Oxo-Phytodienoic Acid-Containing Galactolipids in Arabidopsis: Jasmonate Signaling Dependence[1][W][OA]

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The jasmonate family of phytohormones, as represented by 12-oxo-phytodienoic acid (OPDA), dinor-phytodienoic acid (dn-OPDA), and jasmonic acid in Arabidopsis (*Arabidopsis thaliana*), has been implicated in a vast array of different developmental processes and stress responses. Recent reports indicate that OPDA and dn-OPDA occur not only as free acids in Arabidopsis, but also as esters with complex lipids, so-called arabidopsides. Recently, we showed that recognition of the two bacterial effector proteins AvrRpm1 and AvrRpt2 induced high levels of a molecule consisting of two OPDAs and one dn-OPDA esterified to a monogalactosyl diacylglycerol moiety, named arabidopside E. In this study, we demonstrate that the synthesis of arabidopsides is mainly independent of the prokaryotic lipid biosynthesis pathway in the chloroplast, and, in addition to what previously has been reported, arabidopside E as well as an all-OPDA analog, arabidopside G, described here accumulated during the hypersensitive response and in response to wounding. We also show that different signaling pathways lead to the formation of arabidopsides during the hypersensitive response and the wounding response, respectively. However, the formation of arabidopsides during both responses is dependent on an intact jasmonate signaling pathway. Additionally, we report inhibition of growth of the fungal necrotrophic pathogen *Botrytis cinerea* and in planta release of free jasmonates in a time frame that overlaps with the observed reduction of arabidopside levels. Thus, arabidopsides may have a dual function: as antipathogenic substances and as storage compounds that allow the slow release of free jasmonates.

The jasmonates (cyclic octadecanoids represented by 12-oxo-phytodienoic acid [OPDA], dinor-phytodienoic acid [dn-OPDA], and jasmonic acid [JA]) have been implicated in a vast array of different developmental processes and stress responses (Blee, 2002; Feussner and Wasternack, 2002; Howe and Schilmiller, 2002; Wasternack, 2007). In particular, JA has a well-established function as a signaling molecule in mechanotransduction, senescence, flower development, and responses to necrotrophic pathogens and herbivores (Thomma et al., 2001; La Camera et al., 2004). In addition, OPDA and its C-16 analog dn-OPDA (Weber et al., 1997) have also been found to act as signaling molecules in higher plants (Stintzi et al., 2001; Taki et al., 2005).

Jasmonates are formed from the peroxidation product of α-linolenic acid, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT; Feussner and Wasternack, 2002). The hydroperoxide is cyclized to OPDA by the sequential action of allene oxide synthase (AOS) and allene oxide cyclase in the plastid. The final step, converting OPDA to JA, takes place in the peroxisome and includes the reduction of a double bond and three rounds of β-oxidation to shorten the length of the carboxy-terminal carbon chain. Several reports indicate that OPDA and dn-OPDA not only occur as free acids in Arabidopsis (*Arabidopsis thaliana*) tissue but also as esters with complex lipids (Stelmach et al., 2001; Hisamatsu et al., 2003; Hisamatsu et al., 2005; Andersson et al., 2006a; Buseman et al., 2006). These complex lipids include derivatives of mono- and digalactosyl diacylglycerol (MGDG and DGDG), respectively, containing one or two chains of OPDA and/or dn-OPDA. MGDG and DGDG derivatives

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containing OPDA at both the *sn-1* and *sn-2* positions were given the names arabidopsides B and D, respectively, whereas the derivatives with OPDA at the *sn-1* position and dn-OPDA at the *sn-2* position were named arabidopsides A and C, respectively. Arabidopsis also contains MGDG and DGDG molecular species containing only one OPDA or dn-OPDA moiety at either the *sn-1* or *sn-2* position, respectively (Buseman et al., 2006). MGDG with dn-OPDA esterified to the *sn-2* position was recently given the trivial name arabidopside F (Nakajyo et al., 2006). The different OPDA- and dn-OPDA-containing galactolipids were recently reported to accumulate after wounding of Arabidopsis leaves (Buseman et al., 2006; Böttcher and Weiler, 2007).

The current understanding is that the enzymes in the synthetic pathway of OPDA and JA accept only free fatty acids. Thus, accumulation of OPDA-containing galactolipid derivatives would depend on the release of trienoic fatty acids from membrane lipids and the subsequent re-esterification of OPDA and dn-OPDA to the corresponding glycerolipids. The very fast kinetics of accumulation of OPDA-containing galactolipids has led to the suggestion that the OPDA biosynthesis pathway may also accept lipid-bound fatty acids (Buseman et al., 2006). However, the latter suggestion could not be corroborated by in vitro experiments with the purified enzymes (Stelmach et al., 2001). Arabidopsis is a so-called 16:3 plant and thus has a very active de novo synthesis of galactolipids in the plastids. Non-16:3 plants, on the other hand, depend entirely on the import of diacylglycerol backbones from the endoplasmic reticulum to support chloroplast galactolipid synthesis (Ohlrogge and Browse, 1995). Therefore, the formation of OPDA-containing galactolipids after wounding or elicitation may be caused by the rapid incorporation of newly synthesized OPDA in the plastid lipid biosynthesis pathway. In support of this possibility, OPDA and dn-OPDA appear to be preferentially associated with galactolipids. The only OPDA molecule so far reported to occur in the phospholipid fraction is OPDA esterified to the *sn-1* position of phosphatidylglycerol with trans-hexadecenoic acid at the *sn-2* position; this phospholipid is specifically synthesized within the plastid (Buseman et al., 2006).

Plants can recognize a variety of different molecular patterns associated with pathogenic micro-organisms and mount defense (Nürnberger and Scheel, 2001; Nürnberger et al., 2004). Bacterial pathogens use, among other mechanisms, secreted effector proteins to promote their virulence. However, the presence of some effector proteins can be detected by the plant surveillance system and thus betray the pathogen to the host. The latter case, in which a plant resistance (R) protein recognizes a bacterial avirulence (Avr) protein, often triggers the hypersensitive response (HR; Dangl and Jones, 2001; Nimchuk et al., 2003). The HR is comprised of localized cell death followed by accumulation of anti-pathogenic substances and cell wall fortifications in surrounding cells. The response as a whole efficiently stops the pathogen from colonizing the host. The *Pseudomonas syringae* effector proteins AvrRpt2 and AvrRpm1 are well-characterized examples of bacterial avirulence proteins. AvrRpm1 is detected by the Arabidopsis R protein RPM1 and AvrRpt2 by RPS2. We previously used chemically inducible in planta expression of these avirulence peptides in Arabidopsis to study the effects of their recognition on phospholipids (Andersson et al., 2006b) and oxylipins (Andersson et al., 2006a). Recognition of AvrRpm1 and AvrRpt2, like wounding, induced accumulation of very high levels of galactolipid-bound OPDA and dn-OPDA. In this case, however, most of the lipid-bound OPDA and dn-OPDA was found to be esterified to yet another MGDG derivative, arabidopside E. This lipid consists of MGDG carrying dn-OPDA on the *sn2* position and OPDA on the glycerol *sn1* and the Gal C6′ position.

The proposed function(s) of the arabidopsides and other OPDA-containing galactolipids is presently rather speculative. Arabidopside A appears to have senescence-promoting effects on barley (*Hordeum vulgare*) leaf tissue (Hisamatsu et al., 2006); arabidopsides A, B, and D seem to possess inhibitory effects on root growth (Hisamatsu et al., 2005); and arabidopside E inhibits growth of *P. syringae* in vitro (Andersson et al., 2006a). In addition, it has also been suggested that the arabidopsides may function as a pool for a slow or delayed release of free OPDA, which is then converted to JA (Stelmach et al., 2001; Andersson et al., 2006a; Buseman et al., 2006).

