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Abscisic acid-dependent regulation of capsidiol biosynthesis

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Abscisic acid negatively regulates elicitor-induced synthesis of capsidiol in wild tobacco (Nicotiana plumbaginifolia)

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Abstract

In the solanaceae, biotic and abiotic elicitors induce de novo synthesis of sesquiterpenoid stress metabolites known as phytoalexins. Because plant hormones play critical roles in the induction of defense-responsive genes, we have explored the effect of abscisic acid (ABA) on the synthesis of capsidiol, the major tobacco sesquiterpenoid phytoalexin using wild-type (WT) *Nicotiana plumbaginifolia* versus non-allelic mutants *Npaba2* and *Npaba1* that are deficient in ABA synthesis. *Npaba2* and *Npaba1* mutants exhibited a two-fold higher synthesis of capsidiol than WT plants when elicited with either cellulase or arachidonic acid, or when infected by *Botrytis cinerea*. The same trend was observed for the expression of the capsidiol biosynthetic genes, 5-epi-aristolochene synthase (EAS) and 5-epi-aristolochene hydroxylase (EAH). Treatment of WT plants with fluridone, an inhibitor of the upstream ABA pathway, recapitulated the behaviour of *Npaba2* and *Npaba1* mutants, while the application of exogenous ABA reversed the enhanced synthesis of capsidiol in *Npaba2* and *Npaba1* mutants. Concomitantly with the production of capsidiol, we observed the induction of ABA 8'-hydroxylase (ABAH) in elicited plants. In WT plants, the induction of ABAH coincided with a decrease in ABA content and with the accumulation of ABA catabolic products such as phaseic acid and dihydrophaseic acid, suggesting a negative regulation exerted by ABA on capsidiol synthesis. Collectively, our data indicate that ABA is not required per se for the induction capsidiol synthesis, but is essentially implicated in a stress-response checkpoint to fine-tune the amplification of capsidiol synthesis in challenged plants.
INTRODUCTION

The induction of the synthesis of secondary stress metabolites known as phytoalexins represents part of the metabolic responses induced in plants following the action of abiotic and biotic elicitors (Kuc, 1995). As a consequence of this stress-induced metabolism, sesquiterpenoid phytoalexins are synthesized in solanaceous plants (Kuc, 1982). In pepper and tobacco the bicyclic sesquiterpene capsidiol represents the main type of phytoalexin. Capsidiol is synthesized from farnesyl diphosphate via a two-step process catalyzed by 5-epi-aristolochene synthase (EAS) (Facchini and Chappell, 1992) and 5-epi-aristolochene hydroxylase (EAH) (Ralston et al., 2001). Despite continuing efforts, our understanding of the mechanisms that regulate the amplification of capsidiol synthesis in challenged plants is poorly understood, compared to what we know about camalexin in Arabidopsis (Ren et al., 2008). Reactive oxygen species have been implicated in the expression of EAS in tobacco (Rusterucci et al., 1996; Yin et al., 1997) and in pepper (Maldonado-Bonilla et al., 2008), and in the accumulation of capsidiol in tobacco (Perrone et al., 2003) and in pepper (Arreola-Cortés et al., 2007). In addition, it has been suggested that Ca^{2+}, Calmodulin, Ca^{2+}-dependent protein kinases and phosphoinositide signaling are involved in the regulation of capsidiol synthesis in tobacco (Vogeli et al., 1992; Preisig and Moreau, 1994; Tavernier et al., 1995) and in pepper (Ma, 2008).

Recent investigations have highlighted an essential role of plant hormones in the induction of plant defense responses (Jones and Dangl, 2006; Asselbergh et al., 2008b; Lopez et al., 2008; Spoel and Dong, 2008). In tomato which produces rishitin as the main sesquiterpene phytoalexin, the susceptibility to the phytopathogen fungus Phytophthora parasitica is decreased by salt and water stresses which elevate the concentration of abscisic acid (ABA) (Ristaino and Duniway, 1989). Studies examining loss-of-function mutations in the ABA pathway reveal that ABA deficiency enhances the resistance of tomato to infection by the necrotrophic fungus Botrytis cinerea (Audenaert et al., 2002) and to the bacterial pathogen Erwinia chrysanthemi (Asselbergh et al., 2008a). This trend is reinforced by the fact that tobacco plants treated with ABA become more susceptible to tobacco mosaic virus (Balazs et al., 1973) and to the blue mold pathogen Peronospora tabacina (Salt et al., 1986). Interestingly, the transcription of genes encoding beta-1,3-glucanases isoforms known to be involved in defense responses is down-regulated in tobacco cell cultures treated with...
exogenous ABA (Rezzonico et al., 1998). With respect to phytoalexin, it has been reported that application of exogenous ABA reduces the accumulation of rishitin and lubimin, two sesquiterpenoid stress phytoalexins produced in potato tuber slices infected by incompatible races of *Phytophtora infestans* but not by the compatible races (Henfling et al., 1980). However, the reduction of rishitin and lubimin synthesis by ABA could not be observed if potato tubers were not previously stored at 4°C (Bostock et al., 1983). Analysis of the role of plant hormones on capsidiol synthesis is limited to the fact that jasmonic is not a direct regulator of the capsidiol pathway in tobacco (Mandujano-Chavez et al., 2000) and in pepper (Ma, 2008).

