Requirement of ABA signalling-mediated stomatal closure for resistance of wild tobacco to Alternaria alternata

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Abscisic acid (ABA) is important for mediating abiotic stress responses and also in plant immunity. Its involvement in the resistance of wild tobacco \textit{Nicotiana attenuata} to the pathogenic fungus \textit{Alternaria alternata}, tobacco pathotype, was investigated. After infection, many fungal hyphae were observed to enter leaf tissue through stomata within 24 h, and necrotic lesions were formed after 3–6 days. Importantly, source–sink transition leaves (leaf 0) were more resistant than all fully expanded ones, and this was correlated with more ABA and ABA-related gene transcripts and lower stomatal conductance in leaf 0 after infection. When supplied with ABA or incubated in the dark for 1 day, fully expanded leaves, which accumulated less ABA and showed higher stomatal conductance after infection in comparison to leaf 0, showed increased resistance. Mitogen-activated protein kinase 4-silenced plants, in which ABA-induced stomatal closure responses and resistance to \textit{A. alternata} were highly impaired, did not show stomatal conductance change after infection, and leaf 0 and fully expanded leaves all developed bigger lesions than in wildtype plants. Thus the ABA signalling pathway mediated by NaMPK4 is required for the resistance of \textit{N. attenuata} to \textit{A. alternata}, at least in part through the stomatal closure responses.

\textit{Keywords:} ABA, \textit{Alternaria alternata}, mitogen-activated protein kinase 4 (NaMPK4), \textit{Nicotiana attenuata}, stomata

\textbf{Introduction}

Plants have evolved sophisticated mechanisms to sense and defend against microbial pathogen attacks. Three phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene, are the major regulators for defence responses; each has been shown to assist or antagonize the others to fine-tune the specific response to individual pathogens (Glazebrook, 2005). It is generally accepted that SA plays a major role in activation of defences against biotrophic pathogens, while JA and ethylene are usually associated with defence against necrotrophic pathogens (Glazebrook, 2005; Adie \textit{et al.}, 2007).

In contrast to the overwhelming amount of information with respect to SA, JA and ethylene as important regulators of defence responses to pathogens, abscisic acid (ABA) has only recently been proposed as a key player in plant–pathogen interactions (Mauch-Mani \& Mauch, 2005; Adie \textit{et al.}, 2007). In most cases, ABA behaves as a negative regulator of disease resistance. The ABA-deficient tomato mutant \textit{sitiens} showed enhanced resistance to the necrotroph \textit{Botrytis cinerea} compared to wildtype (WT) tomato plants (Audenaert \textit{et al.}, 2002), while exogenous application of ABA was able to suppress the resistance of \textit{sitiens} to both biotrophic \textit{Oidium neolycopersici} and necrotrophic \textit{B. cinerea} (Achuo \textit{et al.}, 2006). Similarly, \textit{Arabidopsis} ABA biosynthesis mutants \textit{aba2-12} and \textit{aao3-2} also exhibited increased resistance to \textit{B. cinerea} (Adie \textit{et al.}, 2007). In addition, ABA-insensitive mutants \textit{abi2-1} (\textit{ABA insensitive 2-1}) and \textit{abi1-1} (\textit{ABA insensitive 1-1}) were more resistant to the bacterial pathogen \textit{Pseudomonas syringae} compared with wildtype controls, while the ABA hypersensitive mutant \textit{eral1} (\textit{enhanced response to abscisic acid 1}) was more susceptible (de Torres-Zabala \textit{et al.}, 2007; Goritschnig \textit{et al.}, 2008).

However, some studies have suggested a positive role for ABA in pathogen resistance. For example, exogenous ABA application was able to increase the resistance of \textit{rice} (\textit{Oryza sativa}) against the brown spot-causing ascomycete \textit{Cochliobolus miyabeanus} and tomato against \textit{Alternaria solani} (De Vleesschauwer \textit{et al.}, 2010; Song \textit{et al.}, 2011). The \textit{Arabidopsis} ABA biosynthesis mutants \textit{aba2-12}, \textit{aao3-2} and \textit{abi4-1} were more susceptible to the necrotrophs \textit{Pythium irregular} and \textit{Alternaria brassicicola} (Adie \textit{et al.}, 2007).

