The Plant Cell, Vol. 24: 5123–5141, December 2012, www.plantcell.org

Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity[W]

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Metabolic signals orchestrate plant defenses against microbial pathogen invasion. Here, we report the identification of the non-protein amino acid pipecolic acid (Pip), a common Lys catabolite in plants and animals, as a critical regulator of inducible plant immunity. Following pathogen recognition, Pip accumulates in inoculated *Arabidopsis thaliana* leaves, in leaves distal from the site of inoculation, and, most specifically, in petiole exudates from inoculated leaves. Defects of mutants in *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) in systemic acquired resistance (SAR) and in basal, specific, and β-aminobutyric acid–induced resistance to bacterial infection are associated with a lack of Pip production. Exogenous Pip complements these resistance defects and increases pathogen resistance of wild-type plants. We conclude that Pip accumulation is critical for SAR and local resistance to bacterial pathogens. Our data indicate that biologically induced SAR conditions plants to more effectively synthesize the phytoalexin camalexin, Pip, and salicylic acid and primes plants for early defense gene expression. Biological priming is absent in the pipecolate-deficient *ald1* mutants. Exogenous pipecolate induces SAR-related defense priming and partly restores priming responses in *ald1*. We conclude that Pip orchestrates defense amplification, positive regulation of salicylic acid biosynthesis, and priming to guarantee effective local resistance induction and the establishment of SAR.

INTRODUCTION

The plant immune system is multilayered and includes constitutive and inducible defenses to counteract colonization by microbial pathogens (Thordal-Christensen, 2003). In inoculated plant tissue, defense responses are initiated by recognition of microbial structures common to many pathogen types (pathogen-associated molecular patterns [PAMPs]) or effector molecules specific to particular pathogen isolates (Jones and Dangl, 2006). PAMP-triggered immunity (PTI) ensures basal plant resistance to pathogens but is not effective enough to entirely contain infection by a compatible pathogen. Although the signaling pathways leading to effector-triggered immunity (ETI) partially overlap with those of PTI, ETI is usually associated with a hypersensitive response (HR) and provides a more effective resistance than PTI (Jones and Dangl, 2006). Localized inoculation of leaves with compatible or incompatible pathogens also leads to broad-spectrum resistance of the whole plant shoot to subsequent pathogen attack, a phenomenon designated systemic acquired resistance (SAR; Durrant and Dong, 2004; Mishina and Zeier, 2007). Both effector- and PAMP-triggered events have been proposed to contribute to SAR initiation at the site of pathogen inoculation (Cameron et al., 1999; Mishina and Zeier, 2007).

In response to pathogens, plants synthesize a variety of defense-related small metabolites, including the defense hormones salicylic acid (SA) and jasmonic acid (JA), which activate distinct plant defense pathways. JA acts in concert with ethylene to induce resistance against several necrotrophic pathogens, whereas SA-mediated defense responses are effective against hemibiotrophs and biotrophs (Glazebrook, 2005). SA and JA signaling can negatively influence each other (Koornneef and Pieterse, 2008). SA biosynthesis via ISOCHORISMATE SYNTHASE1 (ICS1), its positive regulation by PHYTOALEXIN-DEFICIENT4 (PAD4), and SA downstream signaling via NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is critical for PTI, ETI, and SAR (Jirage et al., 1999; Nawrath and Métraux, 1999; Wildermuth et al., 2001; Durrant and Dong, 2004). Other phytohormones, such as auxin, abscisic acid, and gibberellin, can also influence defense signaling and resistance (Mauch-Mani and Mauch, 2005; Grant and Jones, 2009). In *Arabidopsis thaliana*, the indolic phytoalexin camalexin and several other Trp-derived compounds accumulate in inoculated leaves and can protect against fungal infection (Bednarek et al., 2009; Schlaeppi et al., 2010). Moreover, the unsaturated sterol stigmasterol is synthesized at pathogen inoculation sites, integrates into plant cell membranes, and favors susceptibility to bacterial pathogens (Griebel and Zeier, 2010).