In this study, we utilized wild-type (WT) *Nicotiana plumbaginifolia* and non-allelic mutants *Npaba2* (Marin et al., 1996) and *Npaba1* (Kraepiel et al., 1994) which are impaired in the biosynthesis of ABA, in order to directly investigate if ABA regulates the synthesis of capsidiol following elicitation with cellulase, arachidonic acid (AA) and infection with *Botrytis cinerea*, and to explore the potential mechanisms in which this regulation is achieved. We provide evidence that ABA is not required for capsidiol synthesis but plays a key role in the adjustment of capsidiol production level in plants. We show that ABA down-regulates the transcription of capsidiol biosynthetic genes and subsequently the level of capsidiol production. We also show that in WT plants the biosynthesis of capsidiol is accompanied by decreased bioactive ABA, and the induction of *ABA 8'-hydroxylase (ABAH)* involved in ABA catabolism, consistent with a negative regulation of ABA on capsidiol synthesis. Collectively, our data suggest that ABA plays an essential role in fine-tuning the amplification of capsidiol synthesis in challenged plants.

**RESULTS**

**ABA-deficient Mutants Exhibit Excessive Capsidiol Synthesis**

The capacity of WT *N. plumbaginifolia* plants to produce capsidiol was first tested using cellulase and AA as elicitors, and following infection by *B. cinerea*. The accumulation of total capsidiol was analyzed and measured over time from the leaf discs and the aqueous leaf disc diffusates. The synthesis of capsidiol was induced following cellulase and AA treatments and in response to *B. cinerea* infection, as shown by the typical gas chromatography (GC)-mass spectrometry (GC-MS) profile (Supplemental Fig. S1). The synthesis of capsidiol was
strongly elicited both by cellulase and AA treatments with a maximum value around 35 ± 3 μg g⁻¹ FW and 28 ± 2 μg g⁻¹ FW at 30 h for cellulase and AA. A significant period of time was required for B. cinerea to elicit the synthesis of capsidiol and the response was less intense (15 ± 2μg g⁻¹ FW at 120 h) compared to cellulase and AA, whatever the incubation time. Capsidiol was not produced in measurable amounts in non-elicited leaf discs.

To determine if capsidiol synthesis was dependent upon ABA levels we used WT N. plumaginifolia and two ABA deficient mutants, Npaba2 impaired in zeaxanthin epoxidase (ZEP) (Marin et al., 1996) and Npabal (Kraepiel et al., 1994) deficient in abscisic aldehyde oxidase that catalyzes later steps of ABA synthesis. Equivalent quantities of leaf discs excised from WT plants, Npaba2 and Npabal mutants were treated with cellulase, AA or infected by B. cinerea. The accumulation of capsidiol was analyzed at designated time intervals over a 24 h period for cellulase and AA, and 96 h for plants infected by B. cinerea. Total capsidiol produced by Npaba2 and Npabal mutants nearly doubled to that observed for WT plants (Fig. 1), even in the case of plants infected with B. cinerea, known to produce ABA (Inomata et al., 2004). Based on the magnitude of the quantitative increase of capsidiol, ABA may be a key negative modulator of enhanced capsidiol synthesis in challenged plants. To further test this hypothesis, we made use of the phytoene desaturase inhibitor, fluridone (Bartels and Watson, 1978) to block the upstream branch of the ABA pathway. Previous studies have shown that fluridone blocks de novo ABA biosynthesis in pathogen-challenged plants (Koga et al., 2004). When discs were co-treated with cellulase and fluridone (10 μM) the accumulation of capsidiol in leaf discs was strongly enhanced in WT plants, suggesting an ABA-dependent effect in leaf tissue (Fig. 2A). In contrast, the level of capsidiol in Npaba2 and Npabal mutants remained unchanged under these conditions (Fig. 2A). Very similar results were obtained with AA (Fig. 2B). Thus, the fluridone-induced increase of capsidiol in elicited WT plants recapitulates the effect observed with Npaba2 and Npabal mutants treated with cellulase or AA alone, and hence, is consistent with negative regulation exerted by ABA. This prominent role of ABA is further supported by the fact that co-treatment of WT, Npaba2 and Npabal mutants with ABA (25 μM) and cellulase or AA markedly reduced the induced synthesis of capsidiol in WT plants and in Npaba2 and Npabal mutants (Fig. 2A and B). ABA and fluridone in the absence of cellulase or AA did not induce capsidiol synthesis (data not shown).

To further analyze the mechanism by which ABA modulates capsidiol synthesis in cellulase-elicited plants we determined the expression of EAS and EAH which encode the
enzymes of the later steps of capsidiol synthesis, in addition to \textit{ZEP} and nine-cis-epoxy-carotenoid dioxygenase (\textit{NCED}) which encode enzymes of the upstream pathway of ABA synthesis. We observed that \textit{EAS} and \textit{EAH} transcript accumulation reflected the induced synthesis of capsidiol, in good agreement with previous data obtained from tobacco (Facchini and Chappell 1992; Yin et al., 1997) and pepper (Huguey et al., 1996) (Fig. 3). However, the transcript levels of \textit{EAS} and \textit{EAH} were higher in \textit{Npaba2} and \textit{Npaba1} compared to WT plants (Fig. 3). The expression of the \textit{N. plumbaginifolia} pathogenesis-related protein \textit{PR-Ia} (Payne et al., 1988) monitored by RT-PCR, displayed an expression pattern similar to that of \textit{EAS} and \textit{EAH} (data not shown) and is in agreement with the fact that exogenous ABA down-regulated the accumulation of \(\beta\)-1,3-glucanase (Rezzonico et al., 1998). The accumulation of \textit{ZEP} and \textit{NCED} mRNAs were observed in unelicited discs and during the initial period of elicitation (Fig. 3). During the period of capsidiol accumulation \textit{ZEP} and \textit{NCED} mRNAs decreased (Fig. 3). Although we used a northern blot hybridization procedure to better judge the quality of the mRNAs, we confirmed the expression patterns by two independent semi-quantitative RT-PCR analyses (data not shown). Consistent with these findings, the level of ABA in WT plants was slightly increased during the first period following exposure to cellulase and AA, and decreased thereafter significantly concomitant with capsidiol accumulation (Fig. 4). Considered together, these data suggest that ABA negatively regulates the amplification of capsidiol biosynthesis in challenged plants.