Mitogen-activated protein kinases (MAPKs) are a class of important signalling proteins, usually located downstream of receptors, with functions in numerous developmental processes and in mediation of resistance to biotic and abiotic stresses (Andreasson \& Ellis, 2010; Rodriguez \textit{et al.}, 2010). In \textit{N. attenuata}, NaMPK4 plays a
critical role in stomatal physiology (Hettenhausen et al., 2012). NaMPK4-silenced plants are strongly impaired in ABA-induced stomatal closure responses; thus these plants are highly susceptible to the surface-deposited bacterial pathogen P. syringae pv. tomato (Pst) DC3000. However, when bacteria were directly introduced into leaves by pressure infiltration, NaMPK4 was found to be less important in the resistance to Pst DC3000 located in the apoplast, suggesting a critical role for NaMPK4 in ABA- and stomata-based defence (Hettenhausen et al., 2012).

Stomata represent a major route of entry into plant tissues for many plant pathogens (Gudesblat et al., 2009). However, stomata can react to bacterial attack by recognizing pathogen-derived molecules and probably initiating ABA-mediated stomatal closure as part of an innate immune response. To overcome this response, some bacteria produce specific virulence factors that block ABA-induced stomatal closure, to reopen the stomata and allow bacteria to enter (Melotto et al., 2006). Host plants can also trigger stomatal closure after attack by fungal pathogens by recognizing fungal elicitors such as oligogalacturonic acid and chitosan (Lee et al., 1999).

The tobacco pathotype of Alternaria alternata (Slavov et al., 2004) is a necrotrophic fungus causing brown spot disease in tobacco (Nicotiana tabacum). The disease commonly occurs on senescent leaves near the soil (LaMondia, 2001). Under high humidity conditions, spores germinate, and then fungal hyphae penetrate leaf cells directly through the cuticle or via stomata (Slavov et al., 2004). However, the role of stomata-based defence in resistance to A. alternata is still unresolved (Slavov et al., 2004).

Nicotiana attenuata, a native wild tobacco of the Great Basin Desert of California, Nevada, Idaho and Utah, has been extensively used as a model plant species for studies of herbivore-induced responses using ecological, chemical and molecular approaches (Wu & Baldwin, 2010). Many important defence genes, including NaMPK4 (Hettenhausen et al., 2012), have been silenced in this species, thus providing an excellent Nicotiana species for studying the Nicotiana–A. alternata interaction.

The aim of this study was to examine ABA signalling after challenging leaves of N. attenuata with A. alternata, by assessing quantities of ABA and ABA-related genes in source–sink transition leaves and in fully expanded leaves. The effect of exogenously supplied ABA on stomatal conductance and resistance to A. alternata was studied. NaMPK4-silenced plants, in which ABA-induced resistance to A. alternata and stomatal closure responses are highly impaired, were examined for stomatal conductance changes after infection, and susceptibility to infection.

