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RESEARCH PAPER

Modulation of cellular redox status by thiamine-activated NADPH oxidase confers *Arabidopsis* resistance to *Sclerotinia sclerotiorum*

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Abstract

***Sclerotinia sclerotiorum* can initially suppress host oxidative burst to aid infection establishment, but later promotes reactive oxygen species (ROS) generation as proliferation advances. Here, it was shown that the cellular redox status can be modulated by thiamine to protect *Arabidopsis thaliana* against *Sclerotinia* at the early stages of infection. The initial inhibition of host ROS generation by *Sclerotinia*-secreted oxalate could effectively be alleviated by thiamine. Thiamine pre-treatment and subsequent wild-type *Sclerotinia* invasion induced an increase of ascorbate peroxidase activity concomitant with decreased ascorbate/dehydroascorbate ratios, which led to the cellular transition towards oxidative status in infected tissues. Particularly, it was observed that wild-type *Sclerotinia*, but not oxalate-deficient A2 mutant, could suppress the activity of NADPH oxidase (NOX), which might be an important mechanism underlying the early inhibition of ROS burst. Nevertheless, thiamine pre-treatment followed by wild-type *Sclerotinia* infection promoted NOX-derived ROS accumulation. Further studies showed that cytosolic Ca^{2+} and staurosporine-sensitive protein kinase(s) participated in thiamine-induced activation of NOX. Moreover, thiamine-induced tissue defence responses including callose/lignin deposition and stomatal closure were closely correlated with NOX-derived ROS generation. Additionally, studies with *Brassica* species indicated that the regulation of thiamine is largely conserved upon *Sclerotinia* infection. Collectively, it was concluded that thiamine reverses the initial reducing status through activating NOX-dependent ROS signalling to perturb the disease progress of *Sclerotinia*.**

Key words: Callose; NADPH oxidase; oxalate; redox status; *Sclerotinia*; thiamine.

Introduction

Sclerotinia sclerotiorum, known as white mould, is a necrotrophic fungal pathogen with a broad host range (Purdy, 1979). Early in pathogenesis, *Sclerotinia* secretes up to 10 mM oxalate as host colonization advances (Maxwell and Lumsden, 1970). Oxalate, a simple but versatile molecule, can commandeer programmed cell death (PCD) in plants (Errakhi *et al.*, 2008; Kim *et al.*, 2008), destroy pectin structure by extracting calcium ions (Ca^{2+}) (Bateman and Beer, 1965), and inhibit abscisic acid (ABA)-induced stomatal closure (Guimarães and Stotz, 2004). In addition, *Sclerotinia*-secreted oxalate can inhibit the host's reactive oxygen species (ROS) burst and the consequent defence responses (Cessna *et al.*, 2000). However, reduction of oxalate with a deficient mutant or overexpression of oxalate oxidase leads to ROS generation, which enables the host plant to tune resistance responses (Cessna *et al.*, 2000; Hu *et al.*, 2003; Dong *et al.*, 2008). Interestingly,

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; DPI, diphenyleneiodonium; GR, glutathione reductase; NBT, nitroblue tetrazolium; NOX, NADPH oxidase; O_2^-, superoxide anion; PCD, programmed cell death; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RP-HPLC, reverse-phase high-pressure liquid chromatography; SD, standard deviation; SOD, superoxide dismutase; TPP, thiamine pyrophosphate; XTT, 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

Williams *et al.* (2011) also demonstrated that oxalate-induced inhibition of ROS generation only occurs at the initial stages of infection to aid pathogen compatibility, with promotion of ROS production at the later stages as fungal proliferation advances. It is well known that early recognition of an intruder is critical to halt its invasion. Thus, ROS generation appears to be an important determinant during initial recognition of *Sclerotinia* invasion in plants (Perchepied *et al.*, 2010). However, the mechanism underlying the early inhibition of the ROS burst is unknown.

Among various ROS-generating enzymatic systems in plant, NADPH oxidases (NOX), also known as respiratory burst oxidase homologues (Rbohs), are the most thoroughly studied (Marino *et al.*, 2012). *Rboh* mutants are more susceptible than the wild-type *Arabidopsis* to *Sclerotinia* infection (Perchepied *et al.*, 2010). Diphenyleneiodonium (DPI), an inhibitor of NOX, has been shown to interfere with oxalate-induced plant PCD (Kim *et al.*, 2008). Moreover, ROS production in response to oxalate-deficient *Sclerotinia* is strongly reduced in *AtrbohD* plants, but not in *AtrbohF* mutants (Guo and Stotz, 2010; Perchepied *et al.*, 2010). NOX-mediated ROS signalling may be of prominent significance in plant defence against *Sclerotinia* infection.

The interaction between ROS and antioxidants is crucial for maintaining cellular redox homeostasis, which is a key factor in determining the outcome following pathogen challenge in plants (Foyer and Noctor, 2005, 2011; Sun *et al.*, 2012). Ascorbate and glutathione, as multifunctional metabolites, are important in redox homeostasis and signalling in the plant response to pathogens (Pastori *et al.*, 2003; Parisy *et al.*, 2007). To achieve pathogenic success, the host cell redox homeostasis is perturbed by *Sclerotinia*-secreted oxalate (Williams *et al.*, 2011). However, during the process of infection establishment, the role of ascorbate and glutathione in oxalate-manipulated cell redox status has yet to be determined.

Sclerotinia can readily infect its host and spread rapidly under favourable conditions, which makes it difficult to control the disease (Purdy, 1979). The inducible basal resistance of the plant to the already colonizing pathogen is often too weak and too late to prevent disease. However, the priming induced by some plant activators (e.g. low doses of salicylic acid, β-aminobutyric acid, thiamine) are capable of inducing rapid and effective defence responses to halt the invading pathogen (Thulke and Conrath, 1998; Ton and Mauch-Mani, 2004; Ahn *et al.*, 2005). Thiamine pyrophosphate (TPP) is well known for its role as a co-factor of enzymes involved in the tricarboxylic acid cycle, the pentose phosphate pathway, branched chain amino acid biosynthesis, and isoprenoid biosynthesis (Goyer, 2010). In addition, thiamine also enables plants to counteract various abiotic stresses like salt, heat, and oxidative stress (Rapala-Kozik *et al.*, 2008, 2012; Tunc-Ozdemir *et al.*, 2009). While *in vitro* studies show that thiamine has an antioxidative activity (Jung and Kim, 2003), it is still unclear whether the protective effects of thiamine result from its direct antioxidant effect (Asensi-Fabado and Munné-Bosch, 2010). Furthermore, Ahn *et al.* (2007) reported that the defence signalling induced by thiamine is dependent on hydrogen peroxide (H_2O_2) and removal of H_2O_2 nullifies plant defence responses. It is noteworthy that thiamine apparently influences several signal transduction pathways that are linked to plant defence, including fluxion of Ca^{2+} and upregulation of protein kinase C-like activity (Ahn *et al.*, 2005), which are the master regulators of NOX during stress responses (Sagi and Fluhr, 2001; Jiang *et al.*, 2011). Nevertheless, whether thiamine can activate NOX involved in cellular defence signalling, especially in plant resistance to *Sclerotinia*, remains unclear.

This study aimed to address whether thiamine regulates the signalling pathways of *Arabidopsis* resistance to the necrotrophic pathogen *Sclerotinia*. Our results revealed that thiamine could reverse the initial reducing status through activating NOX-mediated ROS signalling to perturb the disease progress of *Sclerotinia*. The findings contribute to our understanding of plant–*Sclerotinia* interactions, as well as to control of *Sclerotinia* in practice.

Materials and methods

Plant material and chemicals treatment

Arabidopsis ecotype Columbia-0 (Col-0) and mutant seeds *tz-1* (N3375), *AtrbohD* (N9555) and *AtrbohDF* (N9558) were obtained from the European Arabidopsis Stock Centre. For *Brassica napus*, experiments were performed using two cultivars of the susceptible genotype 'Yinong 34' and the partially resistant 'Zhongshuang 9' (Wang *et al.*, 2004). Plants were cultivated in growth cabinets at 22 °C (day) and 18 °C (night), with a 16 h light period (300 μmol m^{-2} s^{-1}). Thiamine, DPI, BAPTA-AM, EGTA, staurosporine, sodium, 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) bezene sulfonic acid hydrate (XTT) were purchased from Sigma-Aldrich. Thiamine treatment was according to Ahn *et al.* (2005). Leaves from 4-week-old plants were sprayed with 250 μg ml^{-1} of Tween 80 (control) or thiamine (dissolved in 250 μg ml^{-1} Tween 80) at 4 h prior to *Sclerotinia* inoculation. For DPI treatment, leaves were inoculated with *Sclerotinia* at 30 min after pre-infiltrated with 100 μM DPI.