The regulatory plant metabolite most closely associated with plant resistance against biotrophic and hemibiotrophic pathogen invasion is SA. In the course of SAR, SA accumulates in inoculated leaves and in systemic leaves distal from the initial inoculation site (Malamy et al., 1990). Although SA has been proposed to be a mobile SAR signal able to travel via the vasculature (Shulaev et al., 1995; Mölders et al., 1996), grafting studies rather suggest that SA is not transported from inoculated to distal leaves but

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[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.112.103564

that its systemic accumulation is critical for SAR (Vernooij et al., 1994). Systemic SA accumulation proceeds via upregulation of *ICS1* and de novo SA biosynthesis (Attaran et al., 2009); this is likely triggered by perception of mobile long-distance signals in the cells of distal leaves (Shah, 2009). Expression of two other SAR regulatory genes, *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*) and *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*), is locally and systemically enhanced in SAR-induced plants. Both genes are not only indispensable for systemic SA accumulation and SAR, but are also critical for full PTI and ETI responses at inoculation sites (Song et al., 2004a; Bartsch et al., 2006; Mishina and Zeier, 2006; Koch et al., 2006). Pathogen-induced expression of *ALD1*, *FMO1*, and *ICS1* in inoculated tissue proceeds independently of SA (Métraux, 2002; Song et al., 2004a; Bartsch et al., 2006; Mishina and Zeier, 2006). This suggests that ALD1 and FMO1 are involved in SA-independent signaling upstream of SA biosynthesis, processes that are required for systemic SA accumulation and SAR. The SAR-induced state provides enhanced pathogen protection via increased defense-related gene expression and is associated with defense priming, enabling the plant to more effectively induce defense responses (Durrant and Dong, 2004; Beckers et al., 2009).

Recent findings indicate significant interconnections between different branches of amino acid metabolism and plant resistance to microbial pathogens or herbivorous insects (van Damme et al., 2009; Liu et al., 2010; Adio et al., 2011; Cecchini et al., 2011; Stuttmann et al., 2011; von Saint-Paul et al., 2011). In this study, we investigate changes in free amino acids that occur during the establishment of SAR in *Arabidopsis*. We show that several free amino acids, including branched-chain amino acids, aromatic amino acids, and Lys enrich in leaves inoculated with SAR-inducing *Pseudomonas syringae* bacteria. Most strikingly, the Lys catabolites α-amino adipic acid (Aad) and pipecolic acid (Pip), which are only faintly detectable in leaves of noninoculated plants, accumulate to high levels at pathogen inoculation sites. We show that pathogen-induced biosynthesis of Aad is dependent on Lys ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH; Galili et al., 2001) but that its production is dispensable for resistance induction. Pip biosynthesis proceeds via a separate pathway involving the ALD1 aminotransferase. Our metabolite and genetic analyses suggest a substantial role for Pip in systemic plant immunity because, unlike other amino acids and metabolites, it strongly accumulates in leaves distal from initial inoculation and in petiole exudates collected from pathogen-inoculated leaves. The critical role for Pip in inducible immunity is corroborated by the findings that defects of *ald1* mutant plants in SAR, PTI, and ETI are associated with a lack of Pip production and that exogenous Pip can complement these resistance defects. Our results also offer mechanistic insights into β-aminobutyric acid (BABA)–induced plant resistance to bacterial pathogen infection that proceeds via Pip accumulation. We show that Pip orchestrates inducible immunity and SAR via defense amplification and priming and demonstrate a role for the Lys catabolite as a novel metabolic defense regulator. Since Pip has neurotransmitter activity in the brain, our study reveals interesting similarities between signaling aspects in plants and animals and might furthermore have applied significance in modern plant protection.