Materials and methods

Plant and fungal material

Seeds of the 21st generation of an inbred line of N. attenuata (Solanaceae) were used as the WT genotype in all experiments. Lines of N. attenuata irNaMPK4, a stably transformed RNAi construct (Hettenhausen et al., 2012), were used as plants that were silenced in the expression of NaMPK4. Seed germination and plant growth were conducted as described by Krügel et al. (2002). Alternaria alternata isolate CN174, a tobacco pathotype, was kindly provided by Dr Zhenyuan Xia, Yunnan Academy of Tobacco Agriculture Science. It was grown at 28°C on potato dextrose agar (PDA) plates for 8–12 days before inoculation. Because accurate and reproducible Alternaria pathogenicity assessment requires precise control of both humidity and temperature, greenhouse inoculation of whole plants was found to be unsatisfactory. Therefore, a detached leaf assay similar to Williams et al. (2011) was adopted to assess resistance. As spore suspensions did not produce consistent and reproducible disease symptoms, and there were concerns about the loss of virulence of the fungus when using spore preparations (Slavov et al., 2004), agar plugs of the fungus were used for all infections. In brief, the source–sink transition leaves (leaf 0), and +3 leaves of rosette-stage plants (before bolting) were excised and inoculated with four PDA plugs (3 mm diameter) containing actively growing A. alternata per leaf, and then closed in a transparent 12 × 12 cm square Petri dish with 100% humidity at 25°C with a 16 h light/8 h dark cycle. Symptom formation was monitored every day. Usually the fungus started to penetrate the leaf at about 20 hours post-infection (hpi), a large amount of hyphae could be observed at 2 days post-inoculation (dpi), and a small lesion usually appeared at 3 dpi. Lesion diameters were measured at 5 dpi as an indicator of the severity of the disease symptoms.

For dark treatment, four leaves were inoculated as described, and placed in a completely dark chamber for 1 day, and then returned to the light chamber (16 h light/8 h dark) at 25°C on the second day. The control leaves were inoculated and kept in the light chamber. The diameter of the lesions was also measured at 5 dpi. The experiment was repeated twice.

Confocal microscopy

Hyphae of A. alternata were stained with the chitin-specific dye WGA-AF488 (Molecular Probes, Invitrogen) as described in Wang et al. (2011). Plant membranes were visualized using propidium iodide (PI; Sigma) (Wang et al., 2011). Samples were incubated in staining solution (20 μg mL⁻¹ PI, 10 μg mL⁻¹ WGA-AF488, 0.02% Tween 20 in phosphate-buffered saline, PBS) for 30 min and washed in PBS (pH 7.4). Confocal images were recorded on an FV1000 laser scanning confocal microscope (Olympus). WGA-AF488 excitation was at 488 nm and detection at 500–540 nm; PI excitation was at 561 nm and detection at 600–700 nm.

ABA treatments and measurements of stomatal conductance

ABA (Sigma), dissolved in ethanol (1 mg mL⁻¹) was diluted to 2 μM and 10 μM with sterile water and supplied via the excised petiole. Sterile water served as the control. After treatment with ABA for 1 h, the stomatal conductance of the abaxial sides of the +3 leaves was measured with a SC-1 leaf porometer (Decagon Devices). For the infected leaves, measurements of the abaxial sides of the infection sites were made.

ABA measurements

ABA was extracted and quantified by LC-MS/MS as described by Wu et al. (2008). Five replicated leaf samples were used for
Real-time PCR assay
cDNA was prepared from 500 ng total RNA with reverse transcriptase (Thermo Scientific). Real-time PCR was performed with a CFX Connect qPCR System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions using gene-specific primers and the following cycle conditions: 10 min at 95°C; and 40 cycles of 30 s at 95°C, 30 s at 60°C. For each analysis, a standard curve of the threshold cycle number versus log designed transcript level was constructed using serial dilution of a specific cDNA sample. The levels of NaOSM1 transcript in all unknown samples were determined according to the standard curve. An N. attenuata actin2 gene, which is a housekeeping gene that has been shown to have constant levels of transcripts by microarray analysis, RNA gel blotting and qRT-PCR (B. Bubner, J. Wu, I. T. Baldwin, Max-Planck Institute of Chemical Ecology, Jena, Germany, personal communication), was used as an internal standard to normalize cDNA concentrations. The relative transcript level of NaOSM1 was obtained by dividing the extrapolated transcript levels of the NaOSM1 genes by the levels of actin2 from the same sample. All primer information is listed in Table S1.