Fungal growth and inoculations

A wild-type isolate (1980) and an oxalate-deficient mutant (A2) of *Sclerotinia* were grown on potato-dextrose agar at 21 °C for 3 d. Agar plugs (diameter 0.3 or 0.8 cm) containing the growing mycelia were used to inoculate the leaves. Infected plants were kept in a clear plastic box under saturating humidity. Oxalate levels were determined following the method of Huang *et al.* (2011). Photographs of the necrotic phenotype were produced using a SONY numeric camera (HDR-XR500E). For lesion size measurement, photographs were captured using a Zeiss inverted microscope installed with a Carl Zeiss AxioCam MRc5 camera. Lesion area was quantified at least in ten leaves with the measurement tool 'outline spline' in AxioVision Rel.4.5 software.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from *Arabidopsis* leaves using Trizol according to the supplier's recommendations. First-strand cDNA was synthesized using the SuperScript II First-Strand Synthesis System (Invitrogen). qRT-PCR was performed using the Roche LightCycler™ 2.0 Real-time PCR Detection System. The expression of target gene was normalized relative to the housekeeping gene

ACTIN2 (Sun *et al.*, 2012). The *RBOHD*, *RBOHF*, *THI1*, *TH1*, and *TPK* primer sequences used have been described elsewhere (Penfield *et al.*, 2006; Rapala-Kozik *et al.*, 2012).

Measurement of thiamine and its phosphate analogues

Thiamine and its phosphate analogues were converted into thiochrome by adding cyanogen bromide, and were measured according to the method of Rapala-Kozik *et al.* (2008, 2012). Samples were quantified by reverse-phase high-pressure liquid chromatography (RP-HPLC). An Agilent ZORBAX Eclipse plus C18 (5 µm) column (150 × 4.6 mm) was used for the separation.

Measurement of superoxide anion (O_2^-) and H_2O_2

O_2^- was measured according to Jiang and Zhang (2002) by analysis of XTT formazan absorbance at 470 nm. Corrections were made for the background absorbance in the presence of superoxide dismutase (SOD; 50 U). The accumulation of O_2^- was also monitored *in situ* by nitroblue tetrazolium (NBT) as described previously (Jabs *et al.*, 1996). Microscopic images were photographed with an Olympus BX51 microscope. For quantitative determination of H_2O_2, an Amplex Red Hydrogen Peroxide Assay kit (Invitrogen) was used.

NOX activity determination

Plasma membranes were isolated as described by Liu *et al.* (2012). For native PAGE, protein samples from each fraction (20 µg per lane) were separated in a 7.5% (w/v) polyacrylamide separating gel and 4% (w/v) stacking gels at 4 °C. The gel was incubated in the dark with NBT solution [50 mM Tris-HCl (pH 7.4), 0.2 mM NBT, 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$] for 20 min. The reaction was initiated by addition of 0.5 mM NADPH and stopped when clear blue formazan bands were observed.

The NOX activity of plasma membrane vesicles was also determined spectrophotometrically by the reduction of XTT (Sagi and Fluhr, 2001). The analysis reaction medium contained 10 µg protein, 0.3 mM XTT, and 0.18 mM NADPH in 1 ml 50 mM Tris-HCl buffer (pH 7.4). The activity was expressed as the difference in XTT formazan absorbance at 470 nm (ΔA_{470}) in the presence or absence of 50 U of SOD.

Enzyme assays, and ascorbate and glutathione determination

The activities of the antioxidant enzymes SOD, ascorbate peroxidase (APX), and glutathione reductase (GR) were measured spectrophotometrically as described previously (Rao *et al.*, 1996). Ascorbate was assayed according to the method of Queval and Noctor (2007) based on the change in absorbance of APX at 265 nm. The levels of glutathione were determined with a reduced glutathione and oxidized glutathione assay kit (Beyotime).

Measurement of cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$)

The method for $[Ca^{2+}]_{cyt}$ detection was based on previous work (Yue *et al.*, 2012). Ca^{2+} was stained with Fluo-3-AM ester (Invitrogen), which is hydrolysed to yield genuine Fluo-3 capable of indicating changes in $[Ca^{2+}]_{cyt}$. The fluorescence intensity of Fluo-3 was measured with a fluorescence spectrometer (excitation at 488 nm, emission at 525 nm).

Cell wall fortification and stomatal aperture measurements

Callose deposition was stained with 0.01% (w/v) aniline-blue and observed using a fluorescence microscope (Sun *et al.*, 2012). Lignin was stained with phloroglucinol as described by Vallet *et al.* (1996), and the content was measured by determining absorbance at 280 nm with a thioglycolic acid assay. Stomatal aperture was monitored as described by Guimarães and Stotz (2004). Epidermal peels were prepared from the areas surrounding necroses, and incubated in the dark for at least 3 h to induce stomatal closing. The stomatal aperture was quantified with AxioVision Rel.4.5 software and is given as width/length.

Results

Thiamine protects Arabidopsis against Sclerotinia invasion

Thiamine has been determined to confer plant resistance to a wide range of pathogens (Ahn *et al.*, 2005), which impelled us to explore whether thiamine was involved in *Arabidopsis* defence against *Sclerotinia*. Thiamine showed a concentration-dependent resistance effect on necrotic lesions, which were reduced by 52% following treatment with 1 mM thiamine relative to untreated plants (Fig. 1A). Since no further increase of resistance effect was observed at higher concentrations, thiamine at 1 mM was used in the following experiments unless otherwise mentioned. Furthermore, pretreatment of thiamine significantly delayed progression of lesion expansion (Fig. 1B, C), but did not directly affect the fungal hyphae growth *in vitro* (Supplementary Fig. S1A, B at *JXB* online).

Since oxalate is responsible for the pathogenicity of *Sclerotinia* (Godoy *et al.*, 1990; Cessna *et al.*, 2000), we evaluated necrotic lesions with an oxalate-deficient *Sclerotinia* mutant (A2) in leaves pre-treated with thiamine. Oxalate accumulation in leaves infected with A2 mutant was much less than that with wild-type *Sclerotinia* (Supplementary Fig. S1C). Compared with wild-type *Sclerotinia*, A2 mutant invasion resulted in restricted necrotic lesions, and 10 mM potassium oxalate (pH 7.0) remarkably restored A2 pathogenicity. However, the recovery of A2 pathogenicity could also be significantly contracted by exogenous application of thiamine (Fig. 1D). Together, these results illustrated that thiamine could induce *Arabidopsis* resistance to *Sclerotinia* by alleviating oxalate-induced necrosis.

The impact of endogenous thiamine on defence against Sclerotinia infection

To assess whether the endogenous thiamine biosynthesis was influenced by *Sclerotinia* infection, we analysed the expression of genes responsible for the biosynthesis of the thiazole moieties of thiamine (*THI1*) and the coupling of 4-methyl-5-(2-hydroxyethyl)thiazole phosphate (HET-P) and 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate (HMP-PP) to form thiamine monophosphate (TMP, *TH1*), as well as the activation of thiamine to TPP (*TPK*). A small scheme showing the different steps in thiamine biosynthesis is given in Supplementary Fig. S2A at *JXB* online. Results showed that the expression of genes *THI1*, *TH1* and *TPK* were upregulated in different degrees after challenged with *Sclerotinia* for 3 h (Fig. 2A). Correlated with the upregulation of these genes, the changes in all three forms of thiamine were also elevated (Fig. 2B), suggesting that plants increased *de novo* thiamine biosynthesis upon *Sclerotinia* infection.