This study addresses the questions of arabidopside formation in response to bacterial pathogens and wounding and provides additional evidence of the function of these compounds in plant cells. Moreover, we show that the synthesis of arabidopsides is independent of the prokaryotic lipid biosynthesis pathway. We also identified the previously uncharacterized arabidopside G, which is an all-OPDA analog of arabidopside E. Arabidopside accumulation during wounding and the HR seems to depend on two different signaling pathways. However, arabidopside formation during both the HR and wounding is dependent on an intact jasmonate signaling pathway. Thus, wounding and HR-specific pathways appear to converge at the level of jasmonate signaling. We also found that the antimicrobial function of arabidopsides E and G extends to the fungal necrotrophic pathogen *Botrytis cinerea* and that arabidopsides are most likely processed to form free jasmonates.

RESULTS

Accumulation of Arabidopsides after Recognition of AvrRpm1

This study analyzed the formation and function of arabidopsides in response to bacterial pathogens and wounding. Therefore, a system for quick and reliable

quantification of arabidopsides in plant tissue was first devised. To this end, we used reversed-phase HPLC (RP-HPLC) of glycolipids isolated from plant tissue. RP-HPLC was previously employed to purify arabidopside E after partial purification on thin-layer silica plates (Andersson et al., 2006a). We used a simple gradient system consisting of acetonitrile:water and propylalcohol. This gradient efficiently eluted all galactolipids containing normal fatty acids from the glycolipid fraction obtained by silica column fractionation of Arabidopsis leaf lipid extract (not shown). To detect OPDA- and dn-OPDA-containing galactolipids, absorption at 220 nm was monitored. The HPLC system reliably resolved arabidopsides A, B, and E, as determined with authentic lipid standards. The content of arabidopsides A, B, and E in healthy unwounded Arabidopsis leaf tissue ranged from below detection level to approximately 2 nmol g fresh weight $(FW)^{-1}$ for arabidopside E and 10 to 20 nmol g FW^{-1} for arabidopside A.

To validate this methodology, we measured the accumulation of arabidopsides using the dexamethasone (Dex)-inducible expression of AvrRpm1 in planta (compare with "Materials and Methods"). The expression of the bacterial avirulence peptide AvrRpm1 in the wild-type background caused a massive accumulation of arabidopside E (Fig. 1, A and B), whereas the expression of AvrRpm1 in the *rpm1* genetic background, which likely is a protein null mutant and therefore unable to recognize AvrRpm1, had no effect on the content of arabidopsides (Fig. 1A). Arabidopside E accumulated to about 100 nmol g FW^{-1} 4 h after induction of AvrRpm1 expression in the wild-type background, in good agreement with what was previously reported (Andersson et al., 2006a). At a later time point, 24 h postinduction, the amount of arabidopside E had returned to background levels. The recognition of AvrRpm1 also caused an increase in the amount of arabidopside A, which rose to about 20 nmol g FW^{-1} after an initial decrease. Interestingly, the recognition of AvrRpm1 also appeared to induce the accumulation of another strongly UV-absorbing compound that eluted from the column approximately 2 min after arabidopside E (Fig. 1A).

The Arabidopsis mutant *act1* has a strongly reduced activity in plastidial 3-P-glycerol acyl transferase and is thereby only able to synthesize diacylglycerol backbones inside the plastid in minor amounts (Kunst et al., 1988, 1989). As a consequence of this, plastid MGDG in the *act1* mutant contains almost no 16:3 and thus resembles MGDG from 18:3 plants. The expression of AvrRpm1 in the *act1* background had the same visual effect as in the wild-type background. The release of cellular electrolytes, bleaching, and trypan blue positive staining was indistinguishable in AvrRpm1/ecotype Columbia (Col-0) and AvrRpm1/*act1* leaves treated with Dex (not shown). However, the HPLC profile of the glycolipid fraction obtained from AvrRpm1/*act1* material 4 h after induction of AvrRpm1 expression differed dramatically from that of AvrRpm1/Col-0 (Fig. 1A). The peak representing arabidopside E was completely lost in the AvrRpm1/*act1* material; instead, the slower migrating peak previously observed in AvrRpm1/Col-0 material was considerably.

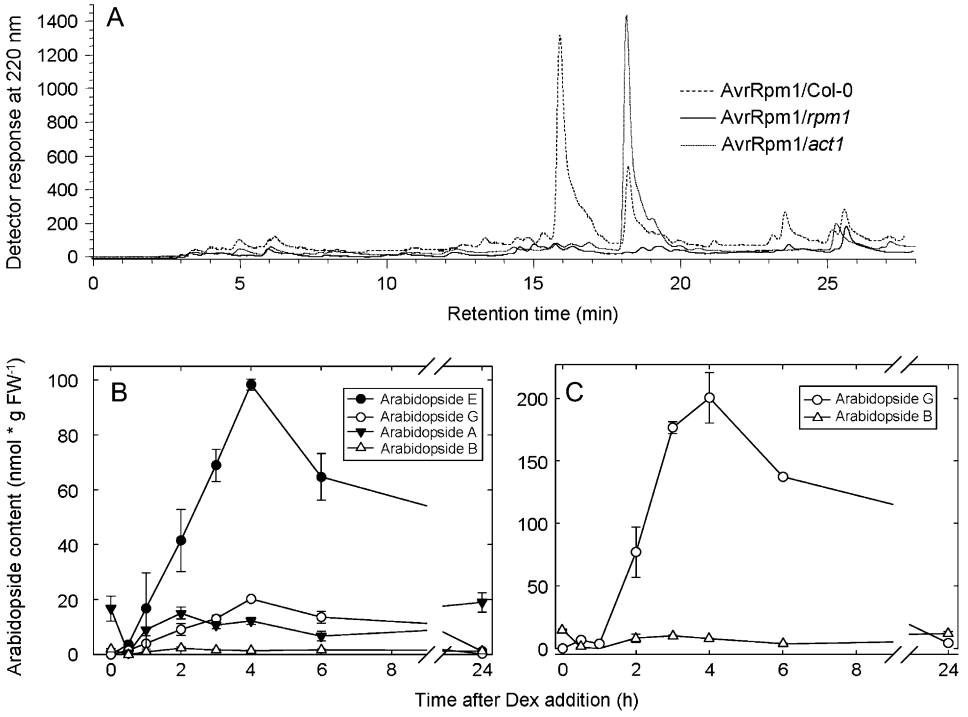

Figure 1. Formation of arabidopsides after recognition of AvrRpm1. Leaf discs obtained from AvrRpm1/Col-0, AvrRpm1/*rpm1*, and AvrRpm1/*act1* plants were incubated with Dex to induce expression of AvrRpm1. A, The lipids were extracted after 4 h, and the glycolipids were separated by RP-HPLC with UV detection at 220 nm. B and C, AvrRpm1/Col-0 (B) or AvrRpm1/*act1* (C) material was extracted at the time points indicated, the glycolipids separated by HPLC, and the amounts of arabidopsides E (black circles), G (white circles), A (black triangles), and B (white triangles) determined. Mean values and range of duplicate samples are shown.

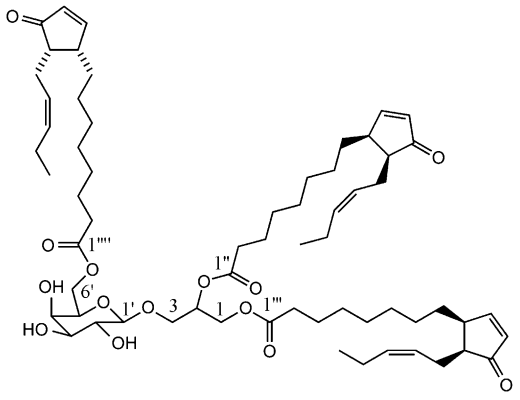

Figure 2. Structure of arabidopside G. A previously uncharacterized OPDA-containing galactolipid was isolated from AvrRpm1/*act1* material incubated with Dex to induce expression of AvrRpm1. The structure was solved with mass spectrometry and NMR spectroscopy. Numbers denote atom numbers for the assigned NMR signals given in "Materials and Methods."

