Abscisic Acid Negatively Regulates Elicitor-Induced Synthesis of Capsidiol in Wild Tobacco

Alexis Samba Mialoundama, Dimitri Heintz, Delphine Debayle, Alain Rahier, Bilal Camara*, and Florence Bouvier

Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique and Université de Strasbourg, 67084 Strasbourg cedex, France

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 wild tobacco (Nicotiana plumbaginifolia) sesquiterpenoid phytoalexin, using wild-type plants versus nonallelic mutants Npaba2 and Npaba1 that are deficient in ABA synthesis. Npaba2 and Npaba1 mutants exhibited a 2-fold higher synthesis of capsidiol than wild-type 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 and 5-epi-aristolochene hydroxylase. Treatment of wild-type plants with fluridone, an inhibitor of the upstream ABA pathway, recapitulated the behavior of Npaba2 and Npaba1 mutants, while the application of exogenous ABA reversed the enhanced synthesis of capsidiol in Npaba2 and Npaba1 mutants. Concomitant with the production of capsidiol, we observed the induction of ABA 8'-hydroxylase in elicited plants. In wild-type plants, the induction of ABA 8'-hydroxylase 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 of capsidiol synthesis but is essentially implicated in a stress-response checkpoint to fine-tune the amplification of capsidiol synthesis in challenged plants.

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 (Capsicum annuum) and tobacco (Nicotiana tabacum), the bicyclic sesquiterpene capsidiol represents the main type of phytoalexin. Capsidiol is synthesized from farnesyl diphasphate 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 with what we know about camalexin in Arabidopsis (Arabidopsis thaliana; 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 Ca2+, calmodulin, Ca2+-dependent protein kinases, and phosphoinositol-tide 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 (Solanum lycopersicum), 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 Duniwai, 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 β-1,3-
ABA-Dependent Regulation of Capsidiol Biosynthesis

The capacity of wild-type *N. plumbaginifolia* plants to produce capsidiol was first tested using cellulase and AA as elicitors and following infection by *B. cinerea*. The accumulation of capsidiol was analyzed at designated time intervals over a 24-h period for cellulase and AA and at 96 h for plants infected by *B. cinerea*. Total capsidiol produced by *Npaba2* and *Npaba1* mutants nearly doubled that observed for wild-type 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 cotreated with cellulase and fluridone (10 μM), the accumulation of capsidiol in leaf discs was strongly enhanced in wild-type plants, suggesting an ABA-dependent effect in leaf tissue (Fig. 2A). In contrast, the level of capsidiol in *Npaba2* and *Npaba1* 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 wild-type plants recapitulates the effect observed with *Npaba2* and *Npaba1* 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 cotreatment of wild-type, *Npaba2*, and *Npaba1* plants with ABA (25 μM) and cellulase or AA markedly reduced the induced synthesis of capsidiol in wild-type plants and in *Npaba2* and *Npaba1* mutants (Fig. 2). 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 *ZEP* and 9-cis-epoxycarotene dioxygenase (*NCED*), which encode enzymes of the upstream pathway of ABA synthesis. We observed that *EAS* and *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 (Hugueney et al., 1996; Fig. 3). However, the transcript levels of EAS and EAH were higher in Npaba2 and Npaba1 compared with wild-type plants (Fig. 3). The expression of the N. plumbaginifolia pathogenesis-related protein gene PR-1a (Payne et al., 1988), monitored by reverse transcription (RT)-PCR, displayed an expression pattern similar to that of EAS and EAH (data not shown) and is in agreement with the fact that exogenous ABA down-regulated the accumulation of β-1,3-glucanase (Rezzonico et al., 1998). The accumulation of ZEP and NCED mRNAs was observed in unelicited discs and during the initial period of elicitation (Fig. 3). During the period of capsidiol accumulation, ZEP and 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 semiquantitative RT-PCR analyses (data not shown). Consistent with these findings, the level of ABA in wild-type 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.