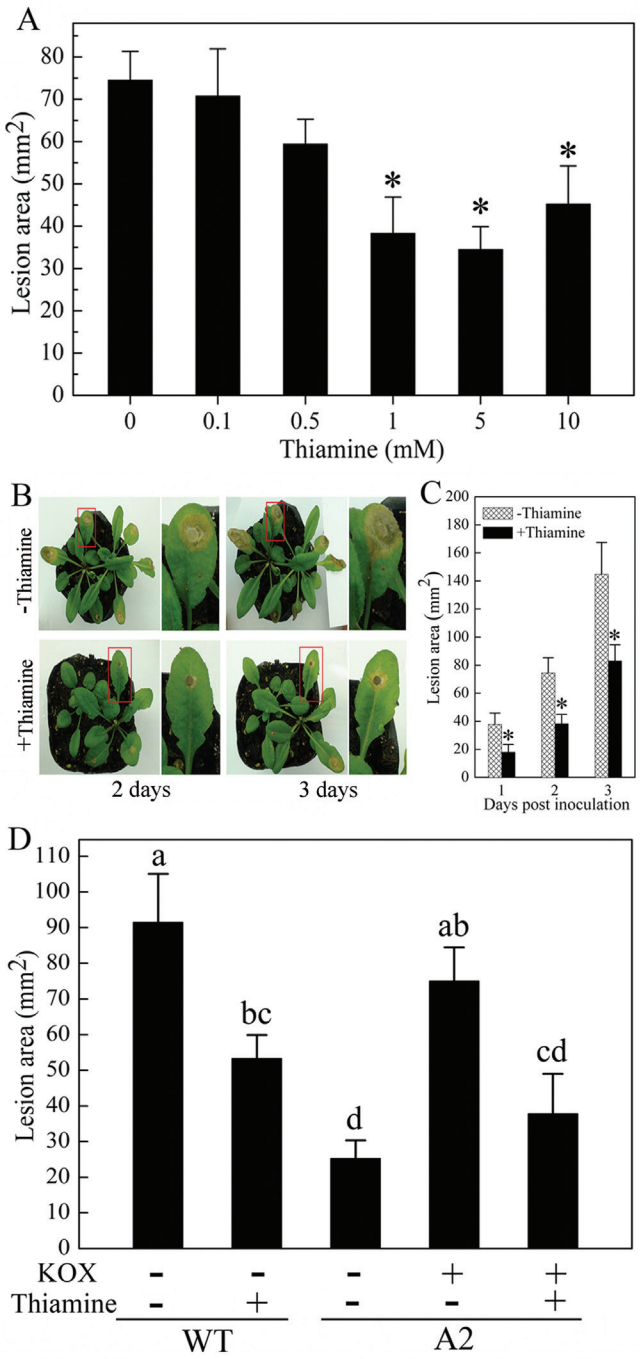

Fig. 1. Thiamine enhances *Arabidopsis* resistance to *Sclerotinia*. (A) Lesion development on *Sclerotinia*-infected leaves pre-treated with 0–10 mM thiamine. Lesion areas were measured at 2 d. (B, C) Disease symptoms (B) and lesion area (C) on *Sclerotinia*-inoculated leaves pre-treated with 1 mM thiamine. (D) Thiamine reduces the recovery of pathogenicity of A2 mutant. Leaves were pre-infiltrated with 10 mM potassium oxalate (KOX) (pH 7.0) to recover the pathogenicity of the A2 mutant. Values represent means ±standard deviation (SD) of at least ten lesions. Asterisks indicate significant differences from thiamine-untreated samples (Student's *t*-test, $P < 0.05$). Different letters indicate statistically significant differences (Duncan's multiple range tests; $P < 0.05$). (This figure is available in colour in *JXB* online.)

We next examined the lesion formation with the thiamine auxotrophic plant *tz-1*, which cannot survive on thiamine-free medium due to mutation of HET-P synthase. RP-HPLC results showed that *tz-1* plants had lower levels of thiamine than Col-0 after 4 weeks grown in soil with 0.01% thiamine (Supplementary Fig. S2B). Thiamine deficiency made plants more susceptible to *Sclerotinia* (Fig. 2C). Unexpectedly, although exogenous application of thiamine increased the resistance of *tz-1* plants, there was a relatively small reduction in lesion size compared with Col-0. This led us to doubt how far endogenous thiamine really contributed to resistance to *Sclerotinia.* We then tested the disease severity with different degrees of recovery of *tz-1* plants that were grown in soil containing 50–300 µM thiamine after germination on agar medium weekly with 0.01% thiamine. The lesion size caused by *Sclerotinia* decreased with increasing of recovery of thiamine concentration in *tz-1* plants (Fig. 2D and Supplementary Fig. S2C). These findings suggested that the elevated level of thiamine in *Arabidopsis* is correlated with enhanced resistance to *Sclerotinia*.

Thiamine alleviates the inhibition of Sclerotinia on host ROS burst

Sclerotinia-secreted oxalate can abolish host defences by suppressing ROS generation at the early stages of infection (Cessna *et al.*, 2000). Consistent with this, wild-type *Sclerotinia*, but not the A2 mutant, inhibited O_2^- and H_2O_2 accumulation at 3 h after inoculation. However, the wild-type *Sclerotinia* provoked augmented accumulation of O_2^- and H_2O_2 in leaves pre-treated with thiamine (Fig. 3A, C). Moreover, *in situ* staining with NBT also showed that thiamine pre-treatment increased O_2^- generation when challenged with wild-type *Sclerotinia* for 3 h (Fig. 3B). Similar to the early stages, at 2 d post-inoculation with wild-type *Sclerotinia*, thiamine primed strong NBT staining at the leading edge of necrotic lesions (Supplementary Fig. S3A at *JXB* online). Additionally, wild-type *Sclerotinia*-induced ROS levels showed no discernible differences from samples either with or without thiamine pre-treatment at 12 h (Fig. 3A, C), suggesting that thiamine-alleviated inhibition of host ROS generation occurred at the early pathogenicity stages.

To investigate the effect of thiamine on the changes in cellular redox status, the activities of antioxidant enzymes (SOD, APX, and GR), and the levels of reduced (Asc, GSH) and oxidized (DHA, GSSG) ascorbate and glutathione, respectively, were measured. Both the SOD and APX activities were enhanced by thiamine in wild-type *Sclerotinia*-infected samples (Fig. 3D, E). However, the activities of GR did not significantly change with or without pre-treatment with thiamine (Fig. 3F). Accompany with these enzymes, thiamine pre-treatment and subsequent wild-type *Sclerotinia* infection triggered a reduction of the Asc/DHA ratio (Fig. 3G), reflecting an increasing oxidative status. However, the ratio of GSH/GSSG did not show significant variation under all treatments (Fig. 3H), indicating that thiamine-induced cellular redox changes in wild-type *Sclerotinia*-infected tissues were more specific to the Asc redox state. Additionally, at 12 h post-inoculation, wild-type

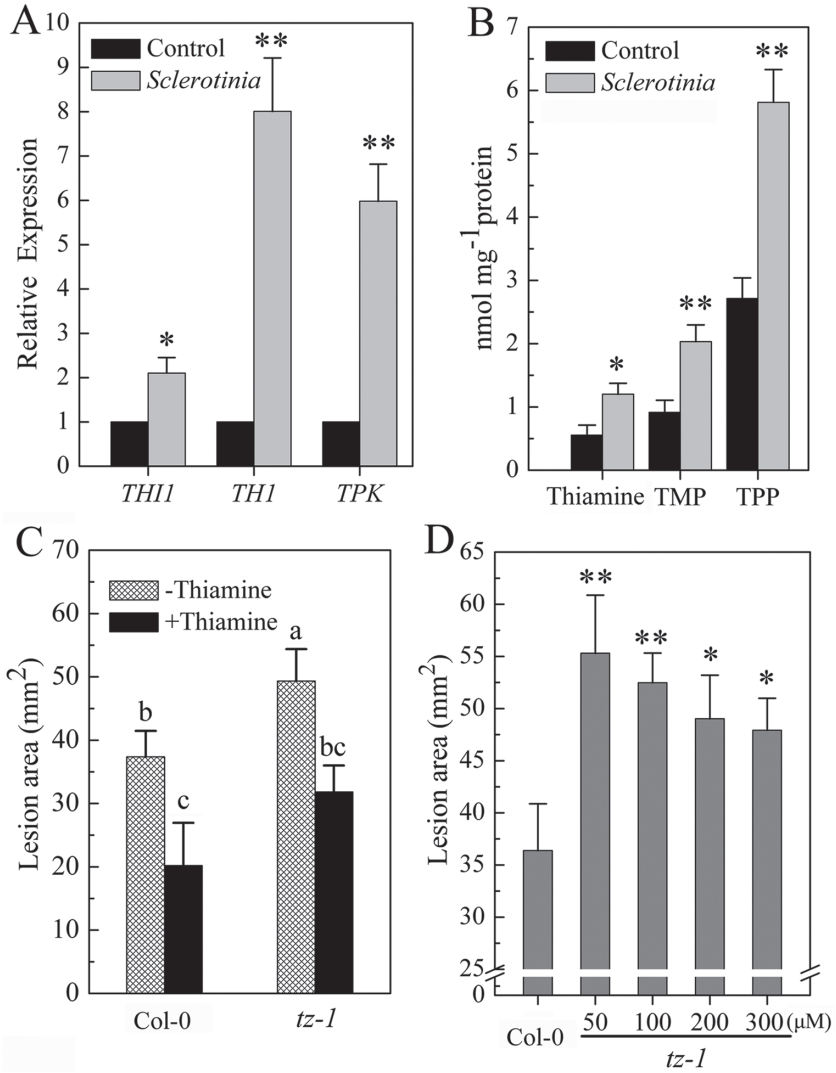

Fig. 2. Thiamine biosynthesis is correlated with the *Arabidopsis* response to *Sclerotinia*. (A, B) Changes in biosynthesis genes and contents of thiamine in *Arabidopsis* 3 h after inoculation with *Sclerotinia*. (C) Lesion size on *tz-1* plants inoculated with *Sclerotinia* for 1 d. (D) Lesion size on recovery of *tz-1* plants with different concentrations of thiamine. Values represent means ±SD of three independent experiments. $^{**}P$ <0.01; $^{*}P$ <0.05 (Student's *t*-test). Different letters indicate statistically significant differences (Duncan's multiple range tests; P <0.05).

