). On the other hand, we also found that the *Arabidopsis sos4* mutant plants showed increased levels of diseases caused by *P. syringae* pv. *tomato* DC3000 and *B. cinerea* in another study (Y. Zhang and F. Song unpublished data), indicating the involvement of the salvage pathway in disease resistance. Therefore, it is also likely that the requirement of the salvage pathway for disease resistance differs among different plant species (e.g., increased level of disease caused by *B. cinerea* in the *Arabidopsis sos4* plants but no alteration in disease phenotype in the tomato *SISOS4*-silenced plants).

The de novo pathway of VB6 involves only two proteins, PDX1 and PDX2, which function together as a glutamine aminotransferase to catalyze the direct formation of the cofactor vitamer (Tambasco-Studart et al. 2005). Surprisingly, only two *SIPDX1* genes, *SIPDX1.2* and *SIPDX1.3*, were identified in the tomato genome, which is similar to the findings in potato (Mooney et al. 2013). Thus, it is likely that the Solanaceae plants encode for two but not three *PDX1* genes. Expression of *SIPDX1.2*, *SIPDX1.3*, and *SISOS4* genes in tomato plants was induced significantly in response to infection by *B. cinerea* (Fig. 4A). We also found that infection by *B. cinerea* increased the VB6 content in tomato plants (Fig. 4B). It was previously found that the transcript levels of tobacco *PDX1* and *PDX2* genes decreased following inoculation with an incompatible pathogen, *P. syringae* pv. *phaseolicola*, which induced an HR (Denslow et al. 2005). These data indicate that the de novo biosynthetic pathway responds differentially to infection by different types of pathogens. This is supported by the fact that silencing of either the *SIPDX1.2* or *SIPDX1.3* gene can reduce the *B. cinerea*-triggered VB6 biosynthesis in tomato plants (Fig. 4B) and facilitate the development of disease caused by *B. cinerea* (Figs. 2 and 3). However, our results are in contrast to a previous observation that excessive VB6 (100 mM) injected into tobacco leaves led to increased disease severity caused by a compatible bacterium (Denslow et al. 2005).

Like the *Arabidopsis* AtPDX1.2 that is not involved directly in VB6 biosynthesis (Tambasco-Studart et al. 2005; Wagner et al. 2006), the tomato *SIPDX1.2* seems also not associated with the de novo pathway of VB6 biosynthesis because silencing of *SIPDX1.2* did not alter the VB6 content in tomato leaf tissues (Fig. 4B). Surprisingly, silencing of *SIPDX1.2* did result in increased level of disease caused by *B. cinerea* (Figs. 2 and 3), indicating a role for *SIPDX1.2* in disease resistance against this pathogen. Recently, it was found that the *Arabidopsis* AtPDX1.2 assembles in planta with either of the other two PDX1 proteins, probably forming higher-order AtPDX1.2-containing complexes, and, thus, affects the de novo VB6 biosynthesis (Leuendorf et al. 2010). In this regard, mechanisms for how *SIPDX1.2* regulates the de novo VB6 biosynthesis and, thus, affects defense response in tomato remain to be explored further.

It is well documented that ROS play important roles in the establishment of infection by some necrotrophic pathogens such as *B. cinerea* (Mengiste 2012). Recently, it was shown that accumulation of anthocyanins in tomato fruit can extend shelf life and reduce susceptibility to *B. cinerea* through increasing total antioxidant capacity (Zhang et al. 2013), indicating the importance of antioxidant capacity in resistance to *B. cinerea*. When compared with those in the control plants, increased disease level and increased accumulation of H₂O₂ and superoxide anion after infection with *B. cinerea* was ob-

served in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants (Figs. 2, 3, and 5). This is similar to the previous observations in some *Arabidopsis* mutants, which showed increased disease severity and higher levels of ROS (Mengiste et al. 2003; Veronese et al. 2006). Markedly, accompanying the *B. cinerea*-triggered increased levels of ROS represented by accumulation of H₂O₂ and superoxide anion, is the pronounced decline of RSC in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants compared with the control plants after infection with *B. cinerea* (Figs. 6 to 8). This pronounced decline of RSC might be caused by multiple factors in the complicated ROS scavenging system in plants. The augmented increase of SOD activity over that in the control plants accelerated enzymatic conversion of the superoxide anion to H₂O₂ and led to further accumulation of H₂O₂ in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants, which showed CAT activity similar to that in the control plants (Fig. 7). *B. cinerea* infection-induced decrease of GSH contents and, thus, GSH/GSSG ratios was previously reported in tomato plants (Kuźniak and Skłodowska 2004, 2005a). However, no significant difference in *B. cinerea*-induced decrease of the GSH contents was observed between the *SIPDX1.2*- and *SIPDX1.3*-silenced plants and the control plants after *B. cinerea*