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 Npaba2 and

(Facchini and Chappell, 1992; Yin et al., 1997) and pepper (Hugueney et al., 1996; Fig. 3). However, the transcript levels of EAS and EAH were higher in Npaba2 and Npaba1 compared with wild-type plants (Fig. 3). The expression of the N. plumbaginifolia pathogenesis-related protein gene PR-1a (Payne et al., 1988), monitored by reverse transcription (RT)-PCR, displayed an expression pattern similar to that of EAS and EAH (data not shown) and is in agreement with
Npaba1 ABA-deficient mutants and in wild-type 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 concomitant to the induced synthesis of capsidiol. The main ABA catabolic pathway is mediated by 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 Arabidopsis challenged with Pseudomonas syringae, not only ABAH but also gibberellin 2-oxidase (GA-2Ox), which encodes a gibberellin catabolic oxidase, were induced (de Torres-Zabala et al., 2007). 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 wild-type plants were transfected with the expression plasmid (NpABAH) or the vector alone (NpΔABAH). At 72 h after 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 (tR) of each chromatographic peak and by tandem mass spectrometry (MS/MS) using 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
Mialoundama et al.

mode (data not shown) and single ion recording-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 monitoring 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<sub>R</sub> 1.54 min, mass-to-charge ratio [m/z] 281, daughter ion m/z 171), PA (t<sub>R</sub> 2.28 min, m/z 279, daughter ion m/z 139), 7'-OH-ABA (t<sub>R</sub> 2.85 min, m/z 279, daughter ion m/z 151), cis-ABA (t<sub>R</sub> 3.03 min, m/z 263, daughter ion m/z 153), and trans-ABA (t<sub>R</sub> 3.38 min, m/z 263, daughter ion m/z 153; Fig. 5; 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 wild-type plants 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) concomitant with the accumulation of EAS and EAH transcripts and the enhanced synthesis of capsidiol (Figs. 1 and 3). Consistent with these findings, ABA catabolites (PA, DPA, and 7'-OH-ABA) could be detected in wild-type plants following elicitation by cellulase or AA (data not shown) or after infection by B. cinerea relative to controls (Fig. 7A). Overall, the observations herein support the 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 plants to pathogens (for reviews, see Mauch-Mani and Mauch, 2005; Robert-Seilaniantz 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 the wild type and Npaba2 and Npaba1 lines of N. plumaginifolia to explore the effect of ABA on the regulation of capsidiol synthesis. First, we determined the capacity of different elicitors, including cellulase, AA, and B. cinereae, to induce the synthesis of capsidiol in challenged N.
plumbaginifolia. Although the synthesis of capsidiol was induced following cellulase and AA treatments and in response to infection by B. cinerea, the amount of capsidiol nearly doubled in Npaba2 and Npaba1 mutant lines compared with wild-type plants. Thus, ABA negatively impacted 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 wild-type, Npaba2, and Npaba1 plants were cotreated with fluridone and cellulase or AA and fluridone, the accumulation of capsidiol nearly doubled in wild-type plants and remained unchanged in Npaba2 and Npaba1 mutant plants (Fig. 2). Interestingly, a significant decrease in ABA content occurred during capsidiol synthesis in wild-type plants (Fig. 4). In a similar vein, ABA level was reduced in soybean (Glycine max) infected by Phytophthora megasperma (Cahill and Ward, 1989). Consistent with this trend, in sugar beet (Beta vulgaris) infected by 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 NCED and ZEP and is consistent with previous studies demonstrating that the geranylgeranyl diphosphate pathway leading to carotenoids is 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 peanut (Arachis hypogaea), drought stress is associated with increased susceptibility to Aspergillus flavus and reduced endogenous phytoalexin (Wotton and Strange, 1987). The synthesis of the two bean (Phaseolus vulgaris) phytoalexins, phaseolin and kievitone, is differently regulated following mercuric chloride elicitation. ABA down-

Figure 6. Northern-blot analysis of ABAH and GA-2Ox transcript levels in N. plumbaginifolia wild-type (WT), Npaba2, and Npaba1 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 Figure 3. rRNA, Ribosomal RNA.