Sclerotinia induced a decrease in the Asc/DHA ratio, but no significant difference was observed relative to samples pre-treated with thiamine (Fig. 3G). These results suggest that pre-treatment with thiamine led to the cellular transition towards oxidative status in the infected tissues at early pathogenesis.

Activation of NOX confers thiamine-induced resistance to Sclerotinia

Since NOX has a dedicated function of generating ROS (Marino *et al.* 2012), we explored whether the activation of NOX was affected by *Sclerotinia* using an *in situ* gel NBT assay. At 3 h post-inoculation, a main activity band was detected in samples infected with the A2 mutant, but only a weak band was observed with wild-type *Sclerotinia* (Fig. 4A). Moreover, NOX activity showed slight upregulation in wild-type *Sclerotinia*-infected leaves and downregulation in A2-inoculated samples after 12 h of infection (Fig. 4A, B). Similar results were obtained by measuring XTT formazan to examine NOX activity (Fig. 4C). These data indicated that inhibition of NOX activity is possibly required for the oxalate-induced reducing environment in the early infection stages.

To test whether NOX was involved in the signal transduction induced by thiamine, the NOX activity was analysed in leaves pre-treated with thiamine following *Sclerotinia* inoculation for 3 h. Compared with wild-type *Sclerotinia* inoculation alone, thiamine pre-treatment and subsequent wild-type *Sclerotinia* infection increased the activity of NOX (Fig. 5A). However, this effect was abolished by the NOX inhibitor DPI (100 μM), and was concomitant with a reduced H_2O_2 generation (Fig. 5B). Moreover, upon wild-type *Sclerotinia* infection, inhibition of NOX with DPI led to a decrease in APX activity, and a reduction of the Asc/DHA ratio in leaves pre-treated with thiamine (Fig. 5C, D).

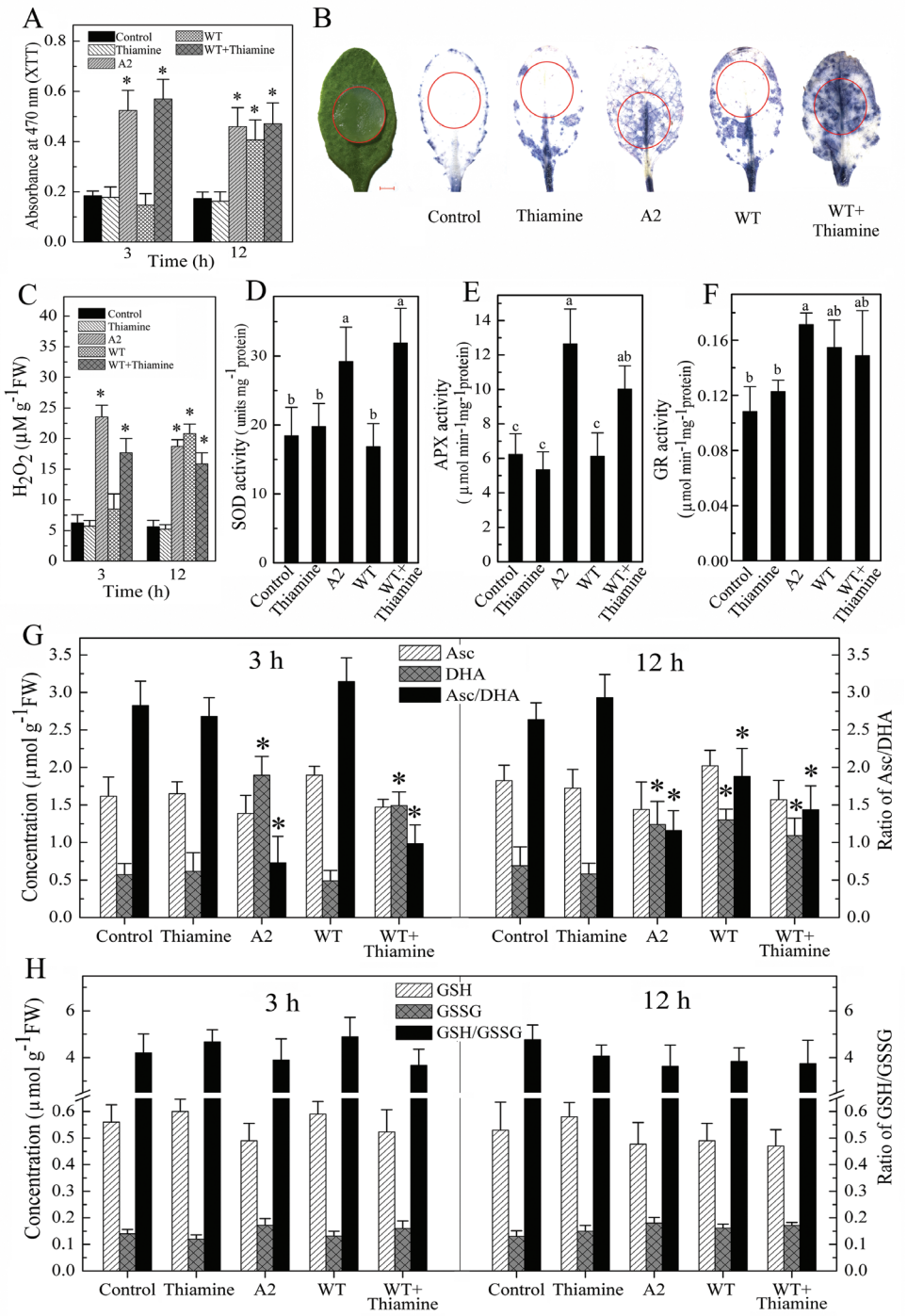

Fig. 3. Thiamine alleviates the inhibitory action of oxalate on the host's initial cellular redox status. (A) Effect of thiamine on O_2^- generation in *Arabidopsis* upon *Sclerotinia* infection. (B) *In situ* detection of O_2^- formation at 3 h after *Sclerotinia* inoculation. Circles represented the location of agar plug (diameter 0.8 cm) inoculation as shown on the left-most leaf. Bar, 0.2 cm. (C) Quantitative determination of H_2O_2 generation. (D–F) Changes in SOD (D), APX (E), and GR (F) activities after inoculation with *Sclerotinia* for 3 h. (G, H) Changes in Asc/DHA (G) and GSH/GSSG (H) ratios in leaves treated with thiamine prior to *Sclerotinia* inoculation. Data are means ±SD of at least three replicates. Asterisks indicate Student's *t*-test significant at $P < 0.05$ versus the control. Different letters indicate statistically significant differences (Duncan's multiple range tests; $P < 0.05$). FW, fresh weight. (This figure is available in colour in *JXB* online.)

