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ABA signaling inhibits oxalate-induced production of reactive oxygen species and protects against *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*

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Abstract Oxalic acid is an essential virulence factor of *Sclerotinia sclerotiorum* that elicits wilting symptoms in infected host plants. Foliar wilting in response to oxalic acid is known to be dependent on an increase in stomatal conductance. To determine whether stomatal regulation controls susceptibility to *S. sclerotiorum*, abscisic acid-insensitive and open stomata mutants of *Arabidopsis thaliana* were analyzed. Whereas abscisic acid-insensitive mutants were hypersusceptible to *S. sclerotiorum*, open stomata mutants were as susceptible as wild type. It was concluded that stomatal regulation does not control susceptibility to *S. sclerotiorum* because open stomata mutants are known to only impair guard cells whereas abscisic acid-insensitive mutants also affect other cell types. Guard cell-independent processes also control sensitivity to oxalic acid because oxalic acid was more toxic to abscisic acid-insensitive mutants than to open stomata mutants. To explore a possible mechanism of toxicity, production of reactive oxygen species was measured in plant cells after exposure to oxalic acid. Oxalic acid was found to elicit reactive oxygen species production independently of abscisic acid. Nevertheless, cancellation of reactive oxygen species elicitation after co-stimulation of wild-type guard cells with oxalic acid and abscisic acid provided evidence for antagonistic interaction between both molecules.

Keywords Disease · Guard cell · H_2O_2 · Hypersusceptible · Stomatal aperture

Abbreviations

ABA	abscisic acid
DAB	3,3′-diaminobenzidine
H_2DCF-DA	2′,7′-dichlorofluorescein diacetate
PCD	programmed cell death
ROS	reactive oxygen species

Introduction

Sclerotinia sclerotiorum is a necrotrophic ascomycete that attacks more than 400 plant species, including *Arabidopsis thaliana* (Boland and Hall 1994). This fungus produces millimolar quantities of oxalic acid in infected host tissues (Purdy 1979; Guimaraes and Stotz 2004). The virulence function of oxalic acid was

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demonstrated by using oxalate-deficient mutants of *S. sclerotiorum*, which are essentially nonpathogenic (Godoy et al. 1990). Genetic reversions that restore the capacity for oxalate production also restore virulence, but the genes that are responsible for oxalate deficiency remain unknown. By comparison, oxalate production of the closely related ascomycete *Botrytis cinerea* is solely dependent on oxaloacetate hydrolase (Han et al. 2007).

Pathogen-produced oxalic acid has multiple modes of action that affect the host. Oxalic acid lowers the pH of infected host tissues (de Bary 1886; Bateman and Beer 1965). This acidification assists in destruction of host cell walls because polygalacturonase and other hydrolytic enzymes have acidic pH-optima. Oxalate is also a strong chelator and able to remove Ca^{2+} ionically bound to pectin. This action further promotes polygalacturonase activity, which is dependent on removal of Ca^{2+} from its substrate (Bateman and Beer 1965).

Oxalate suppresses the elicitor-induced oxidative burst of host plants (Cessna et al. 2000). Oxalate-deficient, but not wild-type, *S. sclerotiorum* elicits superoxide formation in tobacco leaves. In *Arabidopsis*, oxalate-deficient *S. sclerotiorum* elicits hydrogen peroxide accumulation in infected leaves, whereas superoxide accumulates in the wild-type fungus during infection (Guo and Stotz 2007). Differences in observed superoxide accumulation are possibly due to the use of distinct oxalate-deficient strains (Guo and Stotz 2007). Addition of millimolar concentrations of oxalate suppresses oligogalacturonide-induced H_2O_2 production in soybean cell cultures (Cessna et al. 2000). However, oxalate does not influence the transient increase in cytoplasmic calcium after elicitor treatment (Cessna et al. 2000). Overexpression of oxalate oxidase enhances generation of H_2O_2, one of the two catabolic products of oxalate oxidase activity besides CO_2, and activates host defense responses (Hu et al. 2003). Recently, culture fluids from wild-type *S. sclerotiorum* but not from an oxalate-deficient strain were shown to stimulate DNA laddering, a hallmark of programmed cell death (Kim et al. 2008). Diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes including NADPH oxidase (Riganti et al. 2004), was shown to interfere with oxalate-induced DNA laddering and cell death, thus implicating reactive oxygen species (ROS) as signalling intermediates. ROS therefore appear to be an important determinant of interactions between *S. sclerotiorum* and its hosts.

In addition to the above mentioned effects, oxalic acid elicits wilting symptoms in the host via opening of stomatal pores (Guimaraes and Stotz 2004). Regulation of this process is not well understood but it is associated with K^+ accumulation and with starch degradation in guard cells (Guimaraes and Stotz 2004).

In the present study, the possible contribution of guard cells to oxalate sensitivity and pathogen susceptibility was tested using abscisic acid (ABA)-insensitive (*abi1* and *abi2*) and open stomata (*ost1* and *ost2*) mutants (Table 1). Increased stomatal conductance is a characteristic of all four mutants, but both classes of mutants differ in that *abi* mutants alter ABA signalling throughout the plant whereas *ost* mutants impair guard cell regulation without altering ABA signalling in other cell types. Specifically, *ost1* is a loss-of-function mutation in a guard cell-specific Snf1-related protein kinase involved in ABA signaling (Merlot et al. 2002; Belin et al. 2006) and *ost2* is a dominant mutation constitutively activating the major plasma membrane H^+-ATPase (Merlot et al. 2007). In contrast, *abi1* and *abi2* are gain-of-function mutations in homologous protein phosphatases 2C that alter ABA signalling in the entire plant (Leung et al. 1997; Koornneef et al. 1998). This mutant comparison provided evidence for facilitation by ABA of guard cell-independent defences against *S. sclerotiorum*.

Guard cells were still evaluated to gain further insights into oxalate responses and antagonism between oxalic acid and ABA. In response to *S. sclerotiorum* infection, both *abi* mutants were more prone to wilting than wild-type plants although oxalate-induced stomatal opening was independent of ABA. ROS production was studied because it is an important mediator of ABA and oxalate responses (Murata et al. 2001; Kim et al. 2008). Analysis of *rbohD* and *rbohF* mutants with defects in distinct NADPH oxidase isoforms (Table 1) and of *abi* mutants provided evidence for separate but interconnected activation of ROS production by ABA and oxalic acid.