Results

Establishment of N. attenuata–A. alternata pathosystem
To gain insight into the process by which A. alternata infects the fully expanded leaves of N. attenuata, a confocal microscopy study was performed. Fungal material was visualized by WGA-AF488 staining, in contrast with the plant cell membranes stained with PI. At about 20 hpi, the fungus started to grow on the leaf surface, and many fungal hyphae were observed inside the leaf cells at 2 dpi (Fig. 1a,b). Necrotic lesions with diameters c. 0.6–0.7 cm were usually developed on fully expanded leaves after 5 dpi (Fig. 1c). Interestingly, many fungal hyphae just penetrating through stomata were observed at 20 hpi (Fig. 1a). This finding implies that ABA- or stomata-based defence may play a role in the N. attenuata–A. alternata interaction.

Leaf position and resistance to A. alternata
Brown spot disease in tobacco is usually observed on senescent leaves near the soil (LaMondia, 2001), whereas young leaves are more resistant (Zhang et al., 1998; Cheng & Sun, 2001). To see whether the resistance to A. alternata is dependent on leaf developmental stages in N. attenuata, five leaves from rosette-stage plants at different phyllotaxic positions including 0 (source–sink transition leaf), +1 (first fully expanded leaf), +2 (one phyllotaxic position older than the leaf at node +1), +3 and +4, as described by van Dam et al. (2001), were detached and infected with A. alternata for disease rating (Fig. 2a). The results showed that leaves at nodes +1, +2, +3 and +4 developed lesions of about 0.6–0.8 cm in diameter, while the lesion diameters on the leaves at node 0 were significantly smaller, at about 0.3–0.4 cm in diameter (Fig. 2b), which indicated that leaf 0 was more resistant than all the fully expanded leaves.

ABA levels and stomatal conductance after A. alternata infection
The observation that many A. alternata hyphae penetrated leaf cells through stomata led to the hypothesis that ABA may play a role in resistance in N. attenuata. Therefore, ABA levels in leaves of nodes 0 and +3 were analysed by LC-MS/MS before and after infection. The results revealed that ABA levels increased at both leaf positions after infection; however, threefold more ABA

Figure 1 Observations of Nicotiana attenuata fully expanded leaves after inoculation with Alternaria alternata. Confocal microscopy images: (a) penetration of the fungus starts at about 20 hours post-inoculation (hpi); (b) massive fungal growth observed at 48 hpi. Fungal material was stained with WGA-AF488 (green); plant material was stained with propidium iodide (red). White arrows indicate fungal penetration through stomata. Bars: 300 µm. (c) Fully developed disease symptoms at 5 days post-inoculation.
accumulation was observed in leaf 0 than in leaf +3 at 1 dpi (Fig. 3a).

The relative abundance of osmotin 1 (NaOSM1) transcripts, previously shown to be ABA- and drought-inducible (Ré et al., 2011; Hettenhausen et al., 2012), was also measured. Consistent with the induction of ABA, the transcriptional level of NaOSM1 was also highly induced in leaves of both nodes, but the level in leaf 0 was twofold higher than in leaf +3 (Fig. 3b).

Stomatal conductance, usually used as a tool to indicate stomatal aperture by measuring the rate at which water is lost from a given area of leaf tissue, was also measured at 1 dpi. Stomatal conductance significantly decreased in leaves of both nodes, but it was reduced to much lower levels in leaf 0 than in leaf +3 (Fig. 3c), which is correlated with the higher ABA content in leaf 0. Similar results were also obtained 2 dpi, before necrotic lesions appeared (Fig. S1a).

**Effect of exogenous ABA supply on stomatal conductance and fungal resistance**

Because the susceptibility of leaf +3 to A. alternata was associated with lower A. alternata-induced ABA levels, it was speculated that exogenously supplied ABA might enhance resistance to the fungus. Stomatal conductance dramatically decreased 1 h after petiole feeding with 2 or 10 μM ABA to leaf +3 (Fig. 4a), indicating that feeding ABA through petioles is very effective. After 4 h, ABA...
application was stopped. The detached leaves were then inoculated with *A. alternata* agar plugs. The diameters of necrotic lesions were measured at 5 dpi. Compared with the water-treated control, lesion diameters were smaller in leaves pretreated with 2 μM ABA, and were reduced even further in leaves pretreated with 10 μM ABA (Fig. 4b).