RESULTS

Changes in Free Amino Acid Levels during SAR

We performed comparative analyses of free amino acids extracted from locally treated and nontreated, distal (systemic) leaves of mock-control plants and of plants inoculated with the hemibiotrophic, SAR-inducing bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 (*Psm*). Amino acids were analyzed following propyl chloroformate derivatization via gas chromatography–mass spectrometry (GC-MS) (Kugler et al., 2006). The virulent *Psm* strain triggers a robust SAR response in *Arabidopsis* from day 2 after leaf inoculation that is mechanistically indistinguishable from the SAR induced by avirulent *P. syringae* (Mishina and Zeier, 2006, 2007; Attaran et al., 2009; Zhang et al., 2010; Liu et al., 2011). *Psm*-induced SAR is accompanied by massive changes in the contents of free amino acids in pathogen-inoculated leaves (Figure 1, left column). A strong increase, by a factor of 5- to 20-fold, was observed for Lys, the aliphatic amino acids Val, Leu, Ile, and β-Ala, and the aromatic amino acids Phe, Tyr, Trp, and His (Figure 1, highlighted in orange). Moderate but still significant pathogen-triggered increments were detected for GABA, Cys, Asn, Ala, Gly, Ser, and Orn. Notably, two substances that were only faintly present in mock-treated leaves increased by ~70-fold in pathogen-inoculated leaves: Aad and an unknown compound (Figure 1). A conclusion by analogy taking into account the mass spectra of the Lys, Orn, and Pro derivatives strongly suggested the identity of the unknown compound to be Pip (piperidine-2-carboxylic acid, homoproline), the methylene homolog of Pro (see Supplemental Figure 1 online). This assumption was confirmed by examination of the authentic Pip derivative, which gave rise to mass spectra and GC retention times identical with the extract peak (see Supplemental Figures 1 and 2 online). A single amino acid, Asp, showed significant and reproducible decreases after *Psm* inoculation (Figure 1).

Pip and Aad are both catabolites of Lys, an Asp-derived amino acid (Galili et al., 2001; see Supplemental Figure 3 online). The accumulation of Pip, Aad, and Lys and the concomitant decreases of Asp levels after *P. syringae* attack (Figure 1) indicated that Lys biosynthesis and catabolism are pathogen-triggered events. Pip was produced faster in leaves inoculated with the incompatible, HR-inducing *Psm avrRpm1* strain than in leaves infected with the compatible *Psm* strain but accumulated to high levels during later stages of the compatible interaction (Figure 2A). Aad biosynthesis was also triggered upon inoculation with both compatible and incompatible bacteria but trailed behind Pip accumulation (Figure 2B).

Defense Regulation of Local Pip and Aad Accumulation and Induction of Lys Catabolism by Bacterial PAMPs

At inoculation sites, induction of Pip biosynthesis was independent of SA production, since the *ICS1*-defective *sid2/ics1* mutant accumulated Pip to similar levels than the wild type (Figures 2C and 2D). NPR1-mediated signaling seems to positively influence Pip biosynthesis particularly in earlier stages of the *Arabidopsis*–*Psm* interaction because of reduced Pip accumulation in the *npr1* mutant at day 1 but not day 2 after inoculation (Figures 2C and 2D). Moreover, *Psm*-induced Pip production was strongly