The Structure of a Novel OPDA-Containing Lipid, Arabidopside G

The novel compound described above was prepared in large scale from AvrRpm1/*act1* leaf tissue after induction of AvrRpm1 expression. The purified compound possessed a UV absorption spectrum very similar to that of arabidopside E, with an absorption maximum at 220 nm in ethanol. The compound migrated very close to arabidopside E in a thin-layer chromatography system consisting of chloroform: methanol (85:15, v/v), and a sugar-reactive spray reagent demonstrated that the isolated compound contained sugar residues. Hydrolysis of the purified compound and subsequent analysis with gas chromatography coupled to mass spectrometry of methylated hydrolysis products revealed that the purified compound contained OPDA but no detectable dn-OPDA (not shown). An electrospray ionization mass spectrum recorded in the negative mode revealed that the compound had a fragmentation pattern consistent with three OPDA residues and a molecular mass of 1,076 D (Supplemental Fig. S1). The mass spectra and chemical properties of the isolated substance are thus consistent with a molecular species of MGDG harboring three OPDA groups. This structure was confirmed by NMR spectroscopy. These data were reminiscent of those for arabidopside E (two OPDA and one dn-OPDA acyl groups; Andersson et al., 2006a) but of reduced complexity due to increased symmetry of the acyl groups. As for arabidopside E, the placement of ester groups at C-1 and C-2 and the sugar residue at C-3 of the glycerol moiety in this novel compound were evident from downfield chemical shifts for H_2-1 (δ 4.45 and 4.22) and H-2 (δ 5.27 ppm) compared to the relatively upfield shifts for H_2-3 (δ 3.90 ppm, 3.76 ppm). Coupling constants and NOE correlations between methine protons around the sugar pyran ring were consistent with a Gal residue. The deshielded methylene H_2-6′ (δ 4.33 and 4.21) of this sugar showed HMBC correlations to a carbonyl at δ 175.3, confirming acyl group substitution at the C-6′ alcohol. In a two-dimensional NOESY experiment, a strong NOE correlation from the three-proton multiplet at δ 2.49 (H-13″/13‴/13⁗) to the isochronous protons H-9″, H-9‴, and H-9⁗ (δ 3.06) is consistent with a cis-ring junction in the three OPDA acyl groups. As for arabidopside E, isochronicity of the chemical shifts for the acyl group ω-3 and ω-4 olefinic protons precluded assignment of the double bond geometry by coupling constant analysis. However, the geometry of the ω-3 double bonds in all three acyl groups was evident from the chemical shift of the ω-2 carbon at δ 21.9 (for trans ω-3 olefins, the ω-2 ^{13}C NMR shift would be δ 30–33 ppm; Pfeffer et al., 1977, 1992).

Thus, the isolated compound is an analog of arabidopside E with the dn-OPDA at the *sn-2* position substituted by OPDA (Fig. 2). We propose the trivial name arabidopside G for this previously unreported 1,2-di-*O*-(12-oxophytodienoyl)-3-*O*-(6′-*O*-(12-oxophytodienoyl)-β-D-galactopyranosyl)-sn-glycerol. The content of arabidopside G increased after AvrRpm1 expression in wild-type background in parallel to arabidopside E (Fig. 1B). In the wild-type background, the amount of arabidopside G corresponded to approximately 20% of the amount of arabidopside E, whereas arabidopside G dominated completely and accumulated to approximately 200 nmol g FW^{-1} when AvrRpm1 was expressed in the *act1* background (Fig. 1C). In the latter material, the amount of arabidopside G also decreased back to background levels after 24 h.

HR Induced by AvrRpt2 Expressed by *P. syringae* Promotes Accumulation of Arabidopsides E and G

Accumulation of arabidopsides E and G as a consequence of in planta expression and recognition of AvrRpm1 spurred us to test if this accumulation is also triggered in response to a "real" pathogen expressing an Avr protein. For this purpose, we chose *P. syringae* pv *tomato* (*Pst*) expressing *AvrRpt2*, DC3000(*avrRpt2*). Inoculation of wild-type tissue with DC3000(*avrRpt2*) led to accumulation of primarily arabidopsides E and G (Fig. 3A). Eight hours postinoculation, arabidopside E accumulated to about 50 nmol g FW^{-1}. Arabidopside G accumulated to about 10 nmol g FW^{-1}. In addition, there was also some effect on the amounts of arabidopside A; during the first 2 h, it decreased from about 15 nmol g FW^{-1} to almost below detection level and then subsequently increased again to just below 10 nmol g FW^{-1}. After 8 h, the amount of all detected arabidopsides decreased; however, these values returned to background levels 24 h postinoculation. Interestingly, HR induced by DC3000(*avrRpt2*) did not cause any increase in arabidopside content in systemic leaves. Mock inoculation with only $MgCl_2$ did not affect the arabidopside content throughout the time course investigated (Fig. 3B; data not shown). The

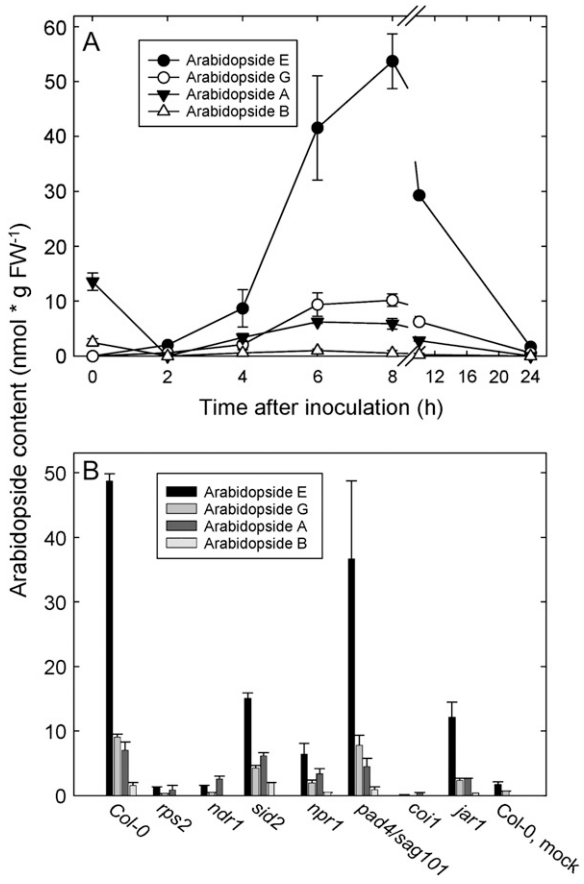

Figure 3. Formation of the arabidopsides during the HR. A, Formation of arabidopsides after elicitation with *P. syringae* DC3000(*AvrRpt2*); leaf discs were punched out and incubated in water for 24 h; the lipids were extracted at the times indicated. The amounts of arabidopsides E (black circles), G (white circles), A (black triangles), and B (white triangles) were determined in the glycolipid fraction. B, Leaves of Col-0, *rps2*, *ndr1*, *pad4sag101*, *sid2*, and *npr1* were infiltrated with *P. syringae* DC3000(*AvrRpt2*) or mock infiltrated with $MgCl_2$, leaf discs were punched out and incubated in water for 8 h, and the lipids were extracted. The amounts of arabidopsides E, G, A, and B were determined in the glycolipid fraction. Mean values and range of duplicate samples are shown.