Materials and methods

Biological material and growth conditions

The *abi2-1*, *rbohD*, *rbohF*, and *rbohD/F* mutants were obtained from Dr. Julian I. Schroeder (Univer-

Table 1 Characterized mutants used in this study. Origins of mutants are listed in the "Materials and methods" section

Mutant	Allele	Mutated gene	AGI code	Phenotypes	References
abi1	*abi1-1*	Protein phosphatase 2C *ABI1*	At4g26080	ABA resistant; reduced seed dormancy; increased leaf transpiration rate	Leung et al. 1994
abi2	*abi2-1*	Protein phosphatase 2C *ABI2*	At5g57050	ABA resistant; reduced seed dormancy; increased leaf transpiration rate	Leung et al. 1997
ost1	*ost1-2*	SNF1-related protein kinase 2E	At4g33950	Impaired in stomatal closure; guard cells non-responsive to ABA	Mustilli et al. 2002
ost2	*ost2-1D*	Plasma membrane proton ATPase 1	At2g18960	Impaired stomatal response to ABA; higher proton extrusion	Merlot et al. 2007
rbohD		NADPH oxidase isoform D	At5g47910	Slightly smaller than wild type; diminuition of ion leakage; impaired ABA signalling	Torres et al. 2002; Kwak et al. 2003
rbohF		NADPH oxidase isoform F	At1g64060	Slightly smaller than wild type; diminuition of ion leakage; impaired ABA signalling	Torres et al. 2002; Kwak et al. 2003

sity of California, San Diego, USA). The *ost1-2* and *ost2-1* mutants were obtained from Dr. Sylvain Merlot (Institut des Sciences du Végétal, Gif-sur-Yvette, France). The *abi1-1* mutant was obtained from the *Arabidopsis* Biological Resource Center. A randomized complete block design was used to grow plants in 72-cell flats. *Arabidopsis thaliana* seeds were sown on wet Sunshine SB40 soil mix (Sun Gro Horticulture, Bellevue, WA, USA) and stratified at 4°C in the dark for 2 days. An MB-60B growth chamber (Percival Scientific, Perry, IA, USA) was used for plant cultivation. A light intensity of 100 μmol m^{-2} s^{-1} and a photoperiod of 11 h light and 13 h darkness were used at 22°C. Fungal strains have previously been obtained from Martin Dickman (Guimaraes and Stotz 2004).

Stomatal aperture bioassays

Rosette leaves from 4- to 6-week-old *Arabidopsis* plants were detached and immersed in incubation buffer containing 10 mM KCl, and 10 mM MES-Tris, pH 6.15 with NaOH in the presence or absence of 10 mM oxalic acid. After 1-hour of incubation leaves were minced with a Waring Blender, and filtered through Miracloth. Epidermal fragments were placed on a microscope slide with a cover slip. The cover slip was sealed to the slide, and stomatal apertures were measured.

Oxalate sensitivity assay

Assays were performed according to published procedures (Guo and Stotz 2007) using Murashige & Skoog (MS) medium with or without 3.5 mM oxalic acid at pH 5.5. Seeds were surface sterilized and eight seeds of each genotype were placed side-by-side on each plate. Petri plates were sealed with surgical tape. The experiments were carried out at the same time with a photoperiod of 16 h light and 8 h darkness at 22°C. Fresh weights were determined by weighing individual seedlings.

Plant inoculation

Wild-type *S. sclerotiorum* isolate 1980 was cultured on minimal medium (1 g NaOH, 3 g DL-malic acid, 2 g NH_4NO_3, 0.1 g $MgSO_4 \times 7H_2O$, 39 g Bacto-agar l^{-1}) to reduce the aggressiveness of the fungus (Cruickshank 1983) prior to inoculation. This precaution was previously shown to be necessary to discern genetic differences in susceptibility of *Arabidopsis* to *S. sclerotiorum* (Guo and Stotz 2007). An agar plug (2 mm in diameter) containing the advancing edge of *S. sclerotiorum* mycelia was removed to inoculate *Arabidopsis* leaves. Two rosette leaves of 4 to 6-week-old *Arabidopsis* plants were inoculated for phenotypic assays. After the inoculation a transparent plastic dome were used to cover the tray and to keep humidity high. Infected plants were kept at 20°C under a 12-h light photoperiod with a light intensity of 34 mmol m^{-2} s^{-1} using fluorescent white lights (Guimaraes and Stotz 2004). Lengths and widths of lesions were measured with a caliper before disease symptoms expanded beyond the inoculated leaves. Lesion diameters were determined by averaging lesion lengths and width.

Detection of ROS

Rosette leaves from 4- to 6-week-old plants were detached, minced with Waring Blender, and filtered through Miracloth. Alternatively, the abaxial epidermis was peeled from the rest of the leaf under water. Tissues were immersed in the incubation medium containing 5 mM KCl, and 10 mM MES-Tris, pH 6.15 (Kwak et al. 2003). After 2 h of incubation, peels were removed, transferred to incubation medium containing 50 μM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) (Lee et al. 1999), and exposed for 10 min at room temperature in the darkness. Excess dye was washed out with incubation medium. Dye-loaded tissues were treated with 0.5% (v/v) ethanol or water as a control, 50 μM ABA, 5 mM oxalic acid, or 50 μM ABA + 5 mM oxalic acid for 20 min. Epidermal fragments were subsequently placed on a microscope slide with a cover slip. The cover slip was sealed to the slide, and guard cells were photographed using a Zeiss Axiovert 100 epifluorescence microscope (Carl Zeiss MicroImaging, Inc). Cellular images were analyzed using the ImageJ program (National Institutes of Health, USA). The same threshold was used across different treatments per genotype.