Fig. 8. Changes in reduced and oxidized glutathione (GSH and GSSG, respectively) contents in *SIPDX1.2*- and *SIPDX1.3*-silenced plants. Two-week-old tomato seedlings were infiltrated with agrobacteria carrying pTRV-SIPDX1.2, pTRV-SIPDX1.3, or pTRV-GUS (as a negative non-silenced control) constructs and foliar spraying inoculated with spore suspension of *Botrytis cinerea* at 3 weeks after virus-induced gene silencing infiltration. GSSG and GSH were analyzed using commercial kits. Data presented are the means $\pm$ standard deviation from three independent experiments and different letters above the columns indicate significant differences at $P < 0.05$.

infection (Fig. 8), indicating that the change of the GSH and GSSG contents is unlikely a cause that led to the pronounced decline of RSC in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants. On the other hand, VB6 has been demonstrated to be an efficient antioxidant capable of quenching ROS (Bilski et al. 2000; Denslow et al. 2005, 2007; Stocker et al. 2003). Coincidence between the pronounced declines in VB6 contents and RSC levels and the augmented increase in ROS accumulation in the *SIPDX1.2*- and *SIPDX1.3*-silenced plants, when compared with those in the control plants, after infection with *B. cinerea* establishes a connection between VB6 as an antioxidant and the cellular RSC. Further direct biochemical and physiological evidence supporting this hypothesis needs to be investigated.

MATERIALS AND METHODS

Plant growth and sample collection.

Tomato (*Solanum lycopersicum*) ‘Suhong 2003’ and ‘micro-Tom’ were used for different purposes in the experiments. Tomato seed were scarified on moist filter paper in petri dishes for 2 days and the sprouted seed were transferred into a mix-

ture of perlite/vermiculite/plant ash (1:6:2) and grown in a growth room at 22°C under a regime of 16 h of light (350 $\mu\text{mol s}^{-1}\text{m}^{-2}$ photons $\text{m}^{-2} \text{s}^{-1}$) and 8 h of darkness. For gene expression and biochemical analyses, 4-week-old tomato plants were inoculated by foliar spraying with a spore suspension of *B. cinerea* (discussed below) or with a similar volume of 4% maltose and 1% peptone solution as mock-inoculation controls. Inoculated plants were covered with a transparent plastic film and kept in a growth chamber under conditions similar to those mentioned above. Leaf samples were collected at indicated time points after inoculation.

Cloning of the tomato VB6 biosynthetic genes and gene expression analysis.

The tomato genomic database was searched to identify the VB6 biosynthetic genes using *Arabidopsis* *PDX* and *SOS4* genes as queries. The predicted *SIPDX* and *SISOS4* genes and their available cDNA sequences were used to design gene-specific primers (Table 1) to amplify full-length cDNAs by RT-PCR. Expression and silencing efficiency of the VB6 biosynthetic genes were analyzed by qRT-PCR using gene-specific primers (Table 1). The PCR reactions were normalized against