Arabidopsis mutant *rps2* is a likely protein null for the R protein responsive to AvrRpt2 and thus highly susceptible to DC3000(*avrRpt2*) (Mindrinos et al., 1994). As expected, inoculation of *rps2* tissue with DC3000(*avrRpt2*) resulted in no formation of arabidopsides E and G after 8 h (Fig. 3B).

The Arabidopsis mutants *non-race-specific disease resistance1* (*ndr1*; Century et al., 1995), *salicylic acid induction-deficient2* (*sid2*)/*isochorismate synthase1* (Nawrath and Metraux, 1999), *nonexpresser of PR genes1* (*npr1*; Cao et al., 1994), and *pad4sag101* (*phytoalexin-deficient4* and *senescence-associated gene101*) double mutant (Feys et al., 2005) are all affected at different levels and branches of disease resistance signaling. The *ndr1* mutant is impaired in R-gene signaling and defense. However, the HR in this mutant is blocked only in the case of recognition of AvrRpt2 and not AvrRpm1. The double mutant *pad4sag101* is blocked in disease resistance signaling originating from a number of other Toll-interleukin receptors but responds like wild type to AvrRpt2. The *sid2* and *npr1* mutants are affected in salicylic acid (SA) production and signaling, respectively, and are thus more susceptible to virulent *Pseudomonas* strains. Both mutants, however, induce wild type like HR after recognition of avirulence peptides (Volko et al., 1998; Nawrath and Metraux, 1999; Clarke et al., 2000).

The accumulation of arabidopsides E and G after inoculation with DC3000(*avrRpt2*) was almost completely absent after 8 h in *ndr1* plants (Fig. 3B). The accumulation of arabidopsides was also severely reduced in *sid2* and *npr1* plants. The *pad4sag101* double mutant, on the other hand, accumulated arabidopsides E and G to levels comparable to those in the wild type after recognition of AvrRpt2.

JA Dependence of Arabidopside Formation during the HR

JA is a well-established phytohormone and a signal molecule in the response by plants to wounding and pathogens. We therefore investigated the involvement of jasmonates in the signal transduction pathway leading to formation of the arabidopsides. For this purpose, we tested the accumulation of arabidopsides in the two JA-insensitive mutants *coronatine insensitive1* (*coi1*; Feys et al., 1994; Xie et al., 1998) and *jasmonate resistant1-1* (*jar1-1*; Staswick et al., 1992) after inoculation with DC3000(*avrRpt2*). The accumulation of arabidopsides E and G was, as compared to the wild type, reduced by about 80% in the *jar1* mutant and completely abolished in the *coi1* mutant 8 h after inoculation (Fig. 3B). The content of arabidopsides in untreated *coi1* and *jar1* was below 2 nmol g FW^{-1} (not shown).

Non-HR-Dependent Bacterial Pathogen Defense Does Not Cause Arabidopside Accumulation

In addition to the avirulent DC3000(*avrRpt2*), we also tested the virulent *Pst* strain (DC3000); the *hrcC* mutant strain, deficient in the type three secretion system; and the *P. syringae* pv *phaseolicola* strain, for which Arabidopsis is a nonhost. None of the three strains induce HR. While DC3000 is highly virulent in the Arabidopsis (Col-0) wild type, the other two strains are completely avirulent in wild-type Arabidopsis. None of the three strains induced any detectable increase in the levels of arabidopside E, G, A, or B in wild-type plants during the first 24 h of infection (not shown).

Formation of Arabidopsides in Wounded Tissue

Because arabidopsides A and B were previously shown to accumulate in Arabidopsis leaf tissue after wounding (Buseman et al., 2006), we tested whether accumulation of arabidopsides E and G could also be

induced by wounding. Wild-type (Col-0) Arabidopsis leaves were wounded with a hemostat, and the effect on the tissue content of arabidopsides A, B, E, and G was monitored (Fig. 4A). Upon wounding, there was an accumulation of all four measured arabidopsides. The amount of all arabidopsides increased transiently from 2 to 8 h and then decreased until 24 h. Arabidopside A accumulated to the highest amounts, up to 140 nmol g FW^{-1}, whereas arabidopside E and G content peaked at about 80 and 10 nmol g FW^{-1}, respectively. Just like during the HR, the accumulation of arabidopsides was restricted to the treated tissue only. The amounts of arabidopsides in adjacent, unwounded tissue and systemic leaves were unaffected (not shown).

When leaves from the *act1* mutant were wounded, only arabidopsides B and G accumulated to levels corresponding more or less to those of arabidopsides E and A in wild-type tissue (Fig. 4B). The disease signaling mutants *ndr1*, *sid2*, *npr1*, and *rps2* all accumulated as much arabidopsides after wounding as did the wild type (Fig. 4B). The two JA-insensitive mutants *coi1* and *jar1*, on the other hand, displayed severely reduced accumulation of arabidopsides after wounding (Fig. 4B). Accumulation of arabidopsides after wounding was reduced by about 70% in *jar1* and completely abolished in *coi1*.

We also tested whether wounding could lead to accumulation of arabidopsides in plants other than Arabidopsis. Leaves from *Brassica napus*, *Nicotiana tabacum*, *Pisum sativum*, *Spinacia oleracea*, *Avena sativa*, and barley were wounded with a hemostat, the lipids extracted after 2 h, and the amounts of arabidopsides A, B, E, and G were quantified. None of the tested species appeared to accumulate any arabidopside A, B, E, or G after wounding under our experimental conditions (not shown).

Accumulation of Free Jasmonates and SA Induced by HR or Wounding

Because the arabidopsides appeared to transiently accumulate during avirulence peptide-induced HR and after wounding, it seems likely that arabidopsides may be degraded to form free jasmonates. To test this, we measured the content of free OPDA, dn-OPDA, and JA in wounded tissue and tissue inoculated with DC3000(*avrRpt2*). Samples were taken at time points where the amounts of arabidopsides were at their peak and during the decline in arabidopside content (see Figs. 3A and 4A). In addition to the jasmonates, we also measured the levels of SA. Because the free acids of the jasmonates and SA might be partially soluble in aqueous solution, the ''bathing'' solution for the leaf discs during bacterial inoculation (see ''Materials and Methods'') was also analyzed for these phytohormones. Much to our surprise, the levels of all four analyzed phytohormones were higher in the aqueous solution than in the leaf tissue (Fig. 5, A and B). Free jasmonates in leaf tissue and dissolved in the bath solution reached their highest level 15 h after inoculation with DC3000(*avrRpt2*) (Fig. 5A). In the leaf tissue, OPDA accumulated to about 25 nmol g FW^{-1} after 15 h. The levels of JA and dn-OPDA were about one-half that of OPDA. After 15 h, the amounts of free jasmonates in the tissue declined. In contrast, the amount of jasmonates dissolved in the bath solution remained fairly constant up to 24 h after inoculation. In the bath solution, OPDA, JA, and dn-OPDA reached approximately 40, 15, and 25 nmol g^{-1} tissue equivalent, respectively. The amount of SA in the tissue and bath solution also increased after inoculation with DC3000(*avrRpt2*) (Fig. 5B). However, in the case of SA, the bath solution contained more than 20 times as much of the phytohormone as the leaf tissue. The amount of SA in the bath solution exceeded 400 nmol g^{-1} tissue equivalent after 24 h. Mock inoculation with $MgCl_2$ did not cause any increase in the content of the tested phytohormones in the leaf tissue. However, the mock inoculation did cause some accumulation of the free acids in the bath solution, but the kinetics