Staining with 3,3′-diaminobenzidine (DAB) was as described (Thordal-Christensen et al. 1997; Guo and Stotz 2007). The experimental conditions for incubation of leaf discs with potassium oxalate and ABA followed published procedures (Kim et al. 2008). Potassium oxalate solutions were adjusted to pH 5.5. Images were processed using background subtraction, selecting light background and a rolling ball radius of 10 pixels. Automatic thresholds were generated using the Process Binary Make Binary routine of ImageJ. Particles were analyzed and Area was used for quantification. All values were normalized to the water control treatment of wild-type leaf discs, which was set to 100%.

Statistical analysis

The SAS program package (Cary, NC, USA) was used for statistical analysis. Specifically, PROC general linear model (GLM) was implemented to analyze disease, oxalate sensitivity, ROS production, and stomatal aperture data. LSMEANS and PDIFF were used to separate significant differences among means that were explained by genotype, treatment, or genotype-by-treatment interaction. Levene's test was used to ascertain homogeneity of variances. Alternatively, *t*-tests were used to determine statistical significance in combination with *F*-tests to decide between equal or unequal variances.

Results

Guard cell-independent ABA signaling opposes susceptibility to *S. sclerotiorum*

It is known that oxalic acid secreted by *S. sclerotiorum* increases stomatal conductance and elicits wilting symptoms. However, the possible role of guard cells in defence against *S. sclerotiorum* has not been tested. Therefore, *Arabidopsis* mutants were used to determine a possible contribution of guard cells to the interaction with this pathogen. Specifically, *ost1* and *ost2* mutants that interfere with stomatal closure without altering ABA responses in cells other than guard cells were tested (Table 1). These mutants were compared to *abi1* and *abi2* mutants, which pleiotropically inhibit ABA responses throughout the plant.

Only *abi1* and *abi2* mutants were more susceptible to *S. sclerotiorum* than wild-type plants as judged by the size of expanding lesions. Differences in the size of local lesions became significant 2-day post-inoculation and continued to express themselves on day 3 post-inoculation (Fig. 1; Supplementary Figure S1). These lesions rapidly expanded beyond the infected leaves of *abi1* and *abi2* mutants on day 4 post-inoculation, but wild-type plants were refractory to infection. In contrast, lesion expansion did not differ between *ost1* and *ost2* mutants and wild-type plants.

ABA-dependent and guard cell-independent oxalate tolerance

ABA-insensitive and open stomata mutants were also used to determine the relative contributions of stomatal regulation and ABA signalling to plant protection against oxalate toxicity.

Oxalic acid caused a reduction of growth and fresh weight in wild-type seedlings, but *abi1* and *abi2* mutants were significantly more sensitive to oxalic acid than wild-type plants (Fig. 2a). In the absence of

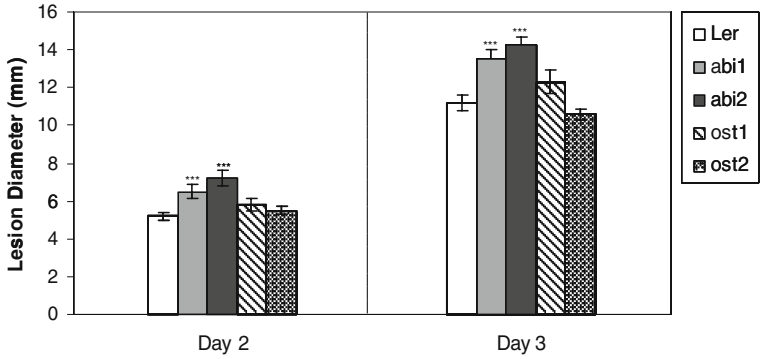

Fig. 1 Comparison of susceptibility to *S. sclerotiorum* isolate 1980 among wild-type and mutant *Arabidopsis*. Diameters of expanding lesions were measured 2 and 3 days post-inoculation. Means and standard errors are shown ($n>5$). Asterisks indicate significant differences ($P<0.05$) by performing Dunnett's test to compare mutant to wild type. The experiment was performed twice with similar results

oxalic acid, root growth of both mutants was enhanced relative to wild type (Fig. 2a), perhaps a reflection of growth limitation in wild-type seedlings under conditions that restrict gas exchange (Israelsson et al. 2006; Nilson and Assmann 2007). Relative to both *abi* mutants, sensitivity of *ost1* and *ost2* mutants to oxalic acid was low and only became apparent after 18 days of growth (Fig. 2b).

Regulation of wilting symptom expression after infection with *S. sclerotiorum*

To study the regulation of wilting symptom expression, responses of *abi1* and *abi2* mutants to inoculation with *S. sclerotiorum* were examined. Both *abi2* (data not shown) and *abi1* mutants, challenged with wild-type *S. sclerotiorum*, were more prone to wilting than inoculated wild-type plants (Fig. 3). This response was dependent on fungal oxalic acid production because the *abi1* mutant inoculated with oxalate-deficient *S. sclerotiorum* did not display wilting symptoms (Fig. 3).

To determine the effect of oxalic acid on guard cells, stomatal apertures were measured in *Arabidopsis* leaves. A significant increase in stomatal apertures was observed after incubation of wild type, *abi1-1* or *abi2-1* mutant plants with oxalic acid (Fig. 4). The increase in stomatal aperture, which was observed in epidermal strips from uninfected leaves of wild-type and *abi1-1* mutant plants inoculated with *S. sclerotiorum*, was quantitatively similar (Supplementary Figure S2). Together, these data clearly show that the oxalate response is intact in both *abi* mutants.

Antagonism between oxalate and ABA-induced ROS production

ROS are signalling intermediates in plant responses to ABA and oxalic acid. ROS production in response to these different stimuli may therefore be a point of signalling crosstalk. We therefore measured ABA and oxalate-elicited ROS accumulation in guard cells.

As reported previously (Lee et al. 1999; Murata et al. 2001), ABA elevated ROS production in guard cells of wild type and *abi2-1* mutant plants but not in guard cells of the *abi1-1* mutant (Fig. 5). In contrast, treatment with oxalic acid increased ROS production in guard cells of wild-type, *abi1* and *abi2* mutant plants (Fig. 5), demonstrating that elicitation of ROS by oxalic acid is independent of ABA signalling. Only guard cells of mutant plants accumulated more ROS after co-treatment with ABA and oxalic acid while guard cells of wild-type plants did not respond to this co-treatment (Fig. 5), indicating that ABA signalling interferes with oxalate-induced ROS production and vice versa.