µg g^{-1} FW	inoculated leaves (2 DAI)			distal leaves (2 DAI)			petiole exudates (6-48 h)		
amino acid	µg g^{-1} FW		ratio	µg g^{-1} FW		ratio	ng ml^{-1} leaf^{-1}		ratio
	$MgCl_2$	*Psm*	P / M	$MgCl_2$	*Psm*	P / M	$MgCl_2$	*Psm*	P / M
Gly	12.8 ± 1.0	21.7 ± 5.4	1.7 *	16.5 ± 1.2	18.0 ± 3.7	1.1	39.2 ± 4.2	55.5 ± 1.2	1.4 ***
Ala	78.4 ± 7.3	141.0 ± 32.5	1.8 **	64.0 ± 7.0	87.9 ± 18.2	1.4	126.9 ± 13.0	161.5 ± 14.3	1.3 *
Val	7.9 ± 1.3	54.4 ± 27.1	6.9 **	6.7 ± 0.4	9.8 ± 1.9	1.5 *	48.5 ± 2.5	55.7 ± 4.5	1.1
β-Ala	2.5 ± 1.2	15.5 ± 7.3	6.2 **	2.5 ± 0.8	3.7 ± 0.7	1.5	1.3 ± 0.1	1.6 ± 0.2	1.2
Leu	3.3 ± 0.6	49.2 ± 26.7	14.8 **	2.5 ± 0.5	4.6 ± 1.6	1.8	50.1 ± 5.9	56.7 ± 3.0	1.1
Ile	2.6 ± 0.4	31.4 ± 16.0	12.0 **	2.6 ± 0.4	3.8 ± 1.3	1.5	54.0 ± 1.9	43.6 ± 3.0	0.8 **
GABA	6.5 ± 0.5	25.1 ± 6.5	3.8 **	5.6 ± 1.5	6.1 ± 0.9	1.1	61.0 ± 7.2	69.0 ± 11.9	1.1
Ser	127.5 ± 17.8	230.5 ± 46.4	1.8 *	110.7 ± 25.5	146.3 ± 41.0	1.3	98.8 ± 10.4	101.2 ± 27.6	1.0
Thr	58.9 ± 13.0	98.5 ± 33.7	1.7	85.8 ± 18.4	122.6 ± 27.1	1.4	104.7 ± 11.1	70.4 ± 15.2	0.7 *
Pro	11.1 ± 1.5	10.0 ± 3.0	0.9	13.5 ± 2.7	12.4 ± 2.4	0.9	8.7 ± 1.6	9.0 ± 1.1	1.0
Pip	0.6 ± 0.2	42.2 ± 15.8	65.3 ***	0.3 ± 0.1	3.1 ± 1.3	10.1 **	1.5 ± 0.2	10.2 ± 1.9	7.0 ***
Aad	0.4 ± 0.1	25.6 ± 6.6	71.2 ***	0.19 ± 0.03	0.42 ± 0.12	2.2 *	0.7 ± 0.2	0.4 ± 0.1	0.5 *
Asp	160.0 ± 46.6	91.7 ± 30.7	0.6 *	145.0 ± 30.1	194.0 ± 57.7	1.3	305.9 ± 10.0	284.1 ± 72.8	0.9
Glu	277.0 ± 63.9	282.5 ± 95.1	1.0	282.0 ± 44.5	347.1 ± 63.5	1.2	281.9 ± 22.2	263.4 ± 88.5	0.9
Asn	28.1 ± 4.3	78.9 ± 22.3	2.8 **	39.6 ± 10.0	41.0 ± 15.2	1.0	47.3 ± 11.0	53.0 ± 17.1	1.1
Gln	254.1± 65.2	374.5 ± 72.0	1.5	358.2 ± 84.2	372.3 ± 78.5	1.0	306.6 ± 89.0	342.1 ± 88.7	1.1
Cys	0.09 ± 0.01	0.25 ± 0.03	2.9 *	0.05 ± 0.02	0.08 ± 0.03	1.5	0.06 ± 0.01	0.06 ± 0.00	1.0
Orn	0.7 ± 0.1	1.1 ± 0.2	1.6 *	1.4 ± 0.3	1.0 ± 0.1	0.7	1.5 ± 0.3	2.3 ± 1.0	1.5
Lys	5.1 ± 1.1	28.8 ± 11.7	5.7 **	5.1 ± 0.6	6.1 ± 1.1	1.2	39.8 ± 4.7	10.6 ± 1.5	0.3 ***
His	0.3 ± 0.2	2.9 ± 2.1	9.4 *	0.42 ± 0.21	0.64 ± 0.25	1.5	0.9 ± 0.2	0.9 ± 0.3	1.0
Phe	3.7 ± 0.2	20.3 ± 4.9	5.5 *	4.2 ± 0.6	9.1 ± 3.3	2.2 *	25.9 ± 2.8	43.2 ± 4.6	1.7 **
Tyr	3.3 ± 1.0	23.9 ± 4.5	7.3 ***	3.3 ± 0.3	5.4 ± 0.8	1.6 *	2.0 ± 0.2	2.2 ± 0.6	1.1
Trp	2.7 ± 0.7	24.7 ± 11.7	9.2 **	2.9 ± 0.3	4.3 ± 0.9	1.5	1.0 ± 0.2	0.9 ± 0.2	1.0

fold change P/M

> 20
5 - 20
3 - 5
1.6 - 3
0.5 – 0.8
< 0.5

Figure 1. Changes in the Levels of Free Amino Acids in *Arabidopsis* Col-0 Plants upon Leaf Inoculation with SAR-Inducing *Psm*.