\textbf{Induction of ABA Catabolism During Elicited Synthesis of Capsidiol}

The above data indicate that the synthesis of capsidiol in elicited plants is up-regulated in \textit{Npaba2} and \textit{Npaba1} ABA deficient mutants, and in WT plants treated with fluridone. These changes were paralleled by a decrease in ABA concentration during the phase of capsidiol accumulation. Based on this evidence, one could suggest that the amplification of capsidiol synthesis requires at least decreased availability of free ABA. How might this occur? One may predict that pathways leading to ABA catabolism could be activated concomitantly to the induced synthesis of capsidiol. The main ABA catabolic pathway is mediated by ABA-8'-hydroxylase (ABAH) to yield the unstable 8'-hydroxy-ABA which spontaneously rearranges to phaseic acid (PA) which produces dihydrophaseic acid (DPA) after reduction (Cutler and Krochko, 1999). We observed by exploiting available genome-wide array analysis that in \textit{Arabidopsis} challenged with \textit{Pseudomonas syringae}, that not only ABAH but also gibberellin 2-oxidase (\textit{GA-2Ox}) which encodes a gibberellin catabolic oxidase were induced (de Torres-
GA-2Ox catalyzes the 2β-hydroxylation of gibberellin to generate biologically inactive gibberellins (Thomas et al., 1999). Similarly, mRNA differential display analysis of genes expressed in tobacco infected by the phytopathogen *Rhodococcus fascians* revealed the expression of putative *ABAH* and *GA-2Ox* genes (Simon-Mateo et al., 2006). Thus, we further analyzed the possible impact of *ABAH* during the elicited synthesis of capsidiol. We cloned the putative *N. plumbaginifolia* cDNA *ABAH* (*NpABAH*) and analyzed its activity using an in vivo transient expression procedure. Leaves from WT plants were transfected with the expression plasmid (*NpABAH*) or the vector alone (*NpΔABAH*). At 72 h post-transfection, leaf discs expressing *NpABAH* and *NpΔABAH* were incubated with exogenous ABA (25 μM) for 20 h prior to the analysis of ABA derivatives. The presence of ABA and ABA catabolites was determined based on the retention time (*t_R*) of each chromatographic peak and by tandem mass spectrometry (MS/MS) using an ultra-performance liquid chromatography coupled with electrospray ionization-mass spectrometry (UPLC/ESI-MS/MS). The examination of the chromatograms obtained in both full scan MS mode (data not shown) and single ion recording (SIR) MS mode revealed that for ABA and ABA derivatives the best ionization mode was the negative mode as reported elsewhere (Chiwocha et al., 2003). The unraveling of the fragmentation patterns obtained by daughter scan (DGS) MS/MS analysis for each of the ABA derivatives permitted the determination of the most intense daughter fragments which have been subsequently used as diagnostic fragments for multiple reaction monitoring (MRM) analysis. As a result of the MRM analysis, key evidence was obtained to confirm the structure of ABA and ABA derivatives. MRM analysis of *NpABAH* leaf extracts allowed the identification of four compounds corresponding to DPA (*t_R* 1.54 min, *m/z* 281, daughter ion *m/z* 171), PA (*t_R* 2.28 min, *m/z* 279, daughter ion *m/z* 139), 7'-OH-ABA (*t_R* 2.85 min, *m/z* 279, daughter ion *m/z* 151), *cis*-ABA (*t_R* 3.03 min, *m/z* 263, daughter ion *m/z* 153) and *trans*-ABA (3.38 min, *m/z* 263, daughter ion *m/z* 153) (Fig. 5 and Supplemental Fig. S2). Although exogenous ABA could induce its own catabolism (Windsor and Zeevaart, 1997; Saito et al., 2004), we observed basal levels or undetectable ABA catabolites both in leaves transiently overexpressing the control vector (*NpΔABAH*) (Fig. 5) and in untransformed leaves (data not shown). Collectively, these data demonstrate that *NpABAH* is functional in vivo.

To further investigate the implication of *ABAH* in the elicited-induced response we analyzed the expression of *ABAH* following elicitation by cellulase and *B. cinerea* infection. Expression of *ABAH* was induced in WT and in *Npaba2* and *Npaba1* mutants, after cellulase
treatment at time points corresponding to the expression of EAS and EAH and capsidiol synthesis (Fig. 6). Along with the induction of ABAH after cellulase treatment, expression of GA-2Ox was also induced (Fig. 6; see also Figs. 1 and 3) concomitantly with the accumulation of EAS, EAH transcripts and the enhanced synthesis of capsidiol (see Figs. 1 and 3). Consistent with these findings, ABA catabolites (PA, DPA, 7'-OH-ABA) could be detected in WT plants following elicitation by cellulase, AA (data not shown) or after infection by B. cinerea relative to controls (Fig. 7 A). Overall, the observations herein support an implication of ABA and possibly GA in the synthesis of capsidiol.

DISCUSSION

Role of ABA in Phytoalexin Production

Phytoalexins are produced in plants in response to pathogen infection or to biotic and abiotic elicitors. Mutations leading to phytoalexin deficiency can increase plant susceptibility (Thomma et al., 1999). A number of studies have documented the role played by hormones in the control of plant stresses and diseases. Exogenous ABA enhances the resistance or the susceptibility of various plant to pathogens (for a review see (Mauch-Mani and Mauch, 2005; Robert-Seilaniertz et al., 2007; Asselbergh et al., 2008b; Lopez et al., 2008). In some extreme cases, as observed in Arabidopsis infected by Ralstonia solanacearum which leads to xylem occlusion, 40 % of the up-regulated genes are linked or belong to the ABA signaling or biosynthetic pathway (Hu et al., 2008). The above mentioned implication of ABA in biotic stresses prompted us to analyze its effect on the biosynthesis of capsidiol, the main sesquiterpenoid phytoalexin produced in tobacco (Kuc, 1982).