Table 1. List of primers used in this study

Primers	Sequences (5′–3′)	Size (bp)
Cloning		
SIPDX1.2- 1F	ATG GAA GAA GAC GGT GCC GTT ACA	918
SIPDX1.2- 1R	CAT CAG TAG TTC TCA TCA CTA ACA	...
SIPDX1.3- 1F	ATG GCC GGA AGT GGT GTC GTT ACA	930
SIPDX1.3- 1R	TCA CTC AGA ACG ATT GGC ATA CCT	...
SIPDX2-1F	ATG GTT GTG GGT GTT CTT GC	756
SIPDX2-1R	CTA CTG GTA TAT GGG AAA ATC	...
SISOS4-1F	ATG TTT GTT TCT CCA CTC ATC A	1,020
SISOS4-1R	TTA GTT ATA TCT TTT AGC TCT A	...
Virus-induced gene silencing (VIGS)		
SIPDX1.2-VIGS-1F	GCG <u>TCT AGA</u> ATG GAA GAA GAC GGT GCC GT (<i>Xba</i> I)	438
SIPDX1.2-VIGS-1R	ATA <u>CTC GAG</u> TAA CGC TTC TCC AAG ATC TC (<i>Xho</i> I)	...
SIPDX1.3-VIGS-1F	GCG <u>TCT AGA</u> ATG GCC GGA AGT GGT GTC GT (<i>Xba</i> I)	398
SIPDX1.3-VIGS-1R	ATA <u>CTC GAG</u> ATG TGG TTC TCA TCG TCA GCA (<i>Xho</i> I)	...
SIPDX2-VIGS-1F	GCG <u>TCT AGA</u> TGA GAA AGC CAG AGC AGT TGC (<i>Xba</i> I)	554
SIPDX2-VIGS-1R	ATA <u>CTC GAG</u> ACT GTG CCA TCG AGT ATC TGC (<i>Xho</i> I)	...
SISOS4-VIGS-1F	GCG <u>TCT AGA</u> CCA GGT GCT TGA AGC AAT TGC A (<i>Xba</i> I)	493
SISOS4-VIGS-1R	ATA <u>CTC GAG</u> TAG GCG TCA GCA TTG AAG CA (<i>Xho</i> I)	...
SIPDS-VIGS-1F	CGG <u>TCT AGA</u> GGC ACT CAA CTT TAT AAA CC (<i>Xba</i> I)	409
SIPDS-VIGS-1R	CGG <u>GGA TCC</u> CTT CAG TTT TCT GTC AAA CC (<i>Bam</i> HI)	...
GUS-VIGS-1F	CGG <u>TCT AGA</u> ACC TGG GTG GAC GAT ATC AC (<i>Xba</i> I)	396
GUS-VIGS-1R	CGG <u>GGA TCC</u> GTG CAC CAT CAG CAC GTT AT (<i>Bam</i> HI)	...
Quantitative reverse-transcriptase polymerase chain reaction		
SIPDX1.2-rt-1F	GAT GCA GCT GGG TTG TGA TG	144
SIPDX1.2-rt-1R	TCC AAA CCA CTG CTA GCC GC	...
SIPDX1.3-rt-1F	CAT GTG CGT TCC GTT ATG GG	140
SIPDX1.3-rt-1R	TGA ACC ACA GGG AGC CTA CC	...
SISOS4-rt-1F	GCG TAT TTC ACG GGA ACT GG	104
SISOS4-rt-1R	CTT GAG ACA GCA AGC TCT GC	...
SIPDX2-rt-1F	CAA AGC TTC GGA ACG CGT TCA A	113
SIPDX2-rt-1R	GTC AAT GAG TAG CCA TTT GAC C	...
SlActin-1F	AGG CAC ACA GGT GTT ATG GT	177
SlActin-1R	AGC AAC TCG AAG CTC ATT GT	...
BcActin-1F	TCC AAG CGT GGT ATT CTT ACC C	117
BcActin-1R	TGG TGC TAC ACG AAG TTC GTT G	...
Yeast complementation		
ScSNZ1-1F	ATA <u>GGA TCC</u> ATG ACT GGA GAA GAC TTT AAG A (<i>Bam</i> HI)	894
ScSNZ1-1R	GCG <u>TCT AGA</u> TCA CCA CCC AAT TTC GGA AAG T (<i>Xba</i> I)	...
ScSNO1-1F	ATA <u>GGA TCC</u> ATG CAC AAA ACC CAC AGT ACA A (<i>Bam</i> HI)	675
ScSNO1-1R	GCG <u>TCT AGA</u> TTA ATT AGA AAC AAA CTG TCT G (<i>Xba</i> I)	...
SIPDX1.2-y-1F	GCG <u>GAG CTC</u> ATG GAA GAA GAC GGT GCC GT (<i>Sac</i> I)	918
SIPDX1.2-y-1R	GCG <u>TCT AGA</u> TCA GTA GTT CTC ATC ACT AAC (<i>Xba</i> I)	...
SIPDX1.3-y-1F	GCG <u>GAG CTC</u> ATG GCC GGA AGT GGT GTC GT (<i>Sac</i> I)	930
SIPDX1.3-y-1R	GCG <u>TCT AGA</u> TCA CTC AGA ACG ATT GGC AT (<i>Xba</i> I)	...
SIPDX2-y-1F	ATA <u>GGA TCC</u> ATG GTT GTG GGT GTT CTT GC (<i>Bam</i> HI)	756
SIPDX2-y-1R	GCG <u>TCT AGA</u> CTA CTG GTA TAT GGG AAA ATC (<i>Xba</i> I)	...