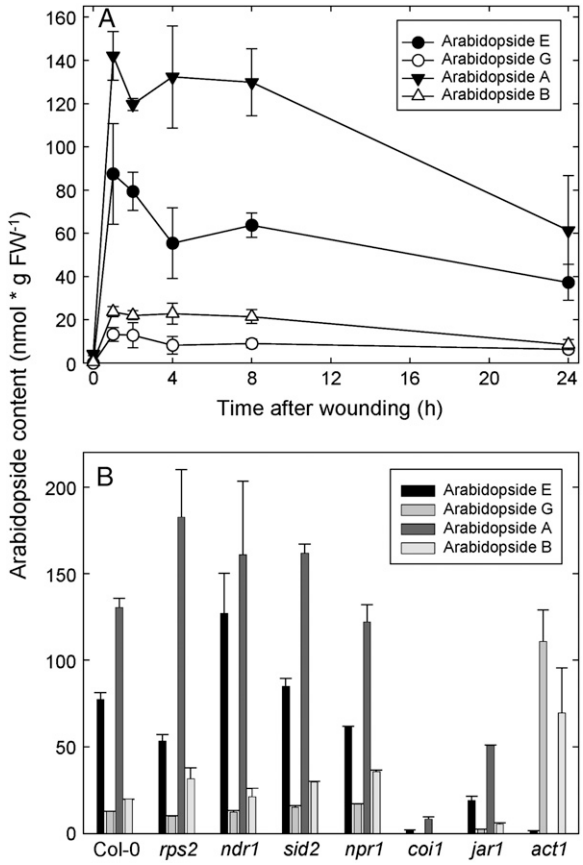

Figure 4. Wounding induces formation of arabidopsides. A, Arabidopsis wild-type Col-0 leaves were wounded with a hemostat and left on the plant for the times indicated. The lipids were extracted and the amounts of arabidopsides E (black circles), G (white circles), A (black triangles), and B (white triangles) were quantified by HPLC. B, Leaves of the indicated genetic backgrounds were wounded with a hemostat and left on the plant for 1 h, the lipids were extracted, and the arabidopsides quantified. Mean values and range of duplicate samples are shown.

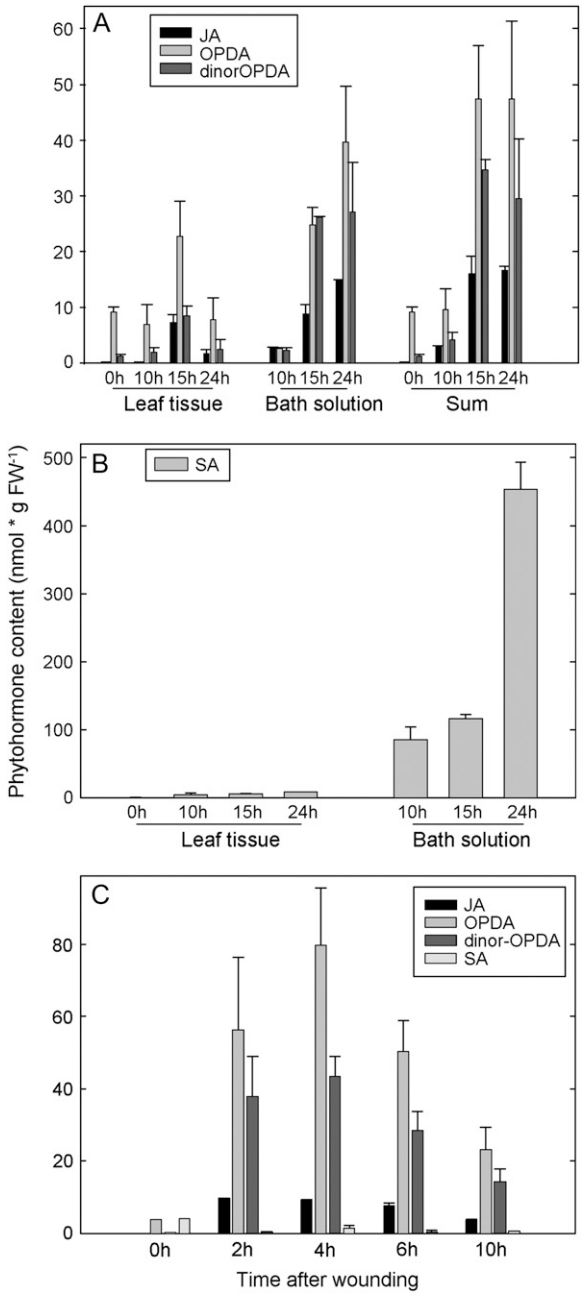

Figure 5. Free jasmonates formed after wounding and recognition of AvrRpt2. Arabidopsis leaves were infiltrated with *P. syringae* DC3000(*AvrRpt2*) or mock inoculated with $MgCl_2$ solution (A and B) or wounded (C) and the lipids were extracted at the indicated times. The amount of free JA, OPDA, dn-OPDA, and SA was determined. In the case of *P. syringae* infiltration (A and B), the solution that the leaf discs were incubated in was also extracted and the amounts of phytohormones analyzed. The levels of the phytohormones in mock-inoculated samples have been subtracted from the data shown in A and B. Mean values and range of duplicate samples are shown.

differed from that observed in the pathogen-inoculated discs. The released free phytohormones in the mock samples are likely to originate from the wounded cells around the rim of the leaf disc. The phytohormone amounts of phytohormones in the bath solutions of mock-inoculated samples were subtracted from the pathogen-inoculated samples. The amount of all three jasmonates increased in wounded tissue up to 4 h (Fig. 5C). Wounding did not cause any accumulation of SA. In wounded tissue, the amount of OPDA, JA, and dn-OPDA reached approximately 80, 10, and 40 nmol g FW^{-1}, respectively, after 4 h.

Arabidopsides E and G Inhibit Growth of a Necrotrophic Pathogen

Arabidopside E has been reported to have a growth-inhibiting effect on the hemibiotrophic bacterial pathogen *P. syringae* (Andersson et al., 2006a). Free OPDA has also been shown to inhibit the growth of several different species of pathogenic fungi (Prost et al., 2005). To test whether arabidopsides E and G have growth inhibitory effects on fungal pathogens, the effects of the arabidopsides on the in vitro growth of the necrotrophic fungal pathogen *B. cinerea* was tested (Fig. 6). Addition of 100 μM of arabidopside E or G to the media decreased the fungal growth to about one-half that of the untreated control. Free OPDA had no apparent effect on the growth of the fungus. The growth inhibition caused by arabidopside E or G was statistically significant at the $P < 0.01$ level.

DISCUSSION

It is becoming increasingly clear that the jasmonate family in Arabidopsis extends far beyond the free acids. A total of nearly 10 different galactolipid species containing OPDA and/or dn-OPDA has now been reported (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005, 2006; Andersson et al., 2006a; Buseman et al., 2006; Nakajyo et al., 2006). However, this and two previous reports (Andersson et al., 2006a; Buseman et al., 2006) demonstrate that four OPDA-containing galactolipids constitute the bulk of the galactolipid-bound OPDA that accumulates during wounding and the HR. Wounding triggers the accumulation of high amounts of arabidopsides A, B, E, and the previously uncharacterized arabidopside G. In contrast, the HR seems to strongly favor the accumulation of arabidopsides E and G, whereas arabidopside A is actually down-regulated during early HR response.