In this report, we took advantage of WT and Npaba2 and Npabal mutant lines of N. plumbaginifolia to explore the effect of ABA on the regulation of capsidiol synthesis. First, we determined the capacity of different elicitors including cellulase, AA and of B. cinerea to induce the synthesis of capsidiol in challenged N. plumbaginifolia. Although the synthesis of capsidiol was induced following cellulase, AA treatments, and in response to infection by B. cinerea, the amount of capsidiol nearly doubled in Npaba2 and Npabal mutant lines compared to WT plants. Thus, ABA was negatively impacting the amplification of capsidiol synthesis. If the effect was due to ABA itself, similar results should be obtained by cotreatment with cellulase or AA and fluridone, a carotenoid desaturase inhibitor that affects the
upstream branch of the ABA pathway. When WT, \textit{Npaba2} and \textit{Npaba1} were co-treated with
fluridone and cellulase or AA and fluridone, the accumulation of capsidiol nearly doubled in
WT plants and remained unchanged in \textit{Npaba2} and \textit{Npaba1} mutant plants (Fig. 2). Interestingly, a significant decrease in ABA content occurred during capsidiol synthesis in
WT plants (Fig. 4). In a similar vein, ABA level was reduced in soybean infected by \textit{Phytophthora megasperma} (Cahill and Ward, 1989). Consistent with this trend, in sugar beet
infected by \textit{Cercospora beticola}, the early stage of the infection is characterized by an
increased production of ABA probably synthesized by the pathogen or the plant itself through
fungal stimulation. Following this initial stage, the concentration of ABA is decreased
(Schmidt et al., 2008). The change in the ABA content that we observed was reflected by the
expression pattern of \textit{NCED} and \textit{ZEP}, and is consistent with previous studies demonstrating
that the geranylgeranyl-PP pathway leading to carotenoids are down-regulated or unaffected
following elicitation or pathogen infection (Hugueney et al., 1996; Truman et al., 2006).
Collectively, our results suggest that ABA represses the amplification of capsidiol synthesis.
Several studies suggest that ABA may have a role in the regulation of phytoalexins in
legumes. In peanuts, drought stress is associated with increased susceptibility to \textit{Aspergillus
flavus} and to reduced endogenous phytoalexin (Wotton and Strange, 1987). The synthesis of
the two bean phytoalexins, phaseollin and kievitone is differently regulated following
mercuric chloride elicitation. ABA down-regulates mercuric chloride-induced kievitone
synthesis while phaseollin synthesis is unaffected (Goossens and Vendrig, 1982). In soybean,
following infection by \textit{P. megasperma} (Ward et al., 1989) or \textit{Phytophthora sojae} (Mohr and
Cahill, 2001) the synthesis of glyceollin is reduced in an ABA-dependent manner (Ward et
al., 1989). In solanaceae, the reduction of rishitin and lubimin sesquiterpenoid phytoalexins
by application of exogenous ABA has been shown in potato tubers infected by \textit{Phytophthora
infestans} (Henfling et al., 1980; Bostock et al., 1983). However the reduction of rishitin and
lubimin could only be demonstrated after a cold pretreatment treatment at 4°C (Bostock et al.,
1983). The role of the cold treatment in the measured responses is presently not understood.

The mechanism whereby ABA mediates its effects on capsidiol synthesis requires
further studies. Because exogenous ABA down-regulates the expression of defense genes
induced by jasmonic acid, ethylene, and salicylic acid (Anderson et al., 2004; Thaler and
Bostock, 2004; Yasuda et al., 2008), the antagonism between ABA and these hormones has
been usually highlighted. However the situation is probably more complex because the
hypersensitivity of the \textit{Arabidopsis enhanced disease resistance1} (\textit{edr1}) mutant to ABA is
associated with enhanced expression of \textit{PR-1 protein} (Frye and Innes, 1998; Wawrzynska et
al., 2008) and salicylate-enhanced resistance to powdery mildew (Frye et al., 2001). Alternatively, it has been suggested that ABA acts via ethylene response factors (ERFs) which regulate GCC box-containing defense genes (Zhou et al., 2008). According to this hypothesis ABA affects the interaction between ERFs and GCC-boxes. High ABA content destabilizes ERFs and GCC-box interactions, and impairs the expression of defense genes while low ABA favors the interaction and their transcription (Zhou et al., 2008). However, although GCC-boxes are indeed present in several pathogenesis related proteins, neither tobacco EAS (Yin et al., 1997) nor pepper EAS have GCC-boxes. Furthermore, the ethylene biosynthetic inhibitor, aminoethoxylvinylglycine, does not inhibit the accumulation of capsidiol in tobacco as elicited by *Phytophthora nicotianae* (Nemestothy and Guest, 1990).

Alternatively, ABA may play a role via cyclic ADP-ribose, an intracellular calcium mobilizer (Wu et al., 1997). Consistent with this hypothesis, the synthesis of sesquiterpenoid phytoalexin rishitin in potato (Zook et al., 1987), and capsidiol in tobacco (Vogeli et al., 1992; Preisig and Moreau, 1994; Tavernier et al., 1995) and in pepper (Ma, 2008) is dependent upon Ca\(^{2+}\). Interestingly, through the use of the calcium sensor aequorin, Ca\(^{2+}\) mobilization could be observed during elicitation in tobacco (Knight et al., 1991).