Oxalate-related ROS production in *rbohD* and *rbohF* mutants

The NADPH oxidase isoforms RBOHD and RBOHF contribute to ROS-mediated signalling leading to stomatal closure in response to ABA (Table 1). In addition, pharmacological data exists on DPI-inhibited ROS production in leaf discs after elicitation with oxalic acid, but the contribution of specific NADPH oxidase isoforms has not been tested. A total of 10

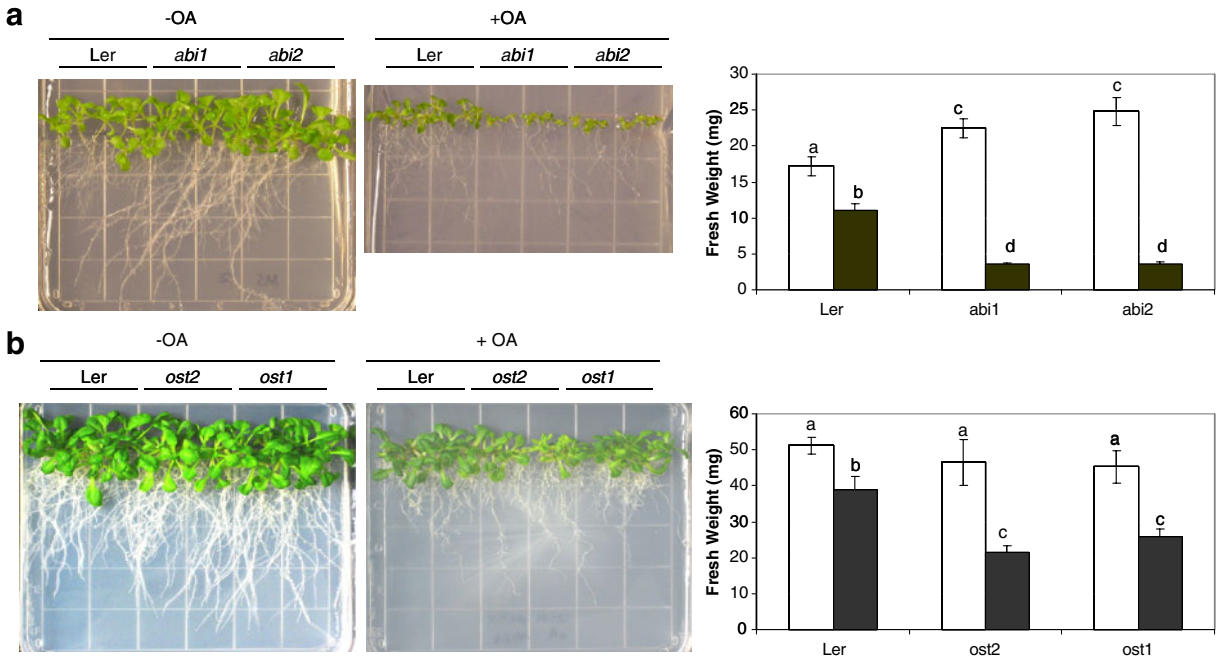

Fig. 2 Hypersensitivity of different mutants to oxalic acid. Images of seedlings grown on MS medium in the absence or presence of 3.5 mM oxalic acid (OA), pH 5.5 and fresh weights are reported. The square size is 1.35 cm. Means and standard errors are shown (n>20). Letters indicate significant differences (P<0.01). (**a**) Comparison among wild type, *abi1-1* and *abi2-1* seedlings grown for 2 weeks. (**b**) Comparison among wild type, *ost1-2* and *ost2-1* seedlings grown for 18 days. The experiment was performed twice with similar results

NADPH oxidase catalytic subunit genes (*RBOHA* through *RBOHJ*) exist in the *Arabidopsis* genome (Kwak et al. 2003). To determine the potential role of two major NADPH oxidase isoforms in oxalate signalling, stimulus-dependent ROS production was studied in *rbohD* and *rbohF* mutants. The cytochemical dye DAB was used to measure oxalate and ABA-induced ROS production in leaf discs.

DAB staining of leaf discs was previously used to demonstrate oxalate-induced production of H_2O_2 (Kim et al. 2008). Our data confirmed that 10 mM oxalate was sufficient to increase of DAB staining in leaf discs relative to control and ABA treatments (Fig. 6a and b). With the exception of 20 mM oxalate, DAB staining in response to all other treatments was reduced in the *rbohD* mutant relative to wild-type and *rbohF* mutant leaves. The least amount of DAB staining was observed in leaf discs of the *rbohD/rbohF* double mutant. Nevertheless, the significant amount of DAB staining observed after oxalate treatment of the *rbohD/rbohF* double mutant suggests that enzymes other than these two NADPH oxidases participate in oxalate-elicited ROS production.

Microbial oxalate production blocks hydrogen peroxide accumulation in *Arabidopsis* leaves after inoculation with wild-type *S. sclerotiorum* (Guo and Stotz 2007). To determine whether pathogen-induced H_2O_2 production depends on the activity of NADPH oxidase, leaves of *rbohD* and *rbohF* single and double mutants were inoculated with an oxalate-deficient mutant of *S. sclerotiorum*, subjected to DAB staining, and compared to wild-type leaves treated the same way.

DAB staining increased in leaves of wild-type and *rbohF* mutant plants challenged with oxalate-deficient *S. sclerotiorum*, but not in challenged leaves of *rbohD* and *rbohD/rbohF* mutant plants (Fig. 7). Despite this difference in H_2O_2 production, susceptibility to *S. sclerotiorum* as judged by measurements of lesion diameters in inoculated leaves was equal among all of the tested *rboh* mutants and wild-type plants irrespective of whether a wild-type or an oxalate-deficient strain was used (data not shown). These results show that pathogen-induced ROS production depends on the activity of *RBOHD* but that this gene is not critical for the interaction with *S. sclerotiorum*.