To genetically determine the role of NOX in the thiamine-induced resistance, mutants of NOX were tested for their response to *Sclerotinia*. Strikingly, thiamine pre-treatment could not effectively protect *AtrbohD* and *AtrbohDF* plants against *Sclerotinia* invasion (Fig. 5E). After 3 h inoculation with *Sclerotinia*, thiamine-induced H_2O_2 generation was reduced by 41% in *AtrbohD*, and by 67% in the double mutant *AtrbohDF* (Fig. 5F). Consistently, APX activity was also

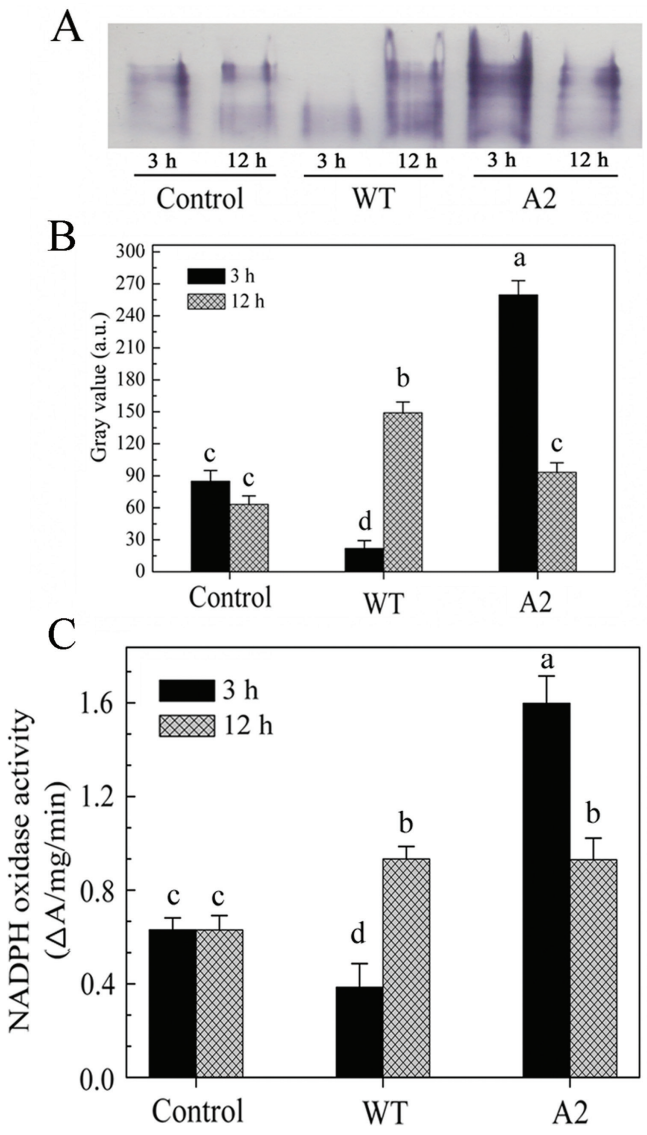

Fig. 4. NOX is activated by the A2 mutant but inhibited by wild-type *Sclerotinia* at the early stages of infection. (A) *In situ* gel NBT assay for NOX activity. Plasma membrane vesicles were used to test the activity of NOX. (B) Grey intensity was quantified with ImageJ software. (C) The O_2^--producing capabilities of NOX were measured based on XTT reduction. Data are means ±SD of three independent experiments. Differences within each treatment were evaluated by Duncan's multiple range test (P <0.05). (This figure is available in colour in *JXB* online.)

significantly downregulated in both *AtrbohD* and *AtrbohDF* plants relative to Col-0 (Fig. 5G). These results indicated that thiamine could enhance NOX activity in the early pathogenesis, which might contribute to alleviate the suppression of initial host oxidative burst caused by *Sclerotinia*.

$[Ca^{2+}]_{cyt}$ and protein kinase(s) participate in thiamine-induced activation of NOX

We analysed *RBOHD* and *RBOHF* gene expression to investigate whether thiamine was attributed to transcriptional regulation of NOX. Compared with the control, no major difference in expression of *RBOHD* or *RBOHF* was observed in samples pre-treated with thiamine at 3 h after *Sclerotinia* infection (Fig. 6A). To explore the possible factors that influenced NOX activation by thiamine, we first measured the changes in $[Ca^{2+}]_{cyt}$ levels with a Ca^{2+}-sensitive fluorescent dye, Fluo-3-AM. As shown in Fig. 6B, relative to samples infected with wild-type *Sclerotinia* alone, the fluorescence intensity of Fluo-3 in leaves pre-treated with thiamine was significantly increased. The effect of $[Ca^{2+}]_{cyt}$ on NOX activity was then investigated by Ca^{2+} scavengers. Thiamine-induced NOX activity was reduced by the addition of 5 mM EGTA (36%, Fig. 6C), and strongly decreased in the presence of 1 mM BAPTA-AM (71%, Fig. 6C). We also determined whether protein phosphorylation participated in thiamine-induced activation of NOX. Following co-treatment with 20 μM staurosporine, a broad-range protein kinase inhibitor, thiamine-induced activation of NOX was markedly inhibited (Fig. 6D). Concomitant with the decreased NOX activity, both BAPTA-AM and staurosporine weakened the initial ROS generation and the subsequent resistant effect induced by thiamine in *Sclerotinia*-infected leaves (Fig. 6E–G). These results suggest that Ca^{2+} and staurosporine-sensitive protein kinase(s) are important signal mediators required for thiamine-induced NOX activation.

Tissue defence responses induced by thiamine

The cell wall fortification by deposition of callose/lignin is a key component of basal defence against pathogen attack (Hardham *et al.*, 2007). We found that callose accumulation was induced by thiamine upon wild-type *Sclerotinia* infection. However, inhibition of NOX with DPI or mutation in *AtrbohD* blocked this effect (Fig. 7A, B). The tissue localization of callose is worth noting. Callose was deposited adjacent to the advancing edge of growing mycelia in A2 mutant-infected tissues. However, there was a 'buffer zone' between the point of thiamine-induced callose deposition and the invading fungal hyphae in leaves inoculated with wild-type *Sclerotinia*. Additionally, lignin was also involved in thiamine-induced cell wall reinforcement, which was further confirmed by quantifying measurement of lignin at 280 nm (Supplementary Fig. S4A, B at *JXB* online). A model of cell wall fortification functions in thiamine-induced resistance to *Sclerotinia* is given in Fig. 7C.

Sclerotinia invasion results in foliar wilting by disturbing guard-cell functions (Guimarães and Stotz, 2004). As shown in Fig. 7C, pre-treatment with thiamine alleviated the wild-type *Sclerotinia*-caused increase of the stomatal aperture under darkness. Interestingly, suppression of NOX either with DPI or by mutation in *AtrbohD* nullified this effect (Fig. 7D, E). Conversely, exogenous application of 0.5 mM H_2O_2 effectively promoted the stomatal closure (Fig. 7C). The results suggested that NOX-derived ROS generation is required for thiamine-induced stomatal closure in *Sclerotinia*-infected tissues.

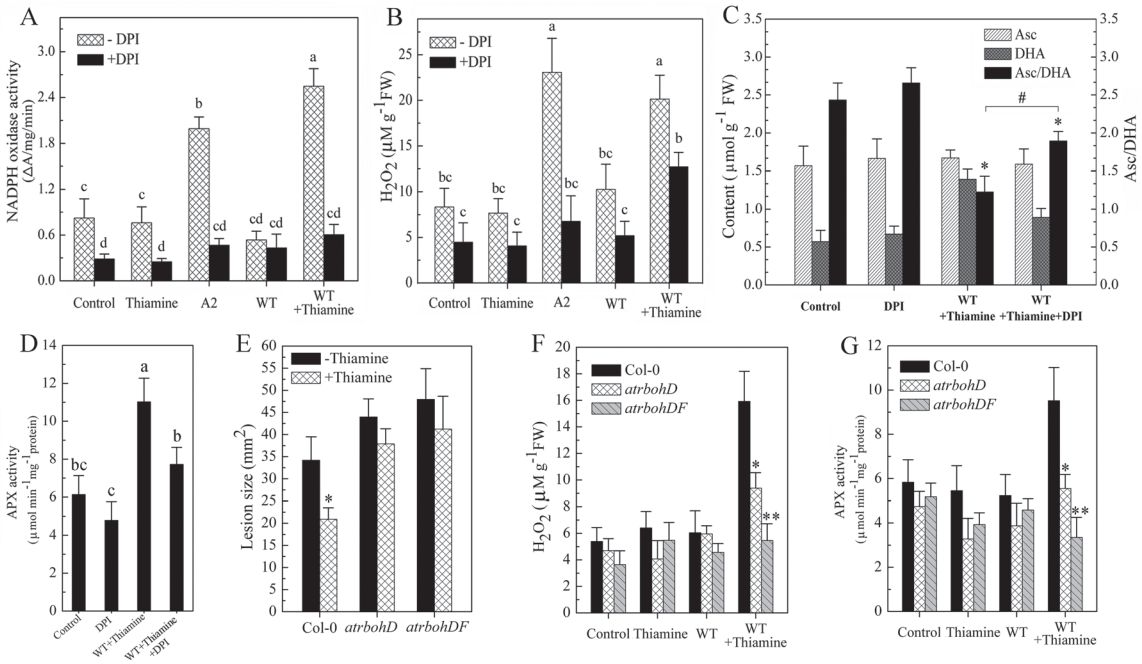

Fig. 5. Thiamine activates NOX at the early stages of *Sclerotinia* infection. (A) Thiamine increased NOX activity in leaves challenged with wild-type *Sclerotinia* for 3 h. (B) Quantitative determination of H_2O_2. (C) Inhibition of NOX with DPI increases the ratio of Asc/DHA. The levels of Asc and DHA were measured from wild-type *Sclerotinia*-infected leaves after 3 h. (D) DPI weakens APX activity in samples pre-treated with thiamine. (E–G) Effect of thiamine on lesion development (E), H_2O_2 production (F), and APX activities (G) in NOX mutants challenged with wild-type *Sclerotinia*. Asterisks indicate significant differences from control or between Col-0 and the NOX mutants: $^{**}P$ <0.01; $^{*}P$ <0.05; #P <0.05 versus the indicated samples (Student's t-test). Different letters indicate significant differences (Duncan's multiple range test, P <0.05).