Left column: Inoculated, lower (1°) leaves at 2 DAI. Central column: Nontreated, upper (distal, systemic, 2°) leaves at 2 DAI. Right column: Petiole exudates from inoculated leaves collected between 6 to 48 h after inoculation. Mean values for leaf samples are given in µg g^{-1} fresh weight (FW) ± SD and for leaf exudate samples in ng mL^{-1} exudate solution leaf^{-1} ± SD from at least four replicate samples. Mock treatments were performed by infiltration of leaves with a 10 mM $MgCl_2$ solution. Asterisks denote statistically significant differences between *Psm* (*P*) and $MgCl_2$ (M) samples (two-tailed *t* test; ***P < 0.001, **P < 0.01, and *P < 0.05).

reduced but not totally blocked, in the *pad4* mutant, indicating a positive regulation of Pip biosynthesis by the lipase-like defense regulator PAD4 (Figures 2C and 2D). By contrast, local accumulation of Pip was independent of the SAR regulator FMO1 (Figure 2C). We even observed overaccumulation of Pip in *fmo1* mutants at day 2 after *Psm* inoculation (Figure 2D). However, the regulation of induced Aad and Pip biosynthesis seems to differ because *Psm*-induced Aad accumulation was partially dependent on ICS1-, NPR1-, PAD4-, and FMO1-mediated defense signaling at 2 d after inoculation (DAI; Figure 2E). Moreover, the basal levels of Pip and particularly of Aad were elevated in the constitutively resistant *cpr5* mutant (Figures 2D and 2E). Pip biosynthesis in response to *Psm* and *Psm avrRpm1* inoculation follows similar mechanistic routes, as shown by the observations that Pip accumulation after leaf infiltration with the avirulent bacterial strain was ALD1 dependent and partially PAD4 dependent but was independent of the SA pathway and FMO1 (see Supplemental Figure 4A online).

Perception of PAMPs can trigger local and systemic defense responses and activate distinct metabolic pathways in *Arabidopsis* (Mishina and Zeier, 2007; Griebel and Zeier, 2010). We thus investigated whether exogenous application of two well-characterized bacterial PAMPs, flagellin, the proteinaceous building unit of the bacterial flagellum, and lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, would be sufficient to activate the biosynthesis of Pip and Aad. Infiltration of 200 nM flg22, a peptide corresponding to the elicitor active epitope of flagellin (Gómez-Gómez et al., 1999), into Columbia-0 (Col-0) leaves indeed