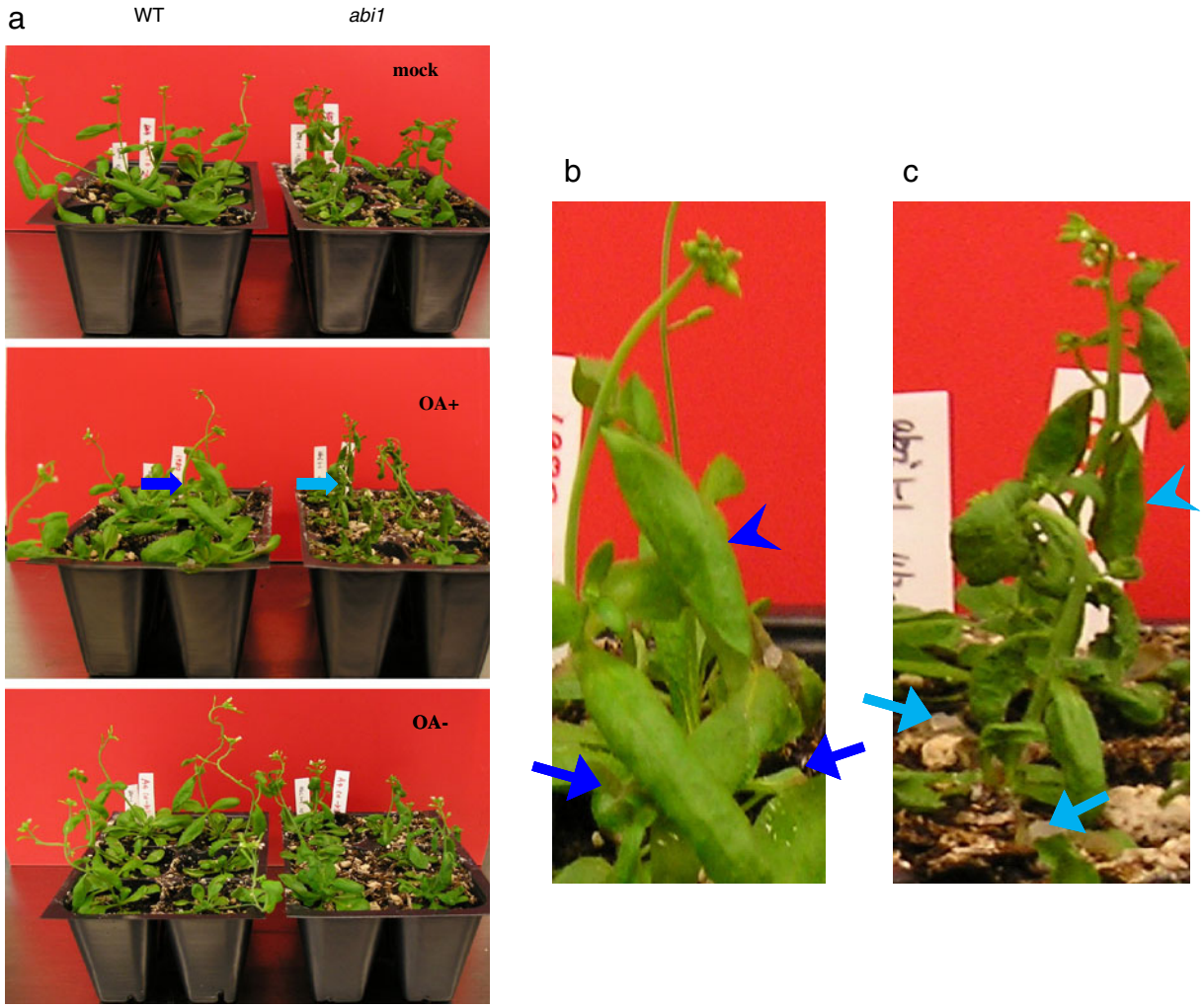

Fig. 3 Wilting symptoms 2 days post-inoculation with *S. sclerotiorum*. Wild-type and *abi1* mutant plants were mock-inoculated or treated with either wild-type (OA+) or oxalate-deficient *S. sclerotiorum* (OA−). Light blue arrows point towards drooping cauline leaves, a sign of foliar wilting, in an *abi1* mutant plant that is infected with the OA+ pathogen. Note that rosette leaves were inoculated, yet cauline leaves show signs of wilting. (**a**) Mock, wild-type (OA+) or oxalate-deficient (OA−) *S. sclerotiorum* inoculation comparison. Arrows point towards plants to visualize enlargement in b and c. (**b**) Enlargement of one wild-type plant inoculated with wild-type (OA+) *S. sclerotiorum*. Dark blue arrows indicated inoculated wild type leaves. Dark blue arrowhead pointed towards an un-inoculated leaf to indicate an un-wilted leaf. Compare with *abi1* mutant plant. (**c**) Enlargement of *abi1* mutant plant inoculated with wild-type (OA+) *S. sclerotiorum*. Light blue arrows indicate inoculated *abi1* mutant leaves. Light blue arrowhead pointed towards one drooping cauline leaf

Discussion

Genetic interactions between *S. sclerotiorum* and its host plants are ill defined, although some host defence and susceptibility components have been identified (Bessire et al. 2007; Guo and Stotz 2007; Wang et al. 2009). Virulence factors of *S. sclerotiorum* are also incompletely understood (Hegedus and Rimmer 2005), but oxalic acid has traditionally been known to play a key role in pathogenesis (de Bary 1886; Godoy et al. 1990). Effects of oxalic acid on plant cells are multifactorial. Oxalate has long been known to assist in degradation of pectin by acidifying host cell walls and by chelating divalent cations like calcium (Bateman and Beer 1965). Oxalate also inhibits elicitor-induced ROS production (Cessna et al.