### Induction of Hormone Catabolic Pathways During Defense Responses

The expression of *ABAH* was induced during capsidiol accumulation (Fig. 6). Because ABAH is a key enzyme of the ABA catabolic pathway, we reasoned that ABAH might affect ABA homeostasis by reducing the level of free ABA concomitantly with capsidiol accumulation. We first assessed the functionality of *N. plumbaginifolia ABAH*. Transient overexpression of ABAH in WT *N. plumbaginifolia* leaf incubated with exogenous ABA resulted in the accumulation of typical ABA catabolic products PA, DPA and 7-OH-ABA (Fig. 5). In WT plants, the implication of ABAH was tested in vivo following elicitation by cellulase and infection by *B. cinerea*. As an indicator of ABAH activity, one may expect that the accumulation pattern of ABAH mRNAs could be correlated to the decrease of free ABA (Fig. 4), and to the accumulation of typical ABA catabolic products (Fig. 7). In addition, we observed that the hormone catabolic pathway is not limited to influence by ABA, but probably affected by the gibberellins as well. GA-2Ox was induced in parallel to the accumulation of capsidiol and EAS and EAH (Fig. 6). GA-2Ox serves as a marker for the catabolism of GA and its activity is in general low under normal conditions (Thomas et al., 1999). Interestingly, it has been shown that two known inhibitors of GA biosynthesis,
prohexadione-calcium and trinexapac-ethyl, enhance the resistance of apple trees to *Rewinia amylovora* concomitantly with an increased expression of genes encoding pathogenesis-related proteins (Maxson and Jones, 2002). In addition, in rice, overexpression of the *elongated uppermost internode* gene which deactivates GA leads to disease resistance (Yang et al., 2008). Thus, the expression pattern we observe for *ABAH* and *GA-2Ox* suggests a regulatory role of ABA, and possibly GA on capsidiol synthesis exerted via ABA and GA catabolic pathways. This hypothesis is supported by data mining of several transcriptomic analyses. For instance, *At4g19230* and *At3g19270* which encode two functionally characterized *ABAHs* and *GA-2Ox*, are induced in *Arabidopsis* infected by *P. syringae* (de Torres-Zabala et al., 2007). Similarly, transcriptomic analysis of *Arabidopsis* infected by *Ralstonia solanacearum* also revealed induction of *GA-2Ox* and *ABAH* (Hu et al., 2008). An mRNA differential display analysis of *N. tabacum* infected by *Rhodococcus fascians* shows overexpression of *ABAH* and *GA-2Ox* (Simon-Mateo et al., 2006). Likewise, decreased expression of the *kaurenoic acid oxidase* is reported in *sitiens*, an ABA-deficient tomato mutant infected by *B. cinerea* (Asselbergh et al., 2007). Interestingly, changes in expression of several auxin and gibberellin modifying enzymes have also been observed in microarray experiments using *Arabidopsis* and the salicylic analog, benzothiadiazole S-methylester (BTH) (Wang et al., 2006; Wang et al., 2007). Finally, it has been shown that rice plants overexpressing *GH3* which is implicated in the conjugation of plant hormones to amino acids, display enhanced resistance to fungal pathogens (Domingo et al., 2009). Although these catabolic pathways are implicated in the negative regulation exerted by ABA and GA in plants subjected to elicitors and pathogens, alternative possibilities may exist. For example, it has been reported that in *Arabidopsis* infected by the pathogen *Blumeria graminis*, a NAC transcription factor designated *ATALF1* down-regulates the accumulation of ABA according to an unknown mechanism, while enhancing the resistance to the pathogen (Jensen et al., 2008).

**Implication for the Role of ABA in Biotic Interactions**

Finally, we show that the induction of capsidiol synthesis per se is not dependent upon ABA itself, but the amplification of capsidiol synthesis is regulated by ABA. ABA attenuates excessive synthesis of capsidiol following elicitation or pathogen invasion. This may account for the elevated level of capsidiol in ABA-deficient plants. We can imagine the in vivo scenario to include that the negative regulation exerted by ABA ensures that adequate level of
capsidiol is synthesized in challenged plants. Once the challenge is mastered, ABA down-regulates the synthesis of capsidiol to restrict its over accumulation that otherwise may lead to tissue injury in the host (Polian et al., 1997), or trigger exhaustive draining of carbon precursors from primary metabolism as observed during the synthesis of amino acid-derived phytoalexins (Schaaf et al., 1995; Zhao and Last, 1996; Batz et al., 1998; Logemann et al., 2000; van der Fits and Memelink, 2000; Ren et al., 2008). ABA could also represent a plant signature sensed by pathogens. Replication and propagation require signals at the pathogen level to adjust a pathogen's sexual cycle and virulence. This non-classic signaling role of ABA is supported by the fact that in the apicomplexan parasite *Toxoplasma gondii*, ABA dictates the transition between the dormant cyst stage and the lytic growth (Nagamune et al., 2008). Because 82% of fungi live in association with terrestrial plants (Schmit and Mueller, 2007), plant-derived signals may play key roles in the modulation and regulation of non-plant organism development. In this context, *Cryptococcus* spp. are lethal fungal pathogens of humans and animals that can complete its life cycle on plant surfaces due to the presence of myo-inositol exsudate from *Arabidopsis* and *Eucalyptus* leaves (Xue et al., 2007). Interestingly, auxin acts synergistically with myo-inositol to stimulate the mating of *Cryptococcus* spp. (Xue et al., 2007). It is interesting to note that although ABA does not directly affect fungal growth (Henfling et al., 1980), it could perturb fungal reproduction. For example, the sexual reproduction of *Hyaloperonospora parasitica* is abolished in the *Arabidopsis* ABA-deficient mutant *aba1-1* (Mauch-Mani and Mauch, 2005). Thus it not surprising that some pathogens "hijack" the ABA biosynthetic or signaling pathway to adapt their level of virulence (de Torres-Zabala et al., 2007; Zhou et al., 2008). In the context of symbiosis, it has been shown that ABA (Ding et al., 2008) and GA (Maekawa et al., 2009) are negative regulators of legume nodulation, suggesting a wider implication of ABA and probably GA in the fine-tuning of biotic interactions through a negative regulatory mode.
MATERIAL AND METHODS