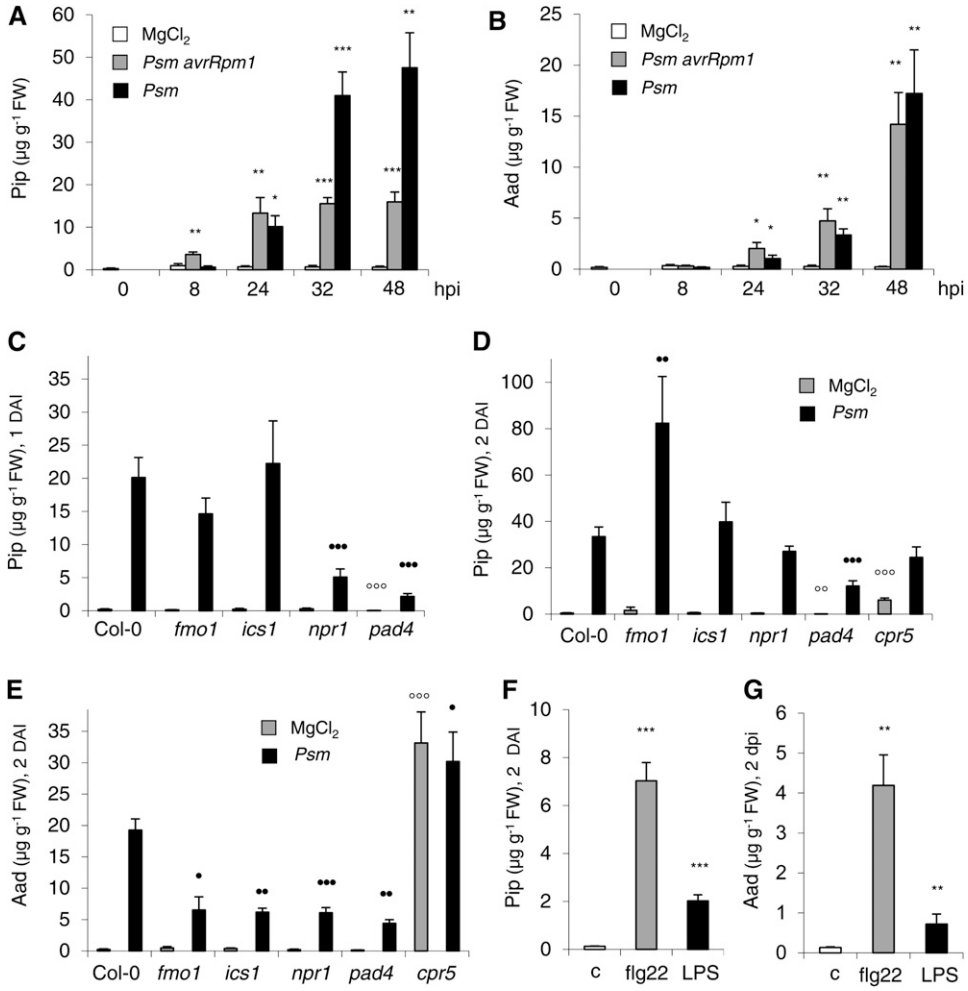

Figure 2. Pip and Aad Accumulation in *P. syringae*–Inoculated and PAMP-Treated *Arabidopsis* Leaves.

(A) and **(B)** Time course of Pip **(A)** and Aad **(B)** accumulation in leaves inoculated with compatible *Psm* and incompatible *Psm avrRpm1*. FW, fresh weight; hpi, hours postinoculation.

(C) and **(D)** Accumulation of Pip in *Psm*-inoculated leaves of wild-type Col-0 and selected defense mutant plants at 1 DAI **(C)** and 2 DAI **(D)**.

(E) Accumulation of Aad in *Psm*-inoculated leaves of wild-type Col-0 and selected defense mutant plants at 2 DAI.

(F) and **(G)** Leaf levels of Pip **(F)** and Aad **(G)** 2 d after leaf treatment with 10 mM $MgCl_2$ (c), 200 nM flg22, and 100 µg mL^{-1} LPS purified from *E. coli*. Data represent the mean ± SD of at least four replicate samples. In **(A)** and **(B)**, asterisks denote statistically significant differences between *P. syringae* and $MgCl_2$ samples and in **(F)** and **(G)** between control and PAMP samples (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; two-tailed *t* test). In **(C)** and **(D)**, open (closed) circles indicate statistically significant differences between an $MgCl_2$ (*Psm*) mutant and the $MgCl_2$ (*Psm*) wild-type sample (two-tailed *t* test).

provoked a considerable accumulation of both Pip and Aad at 48 h after treatment (Figures 2F and 2G). Lower but still significant increases of the Lys catabolites also occurred when leaves were treated with a gel-purified bacterial LPS preparation (Figures 2F and 2G). Thus, onetime treatments of leaves with flagellin or LPS are sufficient to activate Lys catabolism in *Arabidopsis* leaves.