Figure 7. UPLC-MS/MS analysis of ABA catabolites in wild-type N. plumbaginifolia elicited with cellulase or infected by B. cinerea. Leaf discs were elicited with cellulase for 20 h (A), infected with B. cinerea for 96 h (B), or incubated in water (control) for 96 h (C) before extraction and analysis of ABA catabolites. The selected ion recording (SIR)-MS chromatograms were obtained as in Figure 5. RI, Relative intensity; TIC, total ion chromatogram.
regulates mercuric chloride-induced kievitone synthesis, while phaseolin synthesis is unaffected (Goossens and Vendrag, 1982). In soybean, following infection by *P. megasperma* (Ward et al., 1989) or *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 lumbin sesquiterpenoid phytoalexins by application of exogenous ABA has been shown in potato tubers infected by *P. infestans* (Henfling et al., 1980; Bostock et al., 1983). However, the reduction of rishitin and lumbin could only be demonstrated after a cold pretreatment at 4°C (Bostock et al., 1983). The role of the cold treatment in the measured responses is not understood at present.

The mechanism whereby ABA mediates its effects on capsidiol synthesis requires further study. 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 usually been highlighted. However, the situation is probably more complex, because the hypersensitivity of the Arabidopsis enhanced disease resistance1 mutant to ABA is associated with enhanced expression of **PK1** 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, which regulate GCC box-containing defense genes (Zhou et al., 2008). According to this hypothesis, ABA affects the interaction between ethylene response factors and GCC boxes. High ABA content destabilizes ethylene response factors 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** has GCC boxes. Furthermore, the ethylene biosynthetic inhibitor aminoethoxyvinylglycine does not inhibit the accumulation of capsidiol in tobacco as elicited by *Phytophthora nicotianae* (Nemestoathy and Guest, 1990). Alternatively, ABA may play a role via cADP-Rib, an intracellular calcium mobilizer (Wu et al., 1997). Consistent with this hypothesis, the synthesis of sesquiterpenoid phytoalexin rishitin in potato (Zook et al., 1983) 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 concomitant with capsidiol accumulation. We first assessed the functionality of *NpABAH*. Transient overexpression of **ABAH** in wild-type *N. plumaginifolia* leaf incubated with exogenous ABA resulted in the accumulation of the typical ABA catabolic products PA, DPA, and 7-OH-ABA (Fig. 5). In wild-type 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 is affected by the gibberellins as well. **GA-2Ox** was induced in parallel with 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 (*Malus domestica*) trees to *Rewinia amylovora* concomitant with an increased expression of genes encoding pathogenesis-related proteins (Maxson and Jones, 2002). In addition, in rice (*Oryza sativa*), overexpression of the **EAS** 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 *R. solanacearum* also revealed induction of **GA-2Ox** and **ABAH** (Hu et al., 2008). An mRNA differential display analysis of *N. tabacum* infected by *R. 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 (Wang et al., 2006, 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 pos-
sibilities may exist. For example, it has been reported that in Arabidopsis infected by the pathogen *Blumeria graminis*, a NAC transcription factor designated ATAF1 down-regulates the accumulation of ABA according to an unknown mechanism while enhancing the resistance to the pathogen (Jensen et al., 2008).

Implication of 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 the negative regulation exerted by ABA ensuring that an adequate level of capsidiol is synthesized in challenged plants. Once the challenge is mastered, ABA down-regulates the synthesis of capsidiol to restrict its overaccumulation, which 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 nonclassical 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 nonplant organism development. In this context, *Cryptococcus* species are lethal fungal pathogens of humans and animals that can complete their life cycle on plant surfaces due to the presence of myoinositol exudate from Arabidopsis and *Eucalyptus* leaves (Xue et al., 2007). Interestingly, auxin acts synergistically with myoinositol to stimulate the mating of *Cryptococcus* species (Xue et al., 2007). It is interesting 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 is 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 nodule initiation, suggesting a wider implication of ABA and probably GA in the fine-tuning of biotic interactions through a negative regulatory mode.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Elicitation Procedure