Plant Material, Growth Conditions and Elicitation Procedure

*N. plumbaginifolia* WT, *Npaba2* and *Npaba1* seeds were kindly provided by Dr. A. Marion-Poll (INRA, Versailles, France). *Npaba2* of *N. plumbaginifolia* is impaired in the epoxidation of zeaxanthin catalyzed by zeaxanthin epoxidase (Marin et al., 1996) and mutant *Npaba1* in the last step of ABA biosynthesis, i.e., the oxidation ABA-aldehyde into ABA, catalyzed by abscisic aldehyde oxidase (Kraepiel et al., 1994). Plants were grown under greenhouse conditions or in growth chamber saturated at 80% relative humidity for *Npaba2*. Leaf discs (1.75 cm, diameter) were excised with a cork borer from leaves of 75-day-old plants. Leaf discs were floated (the adaxial surface up) on a solution of cellulase (0.5%) from *Trichoderma viride* (Sigma-Aldrich, France) (Hugueney et al., 1996), or 1 mM arachidonic acid (AA) (Sigma-Aldrich, France) (Bloch et al., 1984), to induce the synthesis of capsidiol. A conidial suspension of *Botrytis cinerea* (isolate Flo-07) kindly provided by Drs S. Wiedmann-Merdinoglu and P. Hugueney (INRA Colmar and UDS, France) was used to infect leaf discs as described previously (Asselbergh et al., 2007). Triplicate samples of 10 leaf discs were used for each treatment. ABA (Sigma-Aldrich, France) and fluridone (Duchefa, The Netherlands) solubilized in ethanol/water (90:10, v/v) were added to the elicitation medium at 25 μM and 10 μM concentrations respectively. The incubation was carried out at 25 °C for the indicated period of time.

Capsidiol Analysis

Capsidiol was extracted from elicited leaf discs as described previously (Hugueney et al., 1996) using dichloromethane / methanol (2:1, v/v) and the dried extract was applied to a Pasteur pipette half-filled with silicagel preconditioned with cyclohexane. Apolar compounds were eluted with cyclohexane/ethyl acetate (80:20, v/v) and the capsidiol fraction was eluted with cyclohexane/ethyl acetate (50:50, v/v). The dried sample was analyzed by gas chromatography (GC)-mass spectrometry (GC-MS) as described previously (Hugueney et al., 1996). Alternatively an HPLC procedure was used as described previously (Moreau et al., 1992).

Extraction of ABA Catabolites and Mass Spectrometry Analysis
Freeze-dried *N. plumbaginifolia* leaf discs were ground in liquid nitrogen and extracted three times with 1-propanol:water (80/40, v/v; acidified with 80 µl of concentrated HCl) (Pan et al., 2008). The resulting extract was treated with two volumes of dichloromethane and centrifuged at 10,000 g for 10 min. The dichloromethane extract was subjected to pre-purification by thin layer chromatography on silica gel plate developed with dichloromethane/methanol/water (75:22:3, v/v). The area on the silica (60 F-254) gel plate between Rf 0.05 to 0.6 was scraped from the plate and eluted with 1-propanol:water (80/40, v/v; acidified with 80 µl of concentrated HCl) before liquid chromatography analysis. Characterization of ABA, PA, DPA, 7'-OH-ABA from leaf discs extracts was performed by comparing retention times, MS transitions and MS/MS analysis using an ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). All analyses were performed using a Waters Quattro Premier XE (Waters, Mildorf, MA USA) equipped with an electrospray ionisation (ESI) source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was achieved using an Acquity UPLC BEH C<sub>18</sub> column (100 x 2.1 mm, 1.7µm; Waters), coupled to an Acquity UPLC BEH C<sub>18</sub> pre-column (2.1 x 5 mm, 1.7µm; Waters). The mobile phase consisted of (A) (methanol / water (30/70, v/v; acidified with 0.1% formic acid), (B) (methanol / water (55/45, v/v; 0.1% formic acid), (C) methanol / water (90/10, v/v; 0.1% formic acid) and (D) methanol 100% acidified with 0.1% formic acid. The following gradient was used: solvent A (1 min), linear gradients A to B (2 min), B to C (5 min), C to D (2 min) before isocratic elution using D (2 min) and linear gradient to A (0.8 min) followed by isocratic run using A (3.2 min) to return to initial conditions. The total run time was 15 min. The column was operated at 48°C with a flow-rate of 0.4 ml/min (sample injection volume 3µl). Nitrogen generated from pressurized air in a N2G nitrogen generator (Mistral, Schmidlin-dbs-AG, Switzerland) was used as the drying and nebulizing gas. The nebulizer gas flow was set to approximately 50l/h and the desolvatation gas flow to 900l/h. The interface temperature was set at 400°C and the source temperature at 135°C. The capillary voltage was set at 3 kV and the cone voltage and the ionization mode (positive and negative) were optimised for each molecule. The selected ion recording (SIR) MS mode was used to determined parent mass transition of ABA (m/z: 263); PA (m/z: 279); DPA (m/z: 281); 7'-OH-ABA (m/z: 279). Fragmentation was performed by collision induced dissociation with argon at 1.0 x 10<sup>-4</sup> mbar. The collision energy was optimised for each compound using daughter scan monitoring (DGS) and multiple reaction monitoring (MRM). Mass spectrometry conditions for ABA and ABA catabolites were set after optimization as
follows: polarity ES-, capillary 3kV, cone 25V, for ABA, PA, DPA, 7'-OH-ABA. Low mass and high mass resolution were 13 for both mass analyzers, ion energies 1 and 2 were 0.5V, entrance and exit potential were 2V and 1V and detector (multiplier) gain was 650V. Collision-induced dissociation of deprotonated parent ions, were accomplished with a collision energy of 10V for ABA, PA, DPA, and 20V for 7'-OH-ABA. For ABA and ABA catabolites (PA, DPA, and 7'-OH-ABA). Daughter scan monitoring (DGS) and multiple reaction monitoring (MRM) permitted to identify for each compound the transition from deprotonated parent ion to the predominant daughter fragment ion. The combination of chromatographic retention time, parent mass and unique fragment ion analysis was used to selectively monitor ABA (263>153); PA (279>139); DPA (281>189>171); 7'-OH-ABA (279>151) Data acquisition and analysis were performed with the MassLynx software (ver.4.1) running under Windows XP professional on a Pentium PC.