Arabidopside G, a Novel OPDA-Containing Galactolipid

While testing whether or not the formation of arabidopsides is dependent on the prokaryotic lipid synthesis pathway in the chloroplast, we identified yet another previously unreported OPDA-containing galactolipid. We determined the structure of the compound and found that it represents an all-C18 analog of arabidopside E (Fig. 2). We propose the trivial name arabidopside G for this galactolipid. The presence of arabidopside G is not unexpected, because the all-

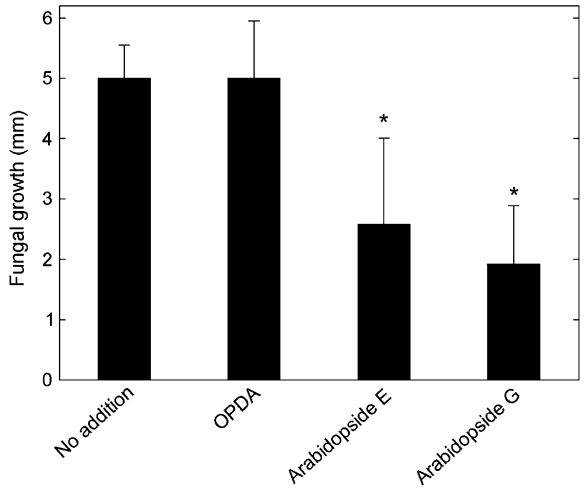

Figure 6. Effects of arabidopsides E and G on growth of *B. cinerea*. Square patches (3 × 3 mm) of *B. cinerea* growing on corn meal agar were cut out and transferred to media supplemented with the indicated compounds (100 μM). The diameter of the oomycete tissue was measured after 3 d. Mean growth and SD of six samples are shown. A star indicates statistically significant difference from the no-addition samples at the $P < 0.01$ level.

OPDA MGDG species, arabidopside B, has already been reported to occur in Arabidopsis (Hisamatsu et al., 2003) and to accumulate after wounding (Buseman et al., 2006).

The Synthesis of OPDA-Containing Galactolipids during Wounding and the HR

It was previously reported that wounding induces the accumulation of OPDA-containing galactolipids in Arabidopsis leaf tissue (Stelmach et al., 2001; Buseman et al., 2006; Böttcher and Weiler, 2007). However, the accumulation of arabidopsides E and G was not yet reported. Wounding of Arabidopsis leaf tissue, in our hands, caused the accumulation of arabidopsides E and G in parallel with arabidopsides A and B. The former accumulated to similar levels as the latter. Why arabidopsides E and G were not reported among the other OPDA-containing galactolipids that accumulate after wounding (Buseman et al., 2006; Böttcher and Weiler, 2007) is not entirely clear. One possibility is that arabidopsides E and G were simply missed because their molecular masses substantially exceed those of the other known OPDA-containing galactolipids. Interestingly, the HR seemed to cause an initial reduction in arabidopside A and B content (Fig. 3A). To our knowledge, this is the first observed reduction of arabidopsides under stress, and this observation supports the suggestion that arabidopsides E and G may be synthesized by the transfer of OPDA from the diacylglycerol backbone of arabidopside A or B to the galactosyl group of another arabidopside A or B molecule (Andersson et al., 2006a). During the HR, arabidopside A or B accumulated to much lower levels. Thus, in this case, the OPDA or dn-OPDA moieties appear to be more efficiently channeled into arabidopsides E and G, respectively, compared to during the wounding response. After wounding, the enzyme that transfers OPDA or dn-OPDA, respectively, to the galactosyl group of arabidopsides may be rate limiting, whereas during the HR the required enzyme may be transcriptionally induced or activated more efficiently.

Exactly how OPDA and dn-OPDA are introduced into the galactolipid pool in planta is an important question. It has been suggested that α-linolenic acid may be converted to OPDA while still attached to a galactolipid (Stelmach et al., 2001; Buseman et al., 2006), but no experimental evidence to support this has been presented. In vitro experiments with purified enzymes (lipoxygenase and AOS) and MGDG reportedly failed to produce any MGDG-bound OPDA (Stelmach et al., 2001). Because almost complete inactivation of the plastidial acyl transferase ACT1 did not inhibit formation of the arabidopsides to the same extent as prokaryotic lipids after wounding or HR, the prokaryotic galactolipid synthesis pathway in the chloroplast appears not to represent a major entry point for free OPDA to galactolipid synthesis. However, the formation of dn-OPDA-containing galactolipids is almost completely blocked in this mutant (Figs. 1, A and C, and 4B). On the other hand, healthy Arabidopsis tissue contains a considerable amount of lipid-bound 13-HPOT, the first intermediate in the synthesis of OPDA and JA (Stenzel et al., 2003; Andersson et al., 2006a). Provided that the following next two enzymes in the synthetic pathway, AOS and allene oxide cyclase, can utilize lipid substrates, the arabidopsides may be synthesized in situ from 13-HPOT bound to a diacylglycerol backbone. The latter is consistent with the recently reported association of AOS with the thylakoid membranes of the chloroplast (Böttcher and Weiler, 2007; Farmaki et al., 2007). Yet another possibility would be that free OPDA is exported from the plastid and enters the acyl lipid synthesis pathway in the endoplasmic reticulum prior to subsequent reimport to the plastid. However, the almost complete absence of esterified OPDA in the eukaryotic phospholipid pool (Andersson et al., 2006a; Buseman et al., 2006) provides evidence against such a suggestion.

Our observation that the synthesis of arabidopsides after wounding and during the HR was largely independent of plastidial acyl lipid synthesis implies that the presence of the arabidopsides might extend to non-16:3 plant species as well. We tested whether wounding induced accumulation of arabidopsides A, B, E, or G in a number of different 16:3 and 18:3 plant species. However, none of the tested species accumulated any detectable levels of arabidopsides when analyzed by HPLC under our experimental conditions, and this observation is supported by a recent report (Böttcher and Weiler, 2007). Therefore, arabidopsides appear to be unique to Arabidopsis or may occur at much lower amounts in other species.

Signaling Pathways Leading to Arabidopside Accumulation

To date, two different stimuli have been found to induce the accumulation of arabidopsides: wounding (Stelmach et al., 2001; Buseman et al., 2006; Böttcher and Weiler, 2007) and recognition of the phytopathogenic bacterial avirulence peptides AvrRpm1 and AvrRpt2 (Andersson et al., 2006a). This study corroborates these findings and provides additional information about the signaling required for the two different stimuli to trigger arabidopside accumulation.

For the plant-bacteria interactions tested in this study, the formation of arabidopsides was observed only in cases where the plant could mount a HR. Exposure to a virulent pathogen resulted in no accumulation of arabidopsides in plant tissue. Interestingly, two different avirulent bacterial strains (*Pst hrcC* and *P. syringae* pv *phaseolicola*) that do not trigger an HR did not induce any accumulation of arabidopsides. Thus, the arabidopsides formation seems to be closely linked to the HR and not to any other types of defense responses to pathogens. The formation of the arabidopsides after elicitation with DC3000(*avrRpt2*) was dependent on the intact resistance protein RPS2 and wild-type NDR1. The latter also indicates that the formation of the arabidopsides is downstream from the NDR1 signaling node. The double mutant *pad4sag101* is blocked in another defense signaling pathway also originating from the recognition of Avr proteins (Aarts et al., 1998). As expected, the double mutant *pad4sag101* caused no significant effect on the accumulation of arabidopsides after the plant recognition of AvrRpt2.

None of the disease resistance or SA signaling mutations (*rps2*, *ndr1*, *pad4sag101*, *sid2*, and *npr1*) tested caused any significant effect on arabidopside accumulation after wounding. Thus, two different signaling pathways appear to trigger arabidopside accumulation during wounding response and AvrRpt2-induced HR. Interestingly, the separate pathways for wounding and defense appear to converge at jasmonate-dependent signaling. The two jasmonate signaling mutations *coi1* and *jar1* strongly reduced arabidopside accumulation after both wounding and AvrRpt2-induced HR. As *ndr1* and SA signaling mutations had no effect on arabidopside accumulation after wounding, the jasmonate convergence must lie downstream of NDR1 and SA in the avirulence peptide-triggered signaling pathway. Both the *coi1* and *jar1* mutants are defective in response to jasmonates. *coi1* mutants are fully insensitive to jasmonates, and COI1 is required for all known jasmonate-dependent responses (Feys et al., 1994; Xie et al., 1998; Devoto and Turner, 2005). The JAR1 gene codes for an enzyme that conjugates JA predominantly to the hydrophobic amino acid Ile (Staswick and Tiryaki, 2004). Thus, strong reduction of arabidopside levels in the *jar1* mutant suggests that JA-amino acid conjugates significantly contribute to the stimulation of arabidopside synthesis. Taken together, the observed degree of involvement of COI1 and JAR1 in arabidopside production highlights the importance of jasmonate signaling in this process, regardless of the inducing stimulus. Arabidopsides, COI1, and jasmonate signaling appear to form a node, unifying wound- and pathogen-triggered signaling pathways. Positive feedback in jasmonate signaling and synthesis has been previously reported (Laudert and Weiler, 1998; Stintzi et al., 2001; Stenzel et al., 2003). Our observations appear to represent another example of this where the detection of free jasmonates induces the formation of the arabidopsides. On a speculative note, one might suggest that the arabidopsides promote a super amplification of the jasmonate signal in Arabidopsis.