*Nicotiana plumbaginifolia* wild-type seeds and *Npaba2* and *Npaba1* mutant seeds were kindly provided by Dr. A. Marion-Poll (INRA). *Npaba2* of *N. plumbaginifolia* is impaired in the epoxidation of zeaxanthin catalyzed by ZEP (Marin et al., 1996), and *Npaba1* in the last step of ABA biosynthesis (i.e., the oxidation of ABA-aldehyde into ABA, catalyzed by ascobic aldehyde oxidase; Knappiel et al., 1994). Plants were grown under greenhouse conditions or in growth chambers saturated at 80% relative humidity for *Npaba2*. Leaf discs (1.75 cm diameter) were excised with a cork borer from leaves of 75-d-old plants. Leaf discs were floated (adaxial surface up) on a solution of cellulose (0.5%) from *Trichoderma viride* (Sigma-Aldrich; Hugueney et al., 1996) or 1 mM AA (Sigma-Aldrich; 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 and Université de Strasbourg) was used to infect leaf discs as described previously (Assenbergh et al., 2007). Triplicate samples of 10 leaf discs were used for each treatment. ABA (Sigma-Aldrich) and fluridone (Duchefa) solubilized in ethanol:water (90:10, v/v) were added to the elicitation medium at 25 and 10 μM concentrations, respectively. The incubation was carried out at 25°C for the indicated periods 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 silica gel preconditioned with cyclohexane. Apolar compounds were eluted with cyclohexanemethyl acetate (80:20, v/v), and the capsidiol fraction was eluted with cyclohexanemethyl acetate (50:50, v/v). The dried sample was analyzed by 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 1 M sodium hydroxide and 0.2% (v/v) acetic acid to a final concentration of 10,000 μM for 10 min. The dichloromethane extract was subjected to prepurification by thin-layer chromatography on a 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 and 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, and 7-OH-ABA from leaf disc extracts was performed by comparing tR, MS transitions, and MS/MS analysis using UPLC-MS/MS. All analyses were performed using a Waters Quattro Premier XE equipped with an ESI source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was achieved using an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters) coupled to an Acquity UPLC BEH C18 precolumn (2.1 × 5 mm, 1.7 μm; Waters). The mobile phase consisted of the following solvents: 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, 100% methanol 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), and C to D (2 min) before isocratic elution using D (2 min) and linear gradient to A (0.5 min), followed by an isocratic run using A (0.5 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⁻¹ (sample injection volume of 3 μL). Nitrogen generated from pressurized air in a N2G nitrogen generator (Mistral; Schmidlin-dbs-AG) was used as the drying and nebulizing gas. The nebulizer gas flow was set to 400 L h⁻¹ and the desolvation gas flow to 900 L h⁻¹.

Plant Physiol. Vol. 150, 2009 1563

Copyright © 2009 American Society of Plant Biologists. All rights reserved.

www.plantphysiol.org on July 18, 2017 - Published by Downloaded from
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 optimized for each molecule. The selected ion recording-MS mode was used to determine parent mass transitions of ABA (m/z 263), PA (m/z 279), DPA (m/z 281), and 7-‘OH-ABA (m/z 279). Fragmentation was performed by collision-induced dissociation with argon at 1.0 × 10−3 mbar. The collision energy was optimized for each compound using data-dependent monitoring and MRM. Mass spectrometry conditions for ABA and ABA catabolites were set after optimization as follows (polarity, ES−; capillary, 3 kV; cone, 25 V) for ABA, PA, DPA, and 7-‘OH-ABA. Low and high mass resolution were 13 for both mass analyzers, ion energies 1 and 2 were 0.5 V, entrance and exit potential were 2 and 1 V, and detector (multiplier) gain was 650 V. Collision-induced dissociation of deprotonated parent ions was accomplished with a collision energy of 10 V for ABA, PA, and DPA and 20 V for 7-‘OH-ABA. For ABA and ABA catabolites (PA, DPA, and 7-‘OH-ABA), daughter scan monitoring and MRM permitted the identification for each compound of the transition from deprotonated parent ion to the predominant daughter fragment ion. The combination of chromatographic bp, parent mass, and unique fragment ion analysis was used to selectively monitor ABA (263 > 153), PA (279 > 139), DPA (281 > 189 > 171), and 7-‘OH-ABA (279 > 151). Data acquisition and analysis were performed with MassLynx software (version 4.1) running under Windows XP Professional on a Pentium personal computer.