Discussion

In this study, we investigated the effectiveness and mode of action of thiamine in *Arabidopsis* resistance against the necrotrophic pathogen *Sclerotinia.* To explore the mechanisms behind thiamine-induced resistance, we examined the involvement of NOX-mediated ROS signalling and the correlated cellular defence responses.

Thiamine-induced resistance against Sclerotinia is based on ROS generation

Accumulating evidence suggests that thiamine is an activator that triggers plant augmented defence responses (Ahn *et al.*, 2005, 2007). Our data show for the first time that thiamine can effectively protect *Arabidopsis* against *Sclerotinia* (Fig. 1). Early in pathogenesis, the plants activated *de novo* thiamine biosynthesis in response to *Sclerotinia* invasion (Fig. 2A, B). Thiamine deficiency, as in the auxotroph *tz-1*, increased plant susceptibility to *Sclerotinia*, but the symptom was partially alleviated by exogenous applying thiamine (Fig. 2C). These results imply that thiamine-induced defence signalling confers plant resistance to *Sclerotinia*.

The role of thiamine in plant resistance and adaptation to environmental stresses is relatively complex. Although thiamine is a potent scavenger of hydroxyl radicals *in vitro* (Jung and Kim, 2003), its contribution to plant adaptation to abiotic stress does not directly involve chemical antioxidants (Tunc-Ozdemir *et al.*, 2009). Moreover, Ahn *et al.* (2007) reported that thiamine-enhanced plant resistance is dependent on H_2O_2 accumulation. These seemingly incongruent characteristics are also found in riboflavin, whose induction of plant resistance requires a H_2O_2 burst (Azami-Sardooei *et al.*, 2010). Upon paraquat-induced oxidative stress, supplementation with thiamine cannot fully abolish ROS accumulation (Tunc-Ozdemir *et al.*, 2009). Interestingly, we found that thiamine partially reduced A2-induced H_2O_2 production, but the level was still higher than that in the control (Supplementary Fig. S5 at *JXB* online). It seems that thiamine more likely functions as a regulator to maintain ROS generation, and keeps it at a relatively reasonable level to induce plant adaptation. Further investigations are needed to address this possibility.

The initial oxidative status is crucial for disturbing Sclerotinia infection

The cellular redox adjustments are central for defence responses during the early stages of *Sclerotinia* infection (Hegedus and Rimmer, 2005; Bolton *et al.*, 2006). The early pathogenicity stage involves production of oxalate to generate a reducing environment that suppresses the host's oxidative burst (Cessna *et al.*, 2000). Reduction of oxalate with the A2 mutant induces high levels of H_2O_2 in infected tissues, whereas induction of an artificial reducing environment nullifies this effect (Williams *et al.*, 2011). Here, we observed that

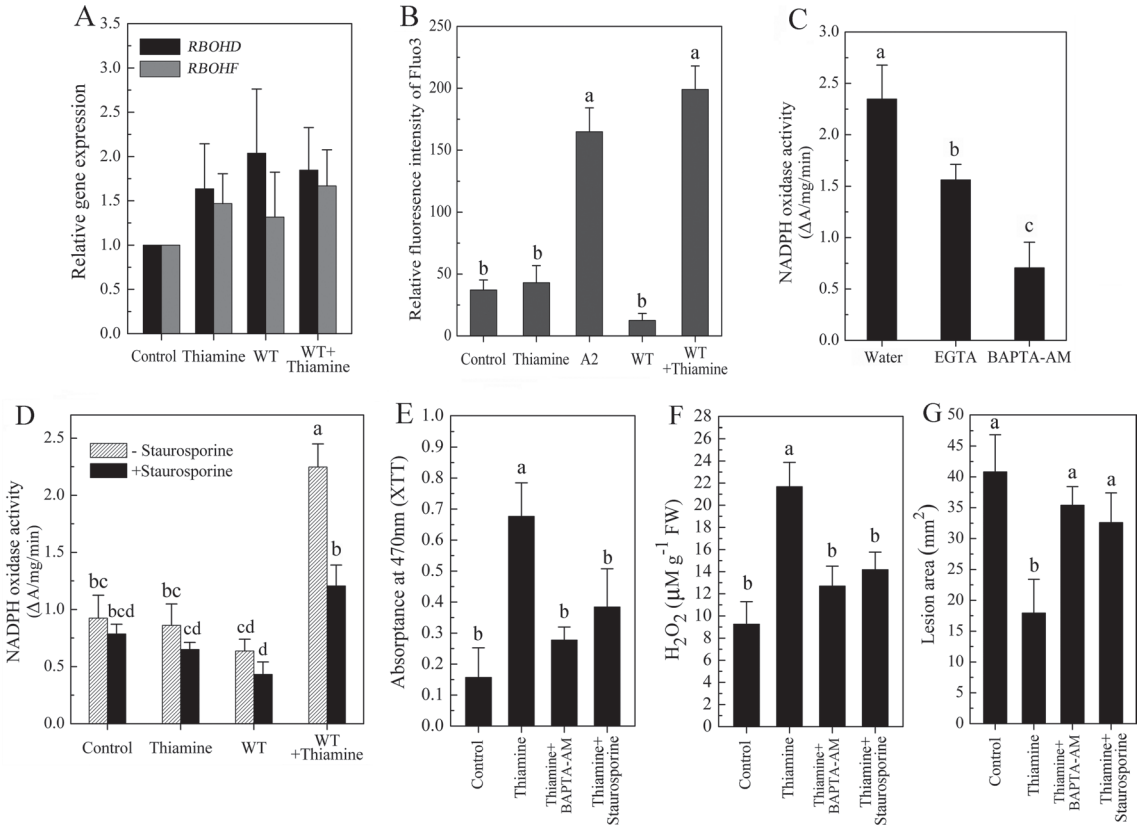

Fig. 6. $[Ca^{2+}]_{cyt}$ and protein kinase(s) participate in thiamine-induced activation of NOX. (A) Changes in *RBOHD* and *RBOHF* expression in samples pre-treated with thiamine at 3 h after *Sclerotinia* infection. (B) Thiamine-induced elevation of $[Ca^{2+}]_{cyt}$ in wild-type *Sclerotinia*-infected leaves. (C) Removal of $[Ca^{2+}]_{cyt}$ restrains thiamine-induced activation of NOX. Samples pre-treated with thiamine were sprayed with water (control), EGTA (5 mM), or BAPTA-AM (1 mM) prior to inoculation with wild-type *Sclerotinia*. (D) Staurosporine suppresses thiamine-induced activation of NOX. (E–G) Both BAPTA-AM and staurosporine abolished thiamine-induced ROS generation and resistance to *Sclerotinia*. Letters indicate significant differences among treatments (Duncan's multiple range test, $P < 0.05$).

thiamine pre-treatment and subsequent *Sclerotinia* infection rapidly induced ROS production (Fig. 3A–C). Interestingly, at the later stages of wild-type *Sclerotinia* infection, no significant difference in ROS generation was observed in samples either with or without thiamine pre-treatment (Fig. 3A, C). Thus, reversion of the initial cellular reducing status may be an effective mechanism for thiamine to disturb the establishment of infection of this fungus.

The interaction between ROS and antioxidants is critical for maintaining cellular redox homeostasis (Foyer and Noctor, 2005, 2011). Wild-type *Sclerotinia* challenged on plants pre-treated with thiamine resulted in reduced Asc redox state, but no significant change in the GSH redox state (Fig. 3G, H). In particular, the alteration of Asc redox state was correlated with the changes in H_2O_2 content and APX activity (Fig. 3C, E). Nevertheless, alone among the expression of antioxidative enzymes, *APX1* transcript levels are not elevated in unstressed *tz-1* plant (Tunc-Ozdemir *et al.*, 2009). Thiamine may therefore alter the Asc redox state by upregulation of APX activity (Fig. 3E), which may partly account for the susceptibility of *tz-1* plant to *Sclerotinia* (Fig. 2C). Together, these data illustrate that the reduced Asc redox is a key determinant of thiamine-reversed initial reducing status.