It has been a long-held belief that jasmonate- and SA-dependent signaling pathways have antagonistic effects on each other (Doherty et al., 1988; Pena-Cortes et al., 1993; Doares et al., 1995; Niki et al., 1998). Our results appear to contradict this notion, at least for these experimental systems. Indeed, other recent reports indicate that the previously suggested antagonism between SA and jasmonate signaling may be more complicated than initially expected (Clarke et al., 2000; Govrin and Levine, 2000; van Wees et al., 2000; Mur et al., 2006). The drastic decrease in SA production in the *sid2* mutant and the inability to relay the SA signal in the *npr1* mutant caused a significant reduction of the arabidopside levels in these plants after inoculation with DC3000(*avrRpt2*). Thus, it appears that the accumulation of arabidopsides after recognition of the bacterial avirulence peptide AvrRpt2 requires SA-mediated potentiation. One particular aspect of jasmonate contra SA signaling that is often overlooked in disease resistance is the timing of the synthesis of different signaling molecules. Many previous studies have tended to place emphasis on much later time points after pathogen inoculation than we report herein. In the case of AvrRpt2 recognition, the levels of both free and galactolipid-bound jasmonates peaks earlier than SA. Importantly, the type of interaction, synergy or antagonism between the SA and JA, has been shown to be concentration dependent (Mur et al., 2006). Taken together, these observations suggest that SA signaling may have very different effects at early and late stages of the HR defense response. Early in the process, a low concentration of SA stimulates the accumulation of jasmonates and arabidopsides, whereas at later time points, the much higher concentrations of SA could have very different signaling properties.

The HR is typically accompanied by the establishment of systemic immunity (systemic acquired resistance [SAR]) to a range of virulent pathogens (Durrant and Dong, 2004). Functional SA signaling is important for local defense responses and essential for the establishment of SAR (Alvarez, 2000). However, the function of SA as the essential mobile signal is debated. Recently, data has emerged implicating JA (Truman et al., 2007) as well as the SA derivative methyl salicylate (Park et al., 2007) as inductive SAR signal/signals. Localized tissue damage in tomato by herbivory also induces

systemic pathogen defense responses, and recently, jasmonates were shown to play a pivotal role in this response (Wasternack et al., 2006). Our observation that a large portion of the assayed jasmonates (JA, OPDA, and dn-OPDA) and more or less all of the SA appear to diffuse out of the tissue undergoing the HR suggests that, in fact, all four phytohormones may serve as tissue mobile signals. Our observations are certainly compatible with the suggestion that jasmonates and/or SA may act alone or in concert as the mobile signals for establishment of systemic defense responses (Park et al., 2007). The different mobility, timing, and concentration of the phytohormones may be envisaged to create complex spatiotemporal gradients in the tissue around localized HR or tissue damage.

The Function of Arabidopsides

A crucial remaining question is the function of the arabidopsides during wound and defense responses. The finding that arabidopsides accumulate in response to two different stimuli and that free OPDA does not simply follow the stream of "normal" fatty acids through the prokaryotic pathway seems to suggest that these compounds are more than just accidental byproducts of hyperactive jasmonate synthesis. The apparent uniqueness of Arabidopsis as an arabidopside hyper-accumulating plant, on the other hand, indicates that the role of the arabidopsides is dispensable or handled by other compound(s) in other plant species.

We have earlier hypothesized that arabidopsides function as antipathogenic substances and as precursors that allow the slow release of more potent products (i.e. free jasmonates). Consistent with our previous finding of the direct antipathogenic effect of arabidopside E (Andersson et al., 2006a) against the pathogenic bacteria *P. syringae*, we now report growth inhibition on a fungal necrotrophic pathogen, *B. cinerea*, by arabidopsides E and G. However, it should be stressed that we cannot exclude an effect of released free OPDA, because an inhibitory effect of OPDA on *B. cinerea* spore germination has been reported (Prost et al., 2005). The transient nature of the accumulation of arabidopsides after wounding and HR indicates that they may serve as a supply of OPDA and dn-OPDA, which are slowly released to sustain a longer lasting signal. This view is further supported by the observed increase in free jasmonates that occurred concomitant with the accumulation of arabidopsides. Arabidopside accumulation seems to be restricted to the local wounded or elicited tissue. Free jasmonates, on the other hand, were found to leak out of the elicited tissue. The later finding is in agreement with the notion that JA may be important as part of the tissue mobile signal in the establishment of SAR (Truman et al., 2007) as well as systemic pathogen defense responses induced by herbivory (Wasternack et al., 2006). An acyl hydrolase involved in basal JA production and plant resistance against *B. cinerea* was recently identified (Yang et al., 2007). This enzyme was shown to have the capacity to release free OPDA from arabidopsides A, B, C, and D. This and/or similar enzymes may be responsible for the slow release of free jasmonates from all arabidopsides, including arabidopsides E and G.

In conclusion, we have demonstrated that the induction of defense responses or wounding leads to the accumulation of OPDA-containing galactolipids, arabidopsides. The synthesis of these OPDA-containing galactolipids is independent of the prokaryotic galactolipid synthesis pathway in the plastid. The triggering of arabidopside accumulation requires different signaling pathways during wounding response and HR. However, in both cases, arabidopside formation is dependent on an intact jasmonate signaling pathway. The arabidopsides appear to act as direct antipathogenic substances and as a pool of jasmonates that are subsequently slowly released in the tissue, and thus, the arabidopsides are potentially important for inducing both local and systemic defense responses.

MATERIALS AND METHODS

Plant Material

Various lines of Arabidopsis (*Arabidopsis thaliana*) were cultivated under short-day conditions (8-h day and 16-h night) at 22°C daytime and 18°C nighttime and 60% relative humidity for 4 to 5 weeks. Transgenic lines harboring the Dex-inducible coding sequence for the *Pseudomonas syringae* avirulence peptide AvrRpm1 were harvested, and the expression of AvrRpm1 was induced with 20 μM Dex as previously described (Andersson et al., 2006b). Various *P. syringae* strains were cultivated on *Pseudomonas* agar (Biolife) for 24 h at room temperature. The bacteria were diluted in 10 mM $MgCl_2$ to an OD600 of 0.1. The bacteria were injected through the abaxial epidermis while the leaf was still attached to the plant using a syringe without a needle. After inoculation, leaf discs 7 mm in diameter were punched out and incubated in water for the times indicated. Each sample consisted of six leaf discs corresponding to approximately 30 mg FW. Wounding was induced with a hemostat on leaves still attached to the plant (Buseman et al., 2006). An entire half of the fully expanded leaves was wounded and care was taken not to injure the midvein. After the times indicated, the wounded tissue was cut out and the lipids extracted. The FW of the unwounded half of the leaf minus the midvein was used as a measure of the FW of the wounded tissue.