**Susceptibility of mitogen-activated protein kinase 4 (NaMPK4)-silenced plants to *A. alternata***

NaMPK4-silenced plants (irNaMPK4) are highly impaired in ABA-induced stomatal closure responses (Hettenhausen *et al.*, 2012). To confirm that irNaMPK4 plants were ABA-insensitive, both ABA-induced stomatal response and lesion development were measured after feeding ABA to irNaMPK4 leaves. Exogenously supplied ABA dramatically reduced the stomatal conductance in WT node +3 leaves 1 h after ABA feeding, whereas it did not induce any stomatal closure responses in irNaMPK4 plants (Fig. 4a). The enhanced resistance to *A. alternata* was not observed in leaf +3 of irNaMPK4 plants after ABA treatment (Fig. 4b). The stomatal conductance, which decreased after *A. alternata* infection in WT plants, did not change in irNaMPK4 plants after fungal challenge (Fig. 5a). A detached leaf assay in leaves 0 and +3 of irNaMPK4 plants was also performed. Both leaves developed significantly larger necrotic lesions in irNaMPK4 plants than in WT plants (Fig. 5b). Thus the data strongly support the requirement for ABA for resistance to *A. alternata*, with ABA exerting its role through NaMPK4.

**Effect of dark treatment on resistance of leaf +3 to *A. alternata***

As part of an innate immune response, stomata can react to pathogen attack by recognizing pathogen-derived...
molecules and initiate stomatal closure (Melotto et al., 2006; Sawinski et al., 2013). Nicotiana attenuata leaves closed their stomata in response to infection by A. alternata (Fig. 3), and this response is NaMPK4-dependent, because NaMPK4-silenced plants did not close their stomata after fungal challenge or ABA treatment (Fig. 4). However, it is still not clear to what extent the stomatal closure responses contribute to resistance against this fungus. It is known that dark can induce stomatal closure responses. Indeed, stomatal conductance dramatically decreased after 1 h of dark treatment, and almost dropped to zero when dark treatments were extended to 3, 24 or 48 h (Fig. 6a). The necrotic lesions were significantly smaller in leaf +3 of control leaves and leaves kept in the dark for 1 day before inoculation. Data represent 16 inoculation sites in four biological replicates. The experiment was repeated twice with similar trends. The asterisks indicate significant differences between control and dark-treated leaves (unpaired t-test: *, P < 0.05; ***, P < 0.005).

Discussion

Brown spot disease is often observed on mature leaves close to the soil in cultivated tobacco (Nicotiana tabacum) (LaMondia, 2001). Zhang et al. (1998) and Cheng & Sun (2001) showed that resistance to A. alternata, decreased as the leaves became mature from the top of the tobacco plant to the bottom. Similarly, in the current study with N. attenuata, the source–sink transition leaves (leaf 0) were more resistant to A. alternata than all the fully expanded leaves (including +3 leaves). The observation that many A. alternata hyphae penetrated fully expanded leaves through stomata led to the hypothesis that ABA- or stomata-based defence might play a role in resistance to A. alternata in wild tobacco.

Interestingly, when N. attenuata plants were inoculated with A. alternata, NaOSM1 (ABA-inducible gene) transcripts and ABA were highly induced in leaf 0 but much less in leaf +3 1 day after inoculation, which indicates that reduced ABA accumulation could account for the increased susceptibility of leaf +3 to A. alternata. Indeed, exogenous application of ABA to leaf +3 before inoculation enhanced the resistance to the fungus, and the resistance rendered by ABA treatments is dosage dependent.