Pip Accumulates Systemically in the Plant and Is Enriched in Petiole Exudates of Inoculated Leaves

Amino acid changes in distal, noninoculated leaves of *P. syringae*–treated plants were less pronounced than at inoculation sites. The only amino acid with a strong, 10-fold systemic increase at 48 h after local *Psm* inoculation was Pip (Figure 1, central column). The systemic levels of Aad, Phe, Tyr, and Val also rose significantly, albeit to much lower amounts (approximately twofold), and the systemic leaf contents of the remaining amino acids did not differ significantly between mock and pathogen treatments. Significantly enhanced systemic levels of Pip but not of SA were already observed at 36 h after *Psm* inoculation (Figures 3A and 3B), suggesting that Pip accumulation precedes SA accumulation in systemic leaves at the onset of SAR. Moreover, the HR-inducing *Psm avrRpm1* strain triggered systemic Pip increases to a similar extent than the compatible *Psm* strain (see Supplemental Figure 4B online). Although the systemic accumulation of Pip was

markedly reduced in the SAR-defective *fmo1* and *ics1* mutants, localized bacterial treatment still provoked significant increases of Pip in distal leaves of these mutants (Figure 3C). By contrast, systemic levels of Pip were not enhanced in the *npr1* and *pad4* mutants (Figure 3C).

The apparently outstanding accumulation pattern of Pip during SAR establishment was corroborated in analyses of petiole exudates collected from inoculated leaves between 6 and 48 h after inoculation (Figure 1, right column). Upon pathogen treatment, levels of Pip increased by sevenfold, whereas other amino acid levels remained at control values and showed less pronounced increases (Phe, Gly, and Ala) or were reduced (Lys, Aad, Ile, and Thr). Compared with the substantial pathogen-induced increase of Pip in exudates, the levels of other defense-related metabolites, such as SA, SA glucoside, methyl salicylate, azelaic acid (AZA), JA, or camalexin in exudates did not change or only modestly increased after pathogen inoculation (see Supplemental Figure 5 online). Along with Pip, several of these metabolites and amino acids did strongly accumulate in inoculated leaves (Figure 1; Malamy et al., 1990; Mishina and Zeier, 2007; Attaran et al., 2009; Jung et al., 2009). The release of Pip out of the petioles of inoculated leaves seems therefore favored over the release of many other accumulating metabolites, at least under the experimental conditions used for exudate collection. Enrichment of Pip in petiole exudates of pathogen-inoculated leaves was also detected in *fmo1* and *ics1* and thus was independent of FMO1- and SA-mediated signaling (Figure 3D).

Pathogen-Induced Activation of Lys Catabolism and *ALD1*-Dependent Biosynthesis of Pip

In plants and animals, Aad biosynthesis occurs via the saccharopine pathway, which is considered a major metabolic route for Lys catabolism (Galili et al., 2001). A bifunctional polypeptide with both LKR and SDH activities catalyzes the first two steps of the saccharopine pathway and converts Lys via saccharopine into α-aminoadipic semialdehyde (see Supplemental Figure 3 online). The aldehyde can be enzymatically oxidized to Aad or spontaneously cyclized to Δ1-piperideine-6-carboxylic acid, which might be reduced to Pip. However, isotope labeling studies suggest that Pip is formed from Lys via ε-amino-α-ketocaproic acid and Δ1-piperideine-2-carboxylic acid (Gupta and Spenser, 1969). This would require an aminotransferase reaction converting Lys into ε-amino-α-ketocaproic acid (see Supplemental Figure 3 online).