Quantitative Analysis of ABA

ABA was extracted as described above using tr-cis-trans-[G-3H]ABA (55 Ci mmol−1; GE Healthcare) to evaluate the recovery and quantified as described previously (Xiong et al., 2001) using an ELISA kit (Phyto detek ABA; AGDIA) according to the manufacturer’s protocol.

Cloning and Functional Characterization of ABAH

NpABAH cDNA was amplified by RT-PCR as described previously (Bouvier et al., 2006) using forward (5′-AGTACATAAATTTTATTTTC-3′) and reverse (5′-GGTTGAAGTGTAGATTTCTTTCCAAAAT3′) primers and Phusion High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer’s instructions. cDNA was cloned into the XhoI site of pKYLX713352 vector (Maiti et al., 1995; Bouvier et al., 2006). Following sequence verification, the resulting plasmid designated pK(Np)ABAH and an empty control vector designated pK(Np)AVAH were transformed into Agrobacterium tumefaciens strain GV3101. Before plant infiltration, Agrobacterium harboring pK(Np)ABAH and pK(Np)AVAH was diluted to an optical density at 600 nm of 0.2 using the infiltration buffer (10 mM MES, pH 5.6, 1 mM sodium phosphate, 0.5% Glc, and 2 mM MgCl2) and injected through the stomata on the lower epidermal surface of leaves of 75-d-old wild-type N. plumbaginifolia plants using a 1-mL plastic syringe without a needle (Bakoto et al., 2000). Expression of the transgene was monitored by RT-PCR using the forward primer (5′-CATACTCTTTGCAGCAAGGCTCT-3′) specific for the vector NpABAH and the reverse primer (5′-GATATGAGTGTGTTTAGAAGTG-3′) specific for NpAVAH to amplify an expected 856-bp band. Ethidium bromide staining of ribosomal RNAs was used as a loading control. At 3 d after infiltration, transcribed plant leaves were incubated with 25 μM ABA for 20 h before extraction and analysis of ABA catabolites.

RNA Gel-Blot Analysis

Total RNA from leaf discs was prepared using the NucleoSpin RNA plant kit from Macherey-Nagel. Following agarose gel electrophoresis and transfer onto nylon membranes, filters were hybridized overnight with cDNA probes and processed as described previously (Bouvier et al., 2006) using forward (5′-GGCAATGAACTCTTGAAGTG-3′) and reverse (5′-CAAACCCCTGAGTATGTTGCT-3′) primers. The sequences reported in this paper have been deposited in the GenBank/EMBL data libraries under the following accession numbers: FM244692 for NpABAH, FM244696 for NpEAH, FM244695 for NpEAS, FM244693 for NpGA-2Ox, and FM244694 for NpNCED.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. GC-MS analysis of capsid produced by N. plumbaginifolia following elicitor treatments or infection with B. cinerea.

Supplemental Figure S2. UPLC-MS/MS analysis using MRM of leaf disc extracts from wild-type N. plumbaginifolia overexpressing NpABAH.

ACKNOWLEDGMENTS

We thank Dr. A. Marion-Poll for the kind gift of seeds of wild-type and ABA-deficient mutants of N. plumbaginifolia and Drs. S. Wiedmann-Merdinoglu and P. Hugueney for B. cinerea cultures. We thank A. Bailly, M. Kerneis, S. Staerck, and R. Wagner for taking care of the plants and M. Alioua for DNA sequencing. The UPLC-MS/MS apparatus was cofinanced by the CNRS, the Université Louis Pasteur, the Région Alsace, the INRA, and the Tepal Company.

Received March 10, 2009; accepted May 1, 2009; published May 6, 2009.

LITERATURE CITED


Bostock RM, Nuckles E, Henfling JWDM, Kuc JA (1983) Effects of potato
tuber age and storage on sesquiterpenoid stress metabolite accumulation, steroid glycolaldehyde accumulation, and response to abscisic and arachidonic acids. Phytopathology 73: 435–438


Maiti IB, Murphy JF, Shaw JG, Hunt AG (1993) Plants that express a polyvirus Vpg:proteinase gene are resistant to virus infection. Proc Natl Acad Sci USA 90: 6110–6114


Rezzonico E, Flury N, Meins F Jr, Beffa R (1998) Transcriptional down-