Activation of NOX is involved in thiamine-induced initial oxidative status

NOX has been reported in plant resistances to *Sclerotinia* (Guo and Stotz, 2010; Perchepied *et al.*, 2010). Inhibition of NOX with DPI interferes with oxalate-induced PCD in plants (Kim *et al.* 2008). Here, we found that NOX activity was inhibited by wild-type *Sclerotinia* but not the A2 mutant at the initial stages of infection (Fig. 4). It is possible that *Sclerotinia*-secreted oxalate inhibits the activity of NOX, thus contributing to the reducing environment establishment in early pathogenesis. Thiamine could effectively alleviate the inhibition of NOX, leading to H_2O_2 generation and oxidative status transition in infected tissues. However, these effects were nullified by suppressing NOX activation with pharmacological and genetic approaches (Fig. 5). These results prove that NOX participates in thiamine-induced initial oxidative status upon *Sclerotinia* infection.

Although we observed that thiamine could enhance NOX activity, the detailed mechanisms downstream of thiamine remains incomplete. Thiamine pre-treatment did not significantly induce change of *RBOHD* or *RBOHF* expression at 3 h after *Sclerotinia* infection (Fig. 6A), suggesting that thiamine perhaps is not attributed to transcriptional regulation

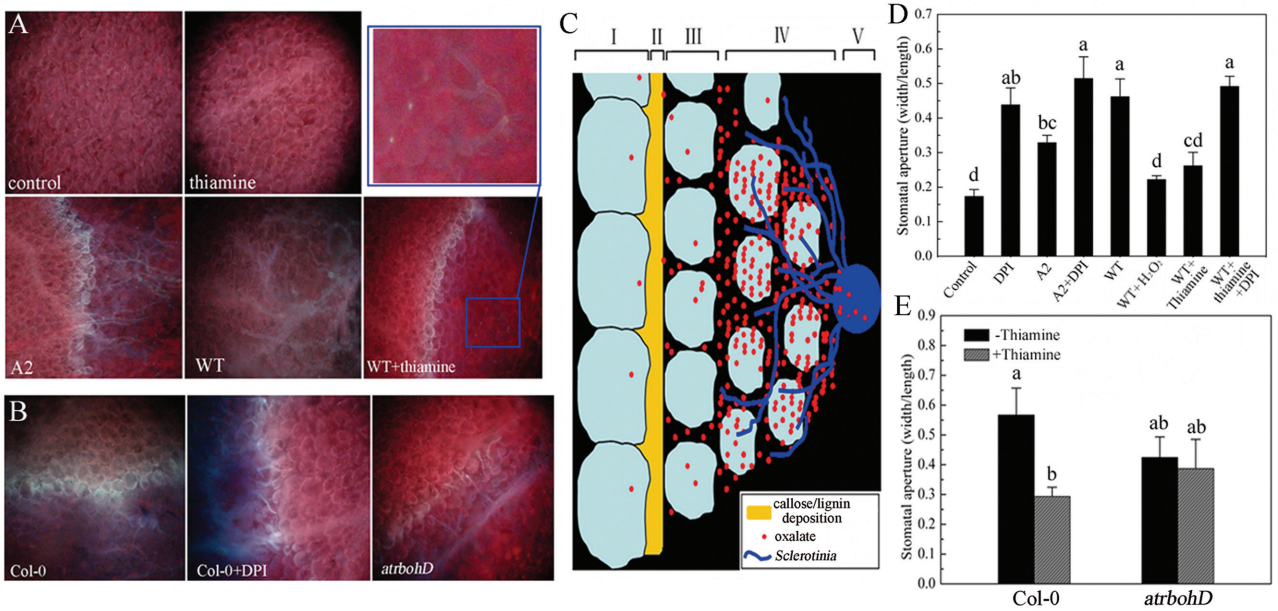

Fig. 7. Tissue defence responses induced by thiamine. (A) Thiamine-induced callose deposited around the advancing edge of necrotic areas. (B) The role of NOX in thiamine-primed callose accumulation. (C) A model of cell wall fortification functions in thiamine-induced resistance to *Sclerotinia*. I, healthy tissue; II, callose/lignin deposition; III, oxalate diffusion; IV, mycelia penetration; V, agar plug containing mycelia. (D, E) Thiamine-induced stomatal closure depends on NOX-derived ROS generation. Values represent means ±SD of at least 30 stomatal apertures. Differences within each treatment were evaluated by Duncan's multiple range test (P <0.05). (This figure is available in colour in *JXB* online.)

of NOX at the early stages of infection. Ahn *et al.* (2005) reported that thiamine could promote fluxion of Ca^{2+} and upregulation of protein kinase C-like activity. Interestingly, these two factors are critical signals upstream of activating NOX activity (Quagliaro *et al.*, 2003). Consistently, both BAPTA-AM and staurosporine inhibited NOX activity and the subsequent ROS generation in samples pre-treated with thiamine upon *Sclerotinia* infection (Fig. 6C–F). These results indirectly demonstrated that Ca^{2+} and staurosporine-sensitive protein kinase(s) participated as important signal mediators in thiamine-induced activation of NOX.

In addition, whether thiamine can interact with multiple phytohormones (e.g. ABA) during the process of NOX activation is unknown. ABA-regulated NOX has been reported in plant resistance to external stresses (Jiang and Zhang, 2002). Recently, we found that *Sclerotinia* could reduce ABA levels by causing a dysfunctional xanthophyll cycle at the initial stages of infection (unpublished data). Interestingly, exogenous application of ABA can increase the transcripts and contents of thiamine (Rapala-Kozik *et al.*, 2012). It will be worth studying thiamine–ABA interactions during the process of NOX activation in future research.

Tissue defence response induced by thiamine requires activation of NOX

Upon pathogen attack, infected cells respond by local reinforcement of the cell wall by rapidly forming callose and lignin (Ton and Mauch-Mani, 2004; Hardham *et al.*, 2007). Tu (1985) reported that the rate of oxalate diffusion in leaf tissue is paralleled by the plant's susceptibility. However, an oxalate-deficient mutant was non-pathogenic to plants (Godoy *et al.*, 1990). Thus, cell wall fortifications with callose/lignin might be an effective barrier to prevent oxalate diffusion. Interestingly, the activation of NOX was necessary for thiamine-primed callose/lignin (Fig. 7A, B, and Supplementary Fig. S4), which were deposited at similar sites to O_2^- accumulation in the early infected tissues (Supplementary Fig. S3). Thiamine might induce callose/lignin deposition to prevent oxalate diffusion and subsequent mycelium penetration via NOX-mediated ROS signalling. Additionally, thiamine-induced stomatal closure was also closely correlated with NOX-derived ROS generation, which might help *Sclerotinia*-infecteded plants protect against foliar wilting (Fig. 7D, E).

Besides the *Arabidopsis* model, analysis of the effect of thiamine in the context of the crop–*Sclerotinia* interaction would give some interesting clues about whether the regulation is conserved. Excitingly, thiamine pre-treatment enhanced the resistance to *Sclerotinia* in both cultivars of susceptible *B. napus* 'Yinong 34' and the partially resistant 'Zhongshuang 9' (Supplementary Fig. S6 at *JXB* online). Moreover, thiamine-induced resistant effects including increased NOX activity, ROS generation, and callose accumulation were also found in *B. napus* upon *Sclerotinia* inoculation. Interestingly, the cultivar 'Zhongshuang 9' showed higher production of H_2O_2 compared with the susceptible cultivar 'Yinong 34' upon *Sclerotinia* infection (Supplementary Fig. S6D). Consistently, similar results were also found in the resistant *Arabidopsis* ecotypes Col-0 and Rbz-1, which generated higher levels of H_2O_2 than the susceptible ecotype Sha in

response to *Sclerotinia* (Perchepied *et al.*, 2010). These data prove that the initiation of an oxidative burst is required for enhancing plant resistance to *Sclerotinia*.

In summary, our results contribute to elucidation of the defence mechanisms induced by thiamine in plant resistance to *Sclerotinia*. We have provided evidence that interfering with the establishment of an initial reducing status is crucial in impeding the pathogenesis of *Sclerotinia*. Early in pathogenesis, thiamine could effectively alleviate the inhibitory action of *Sclerotinia* on NOX activity, which promotes ROS generation and follows a decreased Asc/DHA ratio, reflecting the cellular transition towards oxidative status in infected tissues. Of particular interest is the localization of callose/lignin in infected tissue. Since oxalate diffusion in leaf tissue is crucial for the pathogenesis of *Sclerotinia* (Tu, 1985), we presume that thiamine might induce cell wall fortifications with callose/lignin to prevent oxalate diffusion and disturb pathogen compatibility by activating NOX-mediated ROS signalling.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Thiamine does not affect the growth of *Sclerotinia in vitro*.