Lipid Extraction

The leaf tissue was submerged in boiling isopropyl alcohol for 5 min and dried under a gentle stream of N_2. The lipids were extracted in 2 mL of $CHCl_3$:methanol:water (1:2:0.8, by volume) containing 0.025% of butylated hydroxytoulene by 30 min of sonication (bath type) and 30 min at 4°C. This treatment rendered the leaf tissue completely depigmented. Phase separation was induced by the addition of 0.5 mL of $CHCl_3$ and 0.5 mL of 380 mM K_2SO_4 solution. The lower phase was transferred to a new tube and the aqueous phase was re-extracted with $CHCl_3$. The combined organic phases were dried under N_2, dissolved in a small volume of $CHCl_3$, and applied to a prepacked 500-mg silica column (Supelco). Neutral lipids were eluted with $CHCl_3$:acetone (9:1, by volume), and the arabidopsides were eluted with acetone:methanol (9:1). The glycolipid fraction was dried under N_2, dissolved in 50 μL of methanol, and transferred to an HPLC vial.

Analysis of Arabidopsides

The arabidopsides were separated on a 250- × 4.6-mm 5-μm C18 column (HiChrom) using a binary gradient consisting of acetonitril:water (85:15, by volume, solvent A) and 2-propanol (solvent B). The run consisted of a linear increase from 100% A to 60% B in 30 min. Sixty percent of B was maintained for 5 min, and the gradient was reversed in 10 min. The column was allowed to re-equilibrate for 5 min before the next run. Mobile phase flow was kept

constant at 1 mL min^{-1}. The arabidopsides were detected with UV absorbance detection at 220 nm. Retention times and detector response were verified using authentic lipid standards.

Large-Scale Purification of Arabidopsides

The novel arabidopside induced in AvrRpm1/*act1* material was purified in large scale roughly as previously reported for arabidopside E (Andersson et al., 2006a), except $CHCl_3$ was used instead of ethyl acetate for the extraction. Briefly, a large amount of AvrRpm1/*act1* leaves was incubated with 20 μM Dex and 0.005% silwet L-77. Arabidopsides A and B were purified from wounded Col-0 tissue. Leaf tissue corresponding to 200 g was placed between two sheets of aluminum foil, and a rolling pin was rolled over five times to crush the tissue. The wounded leaves were kept in water for 2 h.

The liquid was filtered off; the leaves were weighed and homogenized in a volume of chilled methanol containing 0.025% butylated hydroxytoulene corresponding to 2.5 times the weight of the leaf tissue. A volume of $CHCl_3$ corresponding to 1.25 times the tissue weight was added, and the mixture was incubated on an orbital shaker for 30 min at 4°C. The mixture was filtered, a volume of $CHCl_3$ and 380 mM KCl solution corresponding to 1.25 times the tissue weight was added, and the mixture was shaken in a separating funnel. The organic phase was recovered and the aqueous phase re-extracted with $CHCl_3$. The combined $CHCl_3$ phases were dried in vacuo and dissolved in a small volume of $CHCl_3$.

The lipids dissolved in $CHCl_3$ were fractionated on a silica column. Neutral lipids were wash-eluted from the column with $CHCl_3$:acetone (9:1, by volume). Arabidopsides E and G were eluted with $CHCl_3$:acetone (7:3, by volume) and arabidopsides A and B with acetone:methanol (9:1, by volume). The eluant was dried and further separated by thin-layer chromatography. Thin-layer plates (Si-60; Merck) were developed with ethyl acetate:acetic acid (70:0.5, by volume), and the arabidopsides were identified by spraying with 0.2% dichlorofluorescein and comparison to authentic standards. The zones containing the arabidopsides were scraped off of the plates and the lipids were eluted in methanol. The lipid was finally purified by HPLC as described (Andersson et al., 2006a) or the method described above.

Structure Determination of a Novel Arabidopside

The structure of the novel arabidopside was determined largely as previously described for arabidopside E (Andersson et al., 2006a). The exact same instrumentation was used and operated in exactly the same way. The only exception was that a position-specific hydrolysis was not required for its structure elucidation, because all three acyl groups were identical. The following NMR signals (referenced to solvent peaks δ_H 4.87 and δ_C 49.15) could be assigned to the novel compound: ^{1}H NMR (CD_3OD, 300 MHz) δ 7.92 (3H, dd, J = 5.9, 2.6 Hz, H-10″/10‴/10⁗), 6.16 (3H, dm, J = 5.9 Hz, H-11″/11‴/11⁗), 5.42 (3H, m, H-16″/16‴/16⁗), 5.40 (3H, m, H-15″/15‴/15⁗), 5.27 (1H, m, H-2), 4.45 (1H, dd, J =12.1, 3.2 Hz, H-1), 4.33 (1H, dd, J = 11.4, 7.7 Hz, H-6′), 4.23 (1H, d, J = 7.2 Hz, H-1′), 4.22 (1H, dd, m, H-1), 4.21 (1H, m, H-6′), 3.90 (1H, dd, J = 11.1, 5.5 Hz, H-3), 3.80 (1H, m, H-4′), 3.76 (1H, m, H-3), 3.72 (1H, m, H-5′), 3.51 (1H, dd, J = 9.8, 7.2 Hz, H-2′), 3.47 (1H, dd, J = 9.8, 3.2 Hz, H-3′), 3.06 (3H, m, H-9″/9‴/9⁗), 2.49 (3H, m, H-13″/13‴/13⁗), 2.45 (3H, m, H-14″/14‴/14⁗), 2.33 (6H, m, H_2-2″/2‴/2⁗), 2.18 (3H, m, H-14″/14‴/14⁗), 2.08 (6H, m, H_2-17″/17‴/17⁗), 1.77 (3H, m, H-8″/8‴/8⁗), 1.61 (6H, m, H_2-3″/3‴/3⁗), 1.44 (6H, m, H_2-5″/5‴/5⁗), 1.36 (12H, m, H_2-6,7″/6,7‴/6,7⁗), 1.34 (6H, m, H_2-4″/4‴/4⁗), 1.18 (3H, m, H-8″/8‴/8⁗), 0.98 (9H, t, J = 7.6 Hz, H_3-18″/18‴/18⁗); ^{13}C NMR (CD_3OD, 75 MHz) gδ 213.6 (C, C-12″/12‴/12⁗), 175.3 (C, C-1″), 175.0/174.6 (C, C-1‴/1⁗), 170.3 (CH, C-10″/10‴/10⁗), 133.9 (CH, C-16″/16‴/16⁗), 133.0 (CH, C-11″/11‴/11⁗), 128.4 (CH, C-15″/15‴/15⁗), 105.7 (CH, C-1′), 74.8 (CH, C-3′), 74.3 (CH, C-5′), 72.3 (CH, C-2′), 71.9 (CH, C-2), 70.4 (CH, C-4′), 69.1 (CH_2, C-3), 64.9 (CH_2, C-6′), 64.0 (CH_2, C-1), 51.2 (CH, C-13″/13‴/13⁗), 46.0 (CH, C-9″/9‴/9⁗), 35.2 (CH_2, C-2″), 35.1 (CH_2, C-2‴/2⁗), 32.0 (CH_2, C-8″/8‴/8⁗), 30.9 (CH_2, C-6″/6‴/6⁗), 30.4 (CH_2, C-4″/4‴/4⁗), 30.30 (CH_2, C-7″), 30.25 (CH_2, C-7‴/7⁗), 28.8 (CH_2, C-5″/5‴/5⁗), 26.1 (CH_2, C-3″/3‴/3⁗), 25.1 (CH_2, C-14″/14‴/14⁗), 21.9 (CH_2, C-17″/17‴/17⁗), 14.6 (CH_3, C-18″/18‴/18⁗).

Other Methods

Free jasmonates and SA were extracted and quantified by GC-MS as previously described using deuterated internal standards (Ochsenbein et al., 2006).

Supplemental Data

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

Supplemental Figure S1. Mass spectrum of arabidopside G.

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