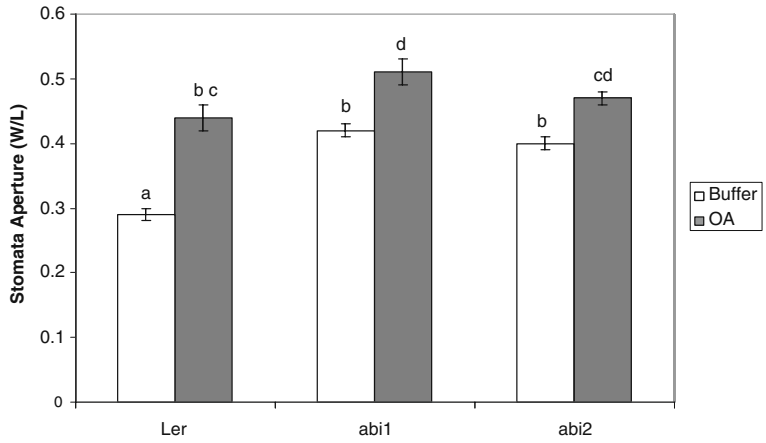

Fig. 4 Comparison of stomatal apertures (width/length) among wild type and *abi* mutants after treatment with buffer or with 10 mM oxalic acid (OA). Means and standard errors are shown ($n>50$). Letters indicate significant differences ($P<0.01$). The experiment was performed twice with similar results

2000; Guo and Stotz 2007) and triggers programmed cell death (PCD) (Kim et al. 2008) via its own ROS production.

In addition, oxalic acid triggers wilting symptoms (Noyes and Hancock 1981), but the possible role of guard cells in facilitating the interaction with *S. sclerotiorum* has not been tested. Open stomata (*ost1* and *ost2*) and ABA-insensitive (*abi1* and *abi2*) mutants make it possible to discriminate guard cell-specific effects from more general dysfunction of ABA signaling on interactions with *S. sclerotiorum*. Only the ABA-insensitive mutants were altered in susceptibility to *S. sclerotiorum* (Fig. 1), which implies that ABA signaling in cells other than guard cells protects the host from pathogen infection. The *abi1* mutant is also hypersusceptible to oxalate-deficient *S. sclerotiorum* (Guimaraes and Stotz 2004) but the *abi2* mutant is not. Thus, oxalic acid appears to enhance virulence in the *abi2* mutant, whereas elevated susceptibility of the *abi1* mutant is independent of oxalic acid production. It is possible that the *abi1* mutation has a broader effect because it acts further upstream in the pathway than the *abi2* mutation. Alternatively, the difference in susceptibil-

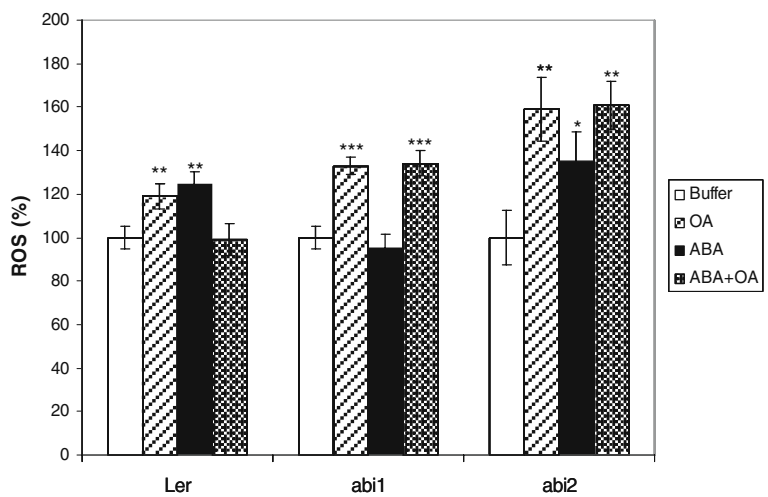

Fig. 5 ROS production in guard cells after treatment with different stimuli. Epidermis of wild type (L*er*) ($n>60$), *abi1* ($n>50$), or *abi2* ($n>20$) mutant plants was exposed to 50 μM ABA and/or 5 mM oxalic acid (OA). EtOH (0.5%) was used as a control treatment. ROS production was measured by image analysis after treatment with the fluorescent dye H_2DCF-DA. Means and standard errors are shown. ROS production after treatment with control buffer was arbitrarily set to 100% and all other treatments were expressed relative to it. *T*-tests indicate significant differences from buffer controls at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). The experiment was performed three times with similar results