Supplementary Fig. S2. Thiamine and its derivatives contents in auxotroph *tz-1 Arabidopsis*.

Supplementary Fig. S3. *In situ* detection of O_2^- formation at 2 d after *Sclerotinia* inoculation.

Supplementary Fig. S4. Thiamine-induced lignin accumulation in *Sclerotinia*-infected leaves.

Supplementary Fig. S5. Effect of thiamine on H_2O_2 generation in leaves inoculated with the A2 mutant.

Supplementary Fig. S6. Effect of thiamine in cultivars of susceptible *B. napus* 'Yinong 34' and the partially resistant 'Zhongshuang 9'.

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References

Ahn IP, Kim S, Lee YH, Suh SC. 2007. Vitamin B1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in *Arabidopsis*. *Plant Physiology* **143,** 838–848.

Ahn IP, Kim S. Lee YH. 2005. Vitamin B1 functions as an activator of plant disease resistance. *Plant Physiology* **138,** 1505–1515.

Asensi-Fabado MA, Munné-Bosch S. 2010. Vitamins in plants: occurrence, biosynthesis and antioxidant function. *Trends in Plant Science* **15,** 582–592.

Azami-Sardooei Z, França SC, Vleesschauwer DD, Höfte M. 2010. Riboflavin induces resistance against *Botrytis cinerea* in bean, but not in tomato, by priming for a hydrogen peroxide-fueled resistance response. *Physiological and Molecular Plant Pathology* **75,** 23–29.

Bateman DF, Beer SV. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotiorum rolfsii*. *Phytopathology* **55,** 204–211.

Bolton MD, Thomma BPHJ, Nelson BD. 2006. Pathogen profile *Sclerotinia sclerotiorum* Lib.de Bary: biology and molecular traits of a cosmopolitan pathogen. *Molecular Plant Pathology* **7,** 1–16.

Cessna SG, Sears VE, Dickman MB, Low PS. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, supresses the oxidative burst of the host plant. *Plant Cell* **12,** 2191–2199.

Dong X, Ji R, Guo X, Foster S, *et al.* 2008. Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape. *Planta* **228,** 331–340.

Errakhi R, Meimoun P, Lehner A, Vidal G, Briand J, Corbineau F, Rona JP, Bouteau F. 2008. Anion channel activity is necessary to induce ethylene synthesis and programmed cell death in response to oxalic acid. *Journal of Experimental Botany* **59,** 3121–3129.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17,** 1866–1875.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology* **155,** 2–18.

Godoy G, Steadman JR, Dickman MB, Dam R. 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiological and Molecular Plant Pathology* **37,** 179–191.

Goyer A. 2010. Thiamine in plants: aspects of its metabolism and functions. *Phytochemistry* **71,** 1615–1624.

Guimarães RL, Stotz HU. 2004. Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiology* **136,** 3703–3711.

Guo XM, Stotz HU. 2010. ABA signaling inhibits oxalate-induced production of reactive oxygen species and protects against *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *European Journal of Plant Pathology* **128,** 7–19.

Hardham AR, Jones DA, Takemoto D. 2007. Cytoskeleton and cell wall function in penetration resistance. *Current Opinion in Plant Biology* **10,** 342–348.

Hegedus DD, Rimmer SR. 2005. *Sclerotinia sclerotiorum*: When "to be or not to be" a pathogen? *FEMS Microbiology Letters* **251,** 177–184.

Hu X, Bidney DL, Yalpani N, *et al.* 2003. Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiology* **133,** 170–181.

Huang YB, Xie XL, Yang L, Zhang J, Li GQ, Jiang DH. 2011. Susceptibility of *Sclerotinia sclerotiorum* strains different in oxalate production to infection by the mycoparasite *Coniothyrium minitans*. *World Journal of Microbiology and Biotechnology* **27,** 2799–2805.

Jabs T, Dietrich RA, Dangl JL. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273,** 1853–1856.

Jiang F, Zhang Y, Dusting GJ. 2011. NADPH Oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. *Pharmacological Reviews* **63,** 218–242.

Jiang MY, Zhang JH. 2002. Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *Journal of Experimental Botany* **53,** 2401–2410.

Jung IL, Kim IG. 2003. Thiamin protects against paraquat-induced damage: scavenging activity of reactive oxygen species. *Environmental Toxicology and Pharmacology* **15,** 19–26.

Kim KS, Min JY, Dickman MB. 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Molecular Plant–Microbe Interactions* **21,** 605–612.

Liu J, Zhou J, Xing D. 2012. Phosphatidylinositol 3-kinase plays a vital role in regulation of rice seed vigor via altering NADPH oxidase activity. *PLoS ONE* **7,** e33817.

Marino D, Dunand C, Puppo A, Pauly N. 2012. A burst of plant NADPH oxidases. *Trends in Plant Science* **17,** 9–15.

Maxwell DP, Lumsden RD. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. *Phytopathology* **60,** 1395–1398.

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F. 2007. Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *The Plant Journal* **49,** 159–172.

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH. 2003. Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *Plant Cell* **15,** 939–951.

Penfield S, Li Y, Gilday AD, Graham S, Graham IA. 2006. *Arabidopsis* ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *Plant Cell* **18,** 1887–1899.

Perchepied L, Balagué C, Riou C, Claudel-Renard C, Rivière N, Grezes-Besset B, Roby D. 2010. Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* **23,** 846–860.

Purdy LH. 1979. *Sclerotinia sclerotiorum*: history, disease and symptomatology, host range, geographic distribution and impact. *Phytopathology* **69,** 875–880.

Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A. 2003. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NADPH-oxidase activation. *Diabetes* **52,** 2795–2804.

Queval G, Noctor G. 2007. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: application to redox profiling during *Arabidopsis* rosette development. *Analytical Biochemistry* **363,** 58–69.

Rao MV, Paliyath G, Ormrod DP. 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiology* **110,** 125–136.

Rapala-Kozik M, Kowalska E, Ostrowska K. 2008. Modulation of thiamine metabolism in *Zea mays* seedlings under conditions of abiotic stress. *Journal of Experimental Botany* **59,** 4133–4143.

Rapala-Kozik M, Wolak N, Kujda M, Banas AK. 2012. The upregulation of thiamine vitamin B1 biosynthesis in *Arabidopsis thaliana* seedlings under salt and osmotic stress conditions is mediated by abscisic acid at the early stages of this stress response. *BMC Plant Biology* **12,** 2.

Sagi M, Fluhr R. 2001. Superoxide production by plant homologues of the gp^{91}phox NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. *Plant Physiology* **126,** 1281–1290.

Sun AZ, Nie SJ, Xing D. 2012. Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by lipopolysaccharides. *Plant Physiology* **160,** 1081–1096.

Thulke O, Conrath U. 1998. Salicylic acid has a dual role in the activation of defence related genes in parsley. *The Plant Journal* **14,** 35–42.

Ton J, Mauch-Mani B. 2004. β-Aminobutyric acid induced resistance against necrotrophic pathogens is based on ABA-dependent priming of callose. *The Plant Journal* **38,** 119–130.

Tu JC. 1985. Tolerance of white bean (*Phaseolus vulgaris*) to white mold (*Sclerotinia sclerotiorum*) associated with tolerance to oxalic acid. *Physiological Plant Pathology* **26,** 111–117.

Tunc-Ozdemir M, Miller G, Song L, *et al.* 2009. Thiamin confers enhanced tolerance to oxidative stress in *Arabidopsis*. *Plant Physiology* **151,** 421–432.

Vallet C, Chabbert B, Czaninski Y, Monties B. 1996. Histochemistry of lignin deposition during sclerenchyma differentiation in alfalfa stems. *Annals of Botany* **78,** 625–632.

Wang H, Liu G, Zheng Y, Wang X, Yang Q. 2004. Breeding of the *Brassica napus* cultivar Zhongshuang 9 with high-resistance to *Sclerotinia sclerotiorum* and dynamics of its important defense enzyme activity. *Scientia Agricultura Sinica* **2004,** 23–28.

Williams B, Kabbage M, Kim HJ, Britt R, Dickman MB. 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathogens* **7,** e1002107.

Yue HY, Nie SJ, Xing D. 2012. Over-expression of *Arabidopsis* Bax inhibitor-1 delays methyl jasmonate-induced leaf senescence by suppressing the activation of MAP kinase 6. *Journal of Experimental Botany* **63,** 4463–4474.