a tomato actin gene (Table 1) for comparative analysis. Total RNA was extracted using TRIZOL and the first-strand cDNA was obtained with RNA PCR kit (Ver. 3.0; TaKaRa, Dalian, China) according to the manufacturer's protocols. qRT-PCR analyses were repeated with three biological replicates by using SYBR Green PCR master mix kit (TaKaRa) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, U.S.A.), according to the manufacturer's instructions. Relative expression was calculated using $2^{-\Delta\Delta CT}$ method, as described (Livak and Schmittgen 2001).

VIGS assays.

For construction of VIGS vectors, gene-specific primers (Table 1) with restriction enzyme sites were designed to amplify fragments of 300 to 500 bp for *SIPDX* and *SISOS4* genes and the amplified fragments were cloned into the vector pYL156 (Liu et al. 2002). The recombinant vectors pTRV-PDX1.2, pTRV-PDX1.3, pTRV-PDX2, and pTRV-SOS4 were confirmed by enzyme digestion and then introduced into *Agrobacterium tumefaciens* GV3101 by electroporation using the GENE PULSER II Electroporation System (Bio-Rad Laboratories). A negative silencing vector containing a *GUS* gene fragment, pTRV-GUS, and a positive silencing vector containing a *PDS* gene fragment, pTRV-PDS, were also constructed and used in the VIGS experiments. *A. tumefaciens* carrying different recombinant vectors was grown with shaking at 200 rpm in YEP (1% yeast extract, 1% tryptone, and 0.5% NaCl, pH7.2) medium with kanamycin at 50 µg/ml, rifampicin at 50 µg/ml, and gentamycin at 25 µg/ml in a 28°C shaker. Cells were harvested by centrifugation at 4,000 × g and 4°C for 10 min and resuspended to a final concentration to an optical density at 600 nm (OD_{600}) = 1.5 in a solution containing 10 mM MgCl₂, 10 mM morpholinoethanesulfonic acid (pH 5.7), and 150 µM acetosyringone and kept in the dark for at least 3 h at room temperature before infiltration (Hartl et al. 2008). Bacterial suspension containing a different pTRV2 construct was mixed with the same volume of agrobacteria containing pTRV1 (pYL196) (Liu et al. 2002). The mixed agrobacterial suspension was infiltrated into the abaxial surface of the 2-week-old seedlings using a 1-ml needleless syringe. Efficiency of the VIGS protocol was evaluated using a *PDS* gene as a marker of silencing in tomato plants, according to Liu and associates (2002). Single VB6 biosynthetic gene-silenced plants in a set of experiments with 100% of pTRV-PDS-silenced plants showing bleaching phenotype were used for disease and biochemical assays. Leaf samples were collected at 20 days after VIGS infiltration and used for analysis of silencing efficiency by qRT-PCR.

Disease assays.

B. cinerea was grown on V8 medium (36% V8 juice, 0.2% CaCO₃, and 2% agar) at room temperature. Spores were collected by passing through two layers of cheesecloth in 4% maltose and 1% peptone buffer, and the spore density was adjusted to 1 × 10⁵ spores/ml. Four-week-old plants were inoculated by foliar spraying with the spore suspension. Alternatively, fully expanded leaves detached from tomato plants were placed onto water-saturated filter paper and inoculated by a drop inoculation method according to a previously reported procedure (AbuQamar et al. 2008). The inoculated plants and detached leaves were covered with a transparent plastic film and kept in a growth chamber with similar conditions as for plant growth. Fungal growth in planta was determined by analyzing *B. cinerea ActinA* transcripts by qRT-PCR. Lesion size was measured 3 dpi. Leaves from at least 10 individual plants were used in each independent experiment.

Measurement of VB6 content.