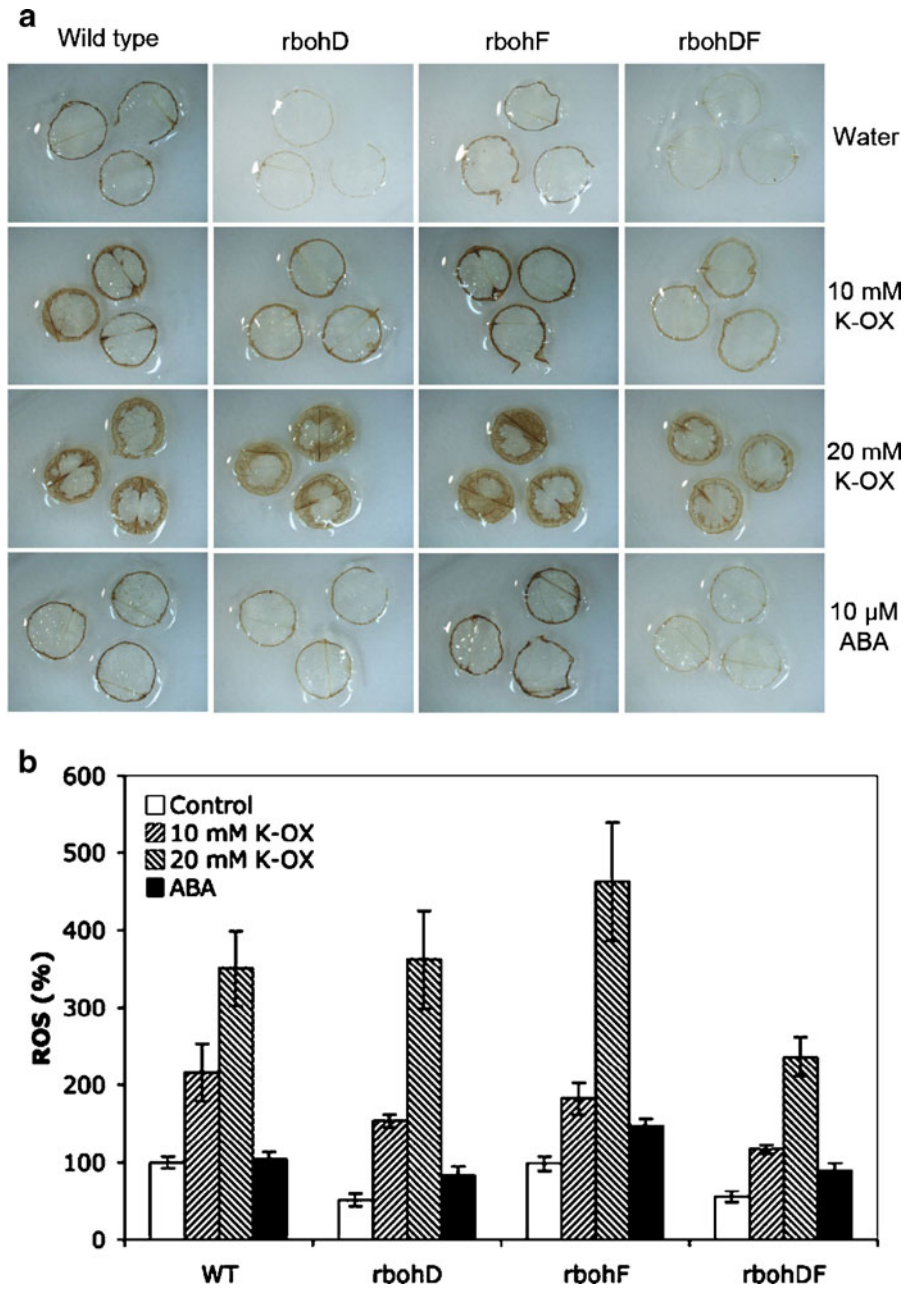

Fig. 6 Oxalate-induced DAB staining in wild type and in *rboh* mutants. (**a**) Leaf discs were stained 24 h after treatment with water, ABA or two different concentrations of potassium oxalate (K-OX). (**b**) Analysis of background-subtracted images shown in (a). DAB staining of wild-type leaves in response to water treatment was arbitrarily set to 100% and the relative intensity of all other treatment-genotype combination was expressed. Means and standard errors are shown. The experiment was performed twice with similar results

ity may be ROS-related. The *abi1* mutant is expected to inhibit ROS production, but the *abi2* mutant should not have such an effect because it acts downstream of ABA-induced ROS production. No effort was made to discriminate between these two possibilities. Differences in pathogen susceptibility between *abi1* and *abi2* mutants are not unique to *S. sclerotiorum* because the *abi1* mutant is also susceptible to the hemibiotrophic fungus *Leptosphaeria maculans*, whereas the *abi2* mutant is as resistant as wild type (Kaliff et al. 2007). Mohr and Cahill (2003) examined the influence of ABA on interactions of Arabidopsis with *Pseudomonas syingae* pv. tomato and *Peronospora parasitica*. They found out that an ABA-deficient mutant of *Arabidopsis*, *aba1-1*, displayed reduced susceptibility to virulent isolates of *P. parasitica* but *abi1-1* did not alter in susceptibility to either *P. syringae* pv. *tomato* or *P. parasitica* when bacteria

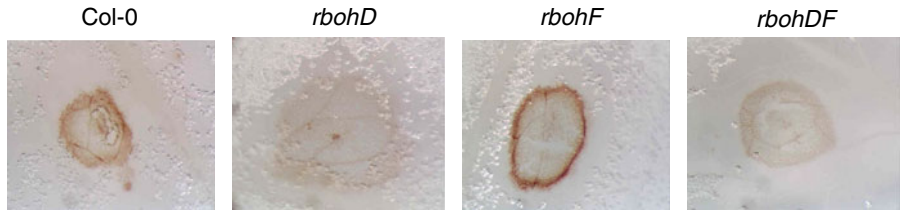

Fig. 7 Dependence of DAB staining in response to infection by oxalate-deficient *S. sclerotiorum* on *rbohD*. Leaves were inoculated with the pathogen and stained with 3,3′-diaminobenzidine. Images were taken 10 days post-inoculation. The experiment was performed twice with similar results

were vacuum infiltrated into leaves. Their results demonstrated that the concentration of endogenous ABA at the time of pathogen challenge is important for the development of susceptibility in *Arabidopsis*. However, surface inoculation with a coronatine-deficient mutant of *P. syringae* pv. *tomato* increased the susceptibility of *ost1* and ABA-deficient *aba3* mutants relative to wild type, clearly demonstrating a role for ABA in plant protection under more natural conditions (Melotto et al. 2006).

Increased susceptibility to *S. sclerotiorum* of both *abi1* and *abi2* mutants correlated with their increased oxalate sensitivity relative to wild type (Fig. 2). In contrast, both *ost1* and *ost2* mutants were not particularly sensitive to oxalic acid and as susceptible to *S. sclerotiorum* as wild type. Increased oxalate sensitivity of the *abi1* and *abi2* mutants is apparently independent of guard cell function. Although both ABA-insensitive mutants displayed oxalate-dependent wilting symptoms in response to infection by *S. sclerotiorum* (Fig. 3), the quantitative increase in oxalate-related stomatal opening was similar in mutant and wild-type plants (Fig. 4). Stomatal opening in response to oxalic acid is therefore primarily dependent on activation of the plasma membrane H^+-ATPase because vanadate, an inhibitor of P-type ATPases, inhibits oxalate-dependent stomatal opening (Supplementary Figure S3).

ROS production is critical for stomatal closure (Murata et al. 2001), whereas ROS are thought to mediate cell death in response to oxalic acid (Kim et al. 2008). Oxalic acid and ABA independently elicit ROS production (Fig. 5). Oxalic acid triggered ROS production in guard cells of wild type and *abi1* and *abi2* mutant plants. Nevertheless, inhibition of oxalate-induced ROS production by ABA was absent in both *abi1* and *abi2* mutants. This ROS-related antagonism between oxalic acid and ABA is reminiscent of the inhibition of elicitor-induced ROS by oxalic acid (Cessna et al. 2000). Oxalic acid elicits ROS production *in vitro* (Kim et al. 2008) but suppresses ROS elicitation *in vivo* (Cessna et al. 2000). The findings of this study suggest that, during infection, pathways are activated that antagonize elicitation of ROS production by oxalic acid. Our data imply that one of those pathways is ABA-related because ABA signalling was shown to inhibit oxalate-induced ROS production in guard cells.