Stomata are major routes of entry into plant tissues for many plant pathogens such as rust fungi, the oomycete Plasmopara viticola and Pst DC3000 (Sawinski et al., 2013). However, stomata can react to pathogen attacks by recognizing pathogen-derived molecules, and initiate different hormone-dependent signalling pathways including ABA signalling in disease resistance.
ABA that mediate stomatal closure as part of an innate immune response (Melotto et al., 2006; Grimmer et al., 2012; Sawinski et al., 2013).

Many *Alternaria* spp. have been observed to penetrate host leaf cells through stomata; for example *Alternaria macrospora* could penetrate through stomata and result in leaf colonization in cotton (Bashan & Levanony, 1987), and penetration of *Withania somnifera* leaves by *A. solani* occurred only through stomata (Alwadi & Baka, 2001). In the current study many hyphae of *A. alternata* were observed entering the leaf cells through stomata at 20 hpi. In response to the fungal attack, *N. attenuata* plants accumulated large amounts of ABA, especially in leaf 0, and closed their stomata, possibly following recognition of pathogen-associated molecular patterns (PAMPs; Sawinski et al., 2013). However, the detailed mechanism by which leaf 0 accumulates more ABA after infection is still unclear. Dark treatment for 1 day, which leads to +3 leaves completely closing their stomata, enhanced the resistance of *N. attenuata* +3 leaves to *A. alternata*. These results suggested that stomata-based defence plays a role in the *N. attenuata*–*A. alternata* interaction.

Recent evidence indicates that MAPK signalling is required for ABA-regulated stomatal closure. AtMPK9 and AtMPK12 proteins are specifically localized in guard cells, and they positively control stomatal closure in response to ABA and hydrogen peroxide treatments (James et al., 2009). Silencing AtMPK3 in *Arabidopsis* also impairs ABA- and H₂O₂-induced guard cell movements (Gudesblat et al., 2007). Similarly, Hettenhausen et al. (2012) showed that NaMPK4-silenced plants were strongly impaired in ABA-induced stomatal closure responses in *N. attenuata*. Indeed, not only ABA-induced stomatal closure responses, but also ABA-induced resistances to *A. alternata*, were blocked in irNaMPK4 plants. As expected, the stomatal conductance did not decrease in irNaMPK4 plants at 1 dpi. More importantly, leaf 0 and +3 leaves became highly susceptible to *A. alternata*, suggesting an important role of *NaMPK4* downstream of ABA in the defence responses to the fungus.

How ABA regulates the resistance of *N. attenuata* to *A. alternata* is still not clear. The data in the current study strongly support the view that ABA affects the resistance, at least in part, through regulating stomatal closure responses. It has been proposed that ABA may exert its role by influencing JA production (Adie et al., 2007). More detailed experiments are needed to identify the specific ABA-induced genes regulated by *A. alternata*.

In summary, the results of this study indicate that the ABA signalling pathway is activated for *A. alternata* infection in *N. attenuata*, and that ABA exerts its role through NaMPK4, then affecting stomatal closure responses and resistance (Fig. 7). The young source–sink transition leaf is more resistant to *A. alternata* than the fully expanded leaves, which is probably at least in part as a result of the higher ABA signalling activated in that leaf.

Acknowledgements

The authors thank Dr Zhenyuan Xia for providing the *A. alternata* isolate; and the Chinese Academy of Sciences for funding (supported by the Knowledge Innovation Project of the Chinese Academy of Sciences).

References


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ABA signalling in disease resistance


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Figure S1. (a) Mean stomatal conductance of mock- (□) and Alternaria alternata-infected (■) Nicotiana attenuata leaves from nodes 0 and +3, measured in six biological replicates at 2 days post-inoculation (dpi). (b) Mean diameter of necrotic lesions on leaf +3 inoculated with A. alternata grown under 16 h light/8 h dark or with A. alternata pretreated with 1-day-dark. Data represent 16 inoculation sites in four biological replicates at 5 dpi. The asterisks indicate significant differences between control and treated samples (unpaired t-test: *, P < 0.05).

Table S1. Real-time PCR primer sequences for NaOSM1 and NaAcitin2.