Quantitative Analysis of ABA

ABA was extracted as described above using DL-cis, trans-[G-3H] abscisic acid (55 Ci/mmol) (GE Healthcare, France) to evaluate the recovery and quantified as described previously (Xiong et al., 2001) using an immunoassay ELISA kit (Phytodetek ABA, AGDIA, Elkhart, IN) according to the manufacturer's protocol.

Cloning and Functional Characterization of ABAH

N. plumbaginifolia ABAH (NpABAH) cDNA was amplified by RT-PCR as described previously (Bouvier et al., 2006) using forward (5’-ATGACTAATTTTGACTTATTTTTC-3’) and reverse (5’-GGTTGAAGTGGTAGATTCTTTCCAAAATC-3’) primers and Phusion™ High-Fidelity DNA Polymerase (Finnzymes, France) according to the manufacturer’s instructions. NpABAH cDNA was cloned into the XbaI site of pKYLX71-35S² vector (Maiti et al., 1993; Bouvier et al., 2006). Following sequence verification, the resulting plasmid designated pKNpABAH and an empty control vector pK(NpΔABAH) were transformed into Agrobacterium strain GV3101. Before plant infiltration Agrobacterium harboring pKNpABAH and pK(NpΔABAH) were diluted to an OD600 of 0.2 using the infiltration buffer (10 mM Mes (pH 5.6), 1 mM sodium phosphate, and 200 µM acetosyringone, 0.5% glucose 2 mM MgCl₂) and injected through the stomata on the lower epidermal surface of leaves of 75-day-old WT N. plumbaginifolia plants using a 1 ml plastic
syringe without a needle (Batoko et al., 2000). Expression of the transgene was monitored by RT-PCR using the forward primer (5’- CACTATCCTTCGCAAGACCCTTC-3’) specific for the vector $NpABAH$ and reverse primer (5’-GATATGAGTGTGATTTGGTACGAATG-3’) specific for $NpABAH$ to amplify an expected 585 bp band. Ethidium bromide staining of ribosomal RNAs was used as a loading control. At 3 days postinfiltration transfected plant leaves were incubated with 25 $\mu M$ ABA for 20 h before extraction and analysis of ABA catabolites.

**RNA Gel Blot Analysis**

Total RNA from leaf discs were prepared using the NucleoSpin RNA plant kit from Macherey-Nagel (Hilden, Germany). Following an agarose gel electrophoresis and transfer onto nylon membranes. Filters were hybridized overnight with $EAS$, $EAH$, $ZEP$, $NCED$, $ABAH$, $GA-2Ox$ cDNA probes and processed as described previously (Bouvier et al., 2006). The hybridization signals were visualized using a Fujifilm-FLA-7000 phosphorimager. Expression levels obtained by northern blots were confirmed by two independent semi-quantitative RT-PCR analyses (Bouvier et al., 2006). The primers used were the following: $EAS$, 5’-CACATGTAAGGACTCATGCTGAC-3’ and 5’-CTTTGTATGGCATCTGTATGC-3’; $EAH$, 5’-CTTTCTTGTGAAATGGAAGA-3’ and 5’-CTACACGTGCTTGGAGTTACC-3’; $ZEP$, 5’-CTTTCTGTTGAGGAAATGGAAGAAC-3’ and 5’-CTTTCTTGTGAAATGGAAGAAC-3’; $NCED$, 5’-GATTTGGTTTTGGACAAATATG-3’ and 5’-CCATTCTTGCTCATTGTGAC-3’; $ABAH$, 5’-CTACATATTTTCTTCCAATACAGGC-3’ and 5’-GCTTTGAAGGCTTCTATAGAAGG-3’; $GA-2Ox$, 5’-GGCTATGGAAACAAGAAGATTGG-3’ and 5’-GCCTTACACTCTTAATCTCCGG-3’.

The expression of $N. plumbaginifolia$ PRI protein (Payne et al., 1988) was assessed by semi-quantitative PCR as described previously (Bouvier et al., 2006) using the forward (5’-TGCTTCTTCAGTTTCATGC-3’) and the reverse (5’-CAAACCACCTGAGTAGGTGTC-3’) primers.

**SUPPLEMENTAL MATERIAL**

**Supplemental Figure S1.** GC-MS analysis of capsidiol produced by $N. plumbaginifolia$ following elicitor treatments or infection with $B. cinerea$. Total ion current chromatogram
(TIC) (A) and mass spectrum (B) of capsidiol (retention time 14.66 and molecular ion at m/z 236) obtained from elicited or infected *N. plumbaginifolia* plants.