A bioassay with a yeast strain (ATCC 9080) auxotrophic for VB6 was used to measure total VB6 content in the leaf tissues. Leaf extracts were prepared with a protocol, as described previously (Denslow et al. 2005). After extraction, pH values of the samples were adjusted to 4.8 and freshly prepared β-glycosidase (5 mg/ml) and acid phosphatase (37.5 mg/ml) was added to deglycosylate and dephosphorylate VB6 vitamers. These leaf extracts in 50-ml falcon tubes were incubated overnight at 37°C with shaking at 70 rpm and then filter sterilized using a 50-ml Millipore's Steriflip Filter Unit (0.22 µm). Yeast cells grown on YPD (1% yeast extract, 2% peptone and 2% dextrose) overnight at 30°C were washed twice with sterile distilled water and pelleted by centrifuging at 4°C and 1,000 × g for 10 min. For bioassay of VB6 content, 20-ml falcon tubes containing 5 ml of pyridoxol Y medium (HB8684; Hope Bio-Technology Co., Qingdao, China) were mixed with 5 × 10⁶ yeast cells and 1 ml of treated leaf extract. A standard curve was made using the same protocol with 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 ng of total PN. All test and standard samples were run in triplicate and incubated at 30°C with shaking at 250 rpm. Yeast growth was measured at 17 and 24 h with a spectrophotometer at OD₅₄₀. The total VB6 content in leaf extracts was calculated by comparison with the standard curve.

Yeast complementation assays.

Yeast (*Saccharomyces cerevisiae*) strains BQS1037 and MML21, which are defective in *ScSNZ1* and *ScSNO1*, respectively, were used (Rodríguez-Navarro et al. 2002; Tambasco-Studart et al. 2005). The coding sequences of *SIPDX1* and *SIPDX2* genes were amplified using gene-specific primers (Table 1) and then cloned into pYES2. The recombinant plasmids containing *SIPDX1.2/1.3* or *SIPDX2* gene were transformed into BQS1037 and MML21, respectively. Complementation was carried out on SC medium with or without VB6 supplement and with the appropriate selection markers (Tambasco-Studart et al. 2005).

In situ detection and quantitative measurement of ROS.

In situ detection of superoxide anion in leaf tissues was performed by nitroblue tetrazolium (NBT) staining (Doke et al. 1983). Leaf samples were collected at 0 (as mock-inoculation control) or 48 hpi with *B. cinerea* and were vacuum infiltrated in 10 mM potassium phosphate buffer, pH7.5, containing 10 mM Na₃N and 0.1% NBT (Sigma, St. Louis) for 30 min at room temperature. Leaves were boiled in 96% ethanol for 10 min before visualizing blue precipitates. Quantitative measurement of H₂O₂ was performed using hydrogen peroxide detection kits (number 064; Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The content of H₂O₂ was determined spectrophotometrically by measuring OD₄₀₅ based on the formation of complexes from reaction of molybdate with H₂O₂ in the samples and calculated according to a standard curve prepared with the same protocol.

Measurements of enzyme activity and antioxidant compounds.

Leaf samples were collected at 0 (as mock-inoculation controls), 24, or 48 hpi with *B. cinerea* and then subjected to prepare leaf extracts for biochemical assays. Activities of total SOD and CAT, RSC, and contents of GSH and GSSG were measured by standard colorimetric assays using commercial kits (Jiancheng Bioengineering Institute), according to the manufacturer's protocols. Briefly, SOD activity was determined colorimetrically by measuring OD₅₃₀ using the superoxide dismutase detection kit (number A001-1), which is based on the oxidation of hydroxylamine to form purple products by

superoxide anions generated from the system of xanthine and xanthine oxidase. One unit of SOD was defined as the amount of SOD required to inhibit the oxidation of hydroxylamine by 50%. Activity of CAT was determined using the catalase detection kit (number A007-2), which measured the consumption of H_2O_2 at OD_{240} for 1 min. The contents of GSH and GSSG were determined using the oxidative glutathione detection kit (number A061-2). GSSG in the samples was first reduced to GSH by glutathione reductase and GSH was determined by measuring OD_{412} using 5,5-dithio-bis-2-nitrobenzoic acid for color development. RSC was assumed colorimetrically using the superoxide anion detection kit (number A052) by measuring OD_{550} , representing the ability of the samples to inhibit the reduction of NBT by superoxide anion generated from reactions of xanthine and xanthine oxidase. Protein concentrations in all samples were determined by the Coomassie brilliant blue method (Jiancheng Bioengineering Institute).

Statistical analysis.

All experiments were performed in triplicate and all data obtained were subjected to statistical analysis according to the Student's *t* test and the probability values of $P < 0.05$ were considered to be significant.

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