DAB staining after treatment of leaf discs with 20 mM oxalate was much stronger than after treatment with 10 mM oxalate (Fig. 6). In particular, cells in the interior of leaf discs responded to 20 mM oxalate but not to the lower concentration. These distinct responses maybe caused by different modes of action. A concentration of 10 mM oxalate still triggers physiological responses like stomatal opening (Guimaraes and Stotz 2004). Higher concentrations of oxalate preferentially trigger PCD (Kim et al. 2008) possibly at the expense of physiological responses.

ROS production in response to 10 mM oxalate and to infection by oxalate-deficient *S. sclerotiorum* was reduced in the *rbohD* but not in the *rbohF* mutant (Figs. 6 and 7). Similarly, the *rbohD* mutation eliminated the majority of ROS elicited by the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000(*avrRpm1*), by the oomycete parasite *Peronospora parasitica* (Torres et al. 2002), and by *B. cinerea* (Torres et al. 2005). *RBOHD* is therefore an important regulator of pathogen-related ROS production. However, no change in lesion development was observed after inoculation with wild-type or oxalate-deficient *S. sclerotiorum*. Whereas the wild-type pathogen spreads systemically, lesions are restricted to leaves challenged with oxalate-deficient *S. sclerotiorum* (Guimarães et al. 2004; Guo and Stotz 2007). Correspondingly, although oligogalacturonides do not stimulate H_2O_2 production in the *rbohD* mutant, the susceptibility of this mutant to *B. cinerea* is not

altered by treatment with the elicitor (Galletti et al. 2008). The authors concluded that defence responses independent of the oxidative burst are effective against this necrotrophic pathogen.

Oxalate-induced ROS was still detected in the *rbohD/rbohF* double mutant. These data suggest that oxalate-induced ROS production is not merely dependent on *RBOHD* and *RBOHF* function. Eight additional *RBOH* genes exist in the Arabidopsis genome, but alternative sources of ROS production cannot be excluded. With respect to the latter possibility, it may be noteworthy that mitochondria of renal cells respond to oxalate stress with an increase in ROS production (Aihara et al. 2003; Jonassen et al. 2003).

Conclusion

Evidence was obtained for a role of ABA signalling in defence against *S. sclerotiorum*. The role of ABA in plant responses to pathogen assault depends on the stage of infection and the particular host-pathogen interaction under investigation (Lopez et al. 2008).

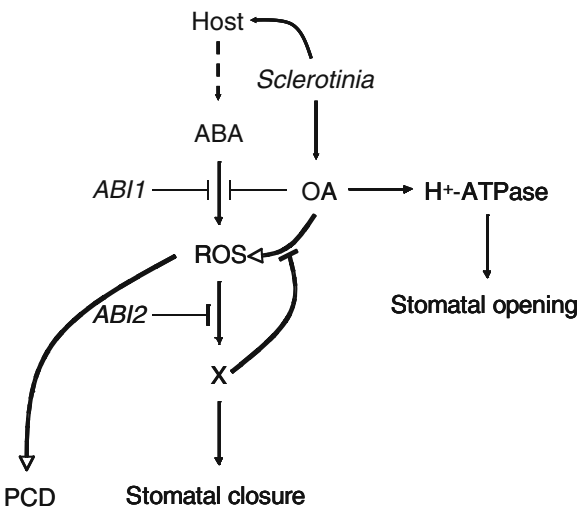

Fig. 8 Proposed model of oxalate action. Our data suggest mutually antagonistic interactions between ABA and oxalic acid (OA). Reactive oxygen species are triggered by both ABA and OA, which affect different outcomes. Oxalate-induced ROS production is proposed to trigger programmed cell death (PCD) (Kim et al. 2008). ABA-induced ROS activates stomatal closure (Pei et al. 2000). OA is also proposed to mediate stomatal opening via activation of the plasma membrane H^+-ATPase

ABA does not activate defence against *B. cinerea* or *Fusarium oxysporum* in *Arabidopsis* (Anderson et al. 2004; AbuQamar et al. 2006). In contrast, ABA-deficient mutants are more susceptible to *Pseudomonas syringae* infection because ABA plays an important role in the activation of stomatal closure, which represents a barrier against bacterial invasion (Melotto et al. 2006). ABA-dependent priming of callose biosynthesis enhances resistance to two necrotrophic pathogens (Ton and Mauch-Mani 2004). Similarly to host defence against *S. sclerotiorum* (Guimaraes and Stotz 2004; Guo and Stotz 2007), ABA, jasmonate, salicylic acid, and ethylene signalling pathways are required for plant defence against the oomycete pathogen *Pythium irregulare* (Adie et al. 2007).

Hypersensitivity of ABA-insensitive mutants to oxalic acid and inhibition of ROS production in guard cells co-treated with ABA and oxalic acid support a model of antagonism between ABA signalling and oxalate action (Fig. 8). In support of previous reports, it is proposed that ROS production in response to ABA and oxalic acid have different fates. Oxalate-induced ROS triggers PCD (Kim et al. 2008), whereas ABA-induced ROS accumulation triggers stomatal closure (Pei et al. 2000). The negative effect of oxalic acid on ABA-induced ROS may explain the interference of oxalic acid with ABA-induced stomatal closure *in vitro* (Guimaraes and Stotz 2004). Conversely, a signalling intermediate downstream of *ABI1* and *ABI2*, perhaps Ca^{2+}, inhibits oxalate-induced ROS production. Support of this idea comes from experiments with transgenic seedlings expressing aequorin that no longer respond to ABA with an increase in calcium when grown in the presence of oxalic acid (Meek and Stotz, unpublished data). Another cellular target of oxalic acid is the P-type H^+-ATPase, but it remains to be determined whether this enzyme is activated directly or indirectly by oxalic acid as a consequence of cytoplasmic acidification.

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