**Supplemental Figure S2.** Ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) analysis using multiple reaction monitoring (MRM) of leaf disc extract from WT *N. plumbaginifolia* overexpressing *NpABAH*. ABAH products were analyzed using 30 discs, following incubation with exogenous ABA (25 μM) for 20 h. The MRM chromatograms, were acquired by monitoring the transition of m/z 263>153 for abscisic acid (ABA), 279> 151 for 7'-hydroxy abscisic acid (7'-OH-ABA), 279> 139 for phaseic acid (PA), and 281>171 for dihydrophaseic (DPA). The cone voltage and the collision energy were 25V and 22eV. The abbreviations refer to the relative intensity (RI).

**ACCESSION NUMBER**

The sequence reported in this paper has been deposited in the EMBL/GenBank data libraries under the accession numbers: FM244692 for *NpABAH*, FM244696 for *NpEAH*, FM244695 for *NpEAS*, FM244693 for *NpGA-2Ox* and FM244694 for *NpNCED*.

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FIGURE LEGEND

**Figure 1.** Capsidiol synthesis in *N. plumbaginifolia* wild type, *Npaba2* and *Npaba1* mutant plants in response to elicitors and infection with *B. cinerea*. Leaf discs were incubated with cellulase (0.5%) (A), arachidonic acid (1 mM) (B), or infected by *B. cinerea* (C) for the indicated times before capsidiol analysis. Data represent the mean ± SEM of triplicate samples.

**Figure 2.** Effect of fluridone and ABA on elicitor-induced capsidiol synthesis in *N. plumbaginifolia* wild type, *Npaba2* and *Npaba1* mutant plants. (A) Leaf discs were co-treated
with cellulase (0.5%) alone, cellulase plus fluridone (10 μM) or cellulase (0.5 %) plus abscisic acid (ABA) (25 μM). (B) Leaf discs were co-treated with arachidonic acid (AA) (1 mM) alone, AA (1 mM) plus fluridone (10 μM) or AA (1 mM) plus ABA (25 μM). After 22h incubation capsidiol synthesis was determined. Data represent the mean ± SEM of triplicate samples. Abbreviations refer to cellulase (Cel) and fluridone (Flu).

**Figure 3.** Northern blot analysis of abscisic acid and capsidiol biosynthetic genes in *N. plumbaginifolia* wild type, *Npaba2* and *Npaba1* mutant plants elicited with cellulase or infected by *B. cinerea*. Leaf discs were incubated with cellulase (0.5%), infected by *B. cinerea* or treated with water for the indicated times before RNA analysis. The ethidium bromide stained gel displaying ribosomal RNA (rRNA) is shown as loading control. Abbreviations refer to transcripts corresponding to genes encoding enzymes of the upstream ABA biosynthesis pathway, *zeaxanthin epoxidase* (ZEP), *nine-cis-epoxy-carotenoid dioxygenase* (NCED) and capsidiol biosynthetic genes *5-epi-aristolochene synthase* (EAS) and 5-*epi-aristolochene hydroxylase* (EAH).

**Figure 4.** Changes in the abscisic (ABA) content of *N. plumbaginifolia* wild type (WT) elicited with cellulase or arachidonic acid (AA). Leaf discs from WT plants were elicited with cellulase (0.5%), AA (1 mM) or incubated with water (Control) for the indicated times and ABA was extracted and quantified using an indirect ELISA method as described in the experimental section. Data represent the mean ± SEM of triplicate samples.

**Figure 5.** Functional analysis of *N. plumbaginifolia* ABAH cDNA (*NpABAH*) and ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) analysis of reaction products. UPLC-MS/MS analysis of extracts from WT plants overexpressing *NpABAH* (A) or the empty vector control (*NpΔABAH*) (B) incubated with exogenous ABA. (C) Chromatogram of ABA standard. *NpABAH* and *NpΔABAH* were expressed in leaves of WT *N. plumbaginifolia* using an *Agrobacterium* transient expression system. At 72 h post-transfection, 30 discs punched out from leaves overexpressing *NpABAH* or *NpΔABAH* were incubated with exogenous ABA (25 μM) for 20 h before chromatographic analysis. UPLC-MS/MS analysis was conducted using a selected ion monitoring (SIR)-MS mode. The SIR-MS chromatograms were
obtained by monitoring the transition of the parent mass \( m/z \): 263 for abscisic acid (ABA), 279 for phaseic acid (PA), 279 for 7'-hydroxy abscisic acid (7'-OH-ABA) and 281 for dihydrophaseic (DPA). The abbreviation refer to the relative intensity (RI).

Figure 6. Northern blot analysis of ABAH and GA-2Ox transcript levels in \( N. \) plumbaginifolia wild type, \( Npaba2 \) and \( Npaba1 \) mutant plants elicited with cellulase or infected by \( B. \) cinerea. Leaf discs were incubated with cellulase or infected by \( B. \) cinerea for the indicated times and processed as described in Fig. 3. Abbreviations refer to ABA 8'-hydroxylase (ABAH) and gibberellin 2-oxidase (GA-2Ox).

Figure 7. UPLC-MS/MS analysis of ABA catabolites in WT \( N. \) plumbaginifolia elicited with cellulase or infected by \( B. \) cinerea. Leaf discs were elicited with cellulase for 20h (A), infected with \( B. \) cinerea for 96 h (B) or incubated in water (control) for 96h (C) before extraction and analysis of ABA catabolites. The SIR-MS chromatograms were obtained as in Fig. 5. Abbreviations refer to abscisic acid (ABA), 7'-hydroxy abscisic acid (7'-OH-ABA), phaseic acid (PA), dihydrophaseic (DPA) and relative intensity (RI).
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