We reasoned that ALD1, an aminotransferase with Lys converting activity (Song et al., 2004b), might be involved in Pip biosynthesis. ALD1 is critical for the activation of local and systemic defenses in *Arabidopsis* (Song et al., 2004a), and *ALD1* transcript levels strongly increased in *Psm*-inoculated Col-0 plants in both infected and in distal leaves (Figures 4A and 4B). Consistent with our hypothesis, the *ald1-T2* (*ald1*) knockout mutant (Song et al., 2004a) not only failed to express *ALD1* in inoculated and distal leaves (see Supplemental Figures 6A and 6C online), but was also fully blocked in *Psm*-induced Pip biosynthesis at inoculation sites (Figure 4C), *Psm*-triggered elevation of Pip levels in systemic leaves (Figure 3C), and enrichment of Pip in petiole exudates (Figure 3D). Our data thus demonstrate that ALD1 is required for pathogen-induced Pip biosynthesis. By contrast, ALD1 is not essential for Aad production, since *ald1* leaves accumulated wild-type levels of Aad upon treatment with *Psm* (Figure 4D).

The strong accumulation of Aad at sites of bacterial infection occurs with a significant enhancement of local *LKR/SDH* transcript levels (Figures 2B and 4A). We isolated two independent, homozygous T-DNA insertion lines with markedly reduced expression of *LKR/SDH* (*lkr-1*, Salk_068769; *lkr-2*, Salk_127160; see Supplemental Figure 6B online). *Psm*-induced Aad production in leaves was strongly attenuated in both *lkr-1* and *lkr-2* (Figure 4D), indicating that LKR/SDH and the saccharopine pathway are involved in pathogen-induced Aad production. By contrast, *Psm*-inoculated leaves of *lkr-1* and *lkr-2* plants accumulated Pip to levels similar to those of the wild type (Figure 4C). Together, these data suggest two distinct biochemical pathways for pathogen-induced Pip and Aad biosynthesis, namely, the ALD1-dependent pipecolate pathway and the LKR/SDH-mediated saccharopine pathway (see Supplemental Figure 3 online). Blockage of either pathway in *ald1* or *lkr-1/2* leads to a hyperaccumulation of the common precursor Lys after pathogen infection (Figure 4E).

L-Pip Is a Metabolic Regulator of Several Forms of Induced Resistance in Plants

The Pip-deficient *ald1* mutant exhibits strongly attenuated basal and specific resistance and is also fully blocked in SAR (Song et al., 2004a; Figure 5A), suggesting that endogenous accumulation of Pip is critical for these different forms of inducible resistance. By contrast, Aad biosynthesis seems not essential for SAR or local resistance to *P. syringae*, since *lkr-1* and *lkr-2* exhibit wild-type-like resistance phenotypes following *Psm* infection (Figure 5A).

We tested whether exogenous application of Pip could complement the attenuated local resistance in *ald1* and its defect in SAR. When racemic D,L-Pip was applied via the root system, a significant uptake into the shoot was observed in both Col-0 and *ald1* plants. Watering of plants with 10 μmol of D,L-Pip 1 d prior to pathogen inoculation led to increases of Pip levels in leaves comparable in magnitude to the pathogen-induced elevations (see Supplemental Figure 7A online). Exogenous Pip also markedly increased resistance of Col-0 wild-type leaves to compatible *Psm* (Figures 5B and 6A to 6C) and incompatible *Psm avrRpm1* (Figure 5D). Quantitatively, this Pip-induced resistance in wild-type plants varied in independent experiments between a threefold and 25-fold reduction in pathogen growth and proved highly significant in each experiment (Figures 5B, 5D, 6B, and 6C; see Supplemental Figures 8A, 8B, and 8E online). Exogenous Pip reproducibly increased resistance when applied via the root but not when infiltrated into the apoplastic space of leaves, presumably due to a lack of efficient uptake of the amino acid via the plasma membrane into the cell interior (see Supplemental Figure 8A online). Remarkably, root application of Pip also chemically complemented *ald1* defects in local resistance, strongly boosting basal and specific resistance of the pipecolate-deficient mutant to wild-type-like levels (Figures 5B to 5D). Along with the strong reduction of apoplastic bacterial growth, a remarkable reduction of disease symptomology was apparent in Pip-treated Col-0 and *ald1* plants (Figure 5C). Application of 1 μmol Pip per plant was sufficient to significantly enhance resistance to *Psm* in Col-0 or *ald1*, with 10 μmol further augmenting

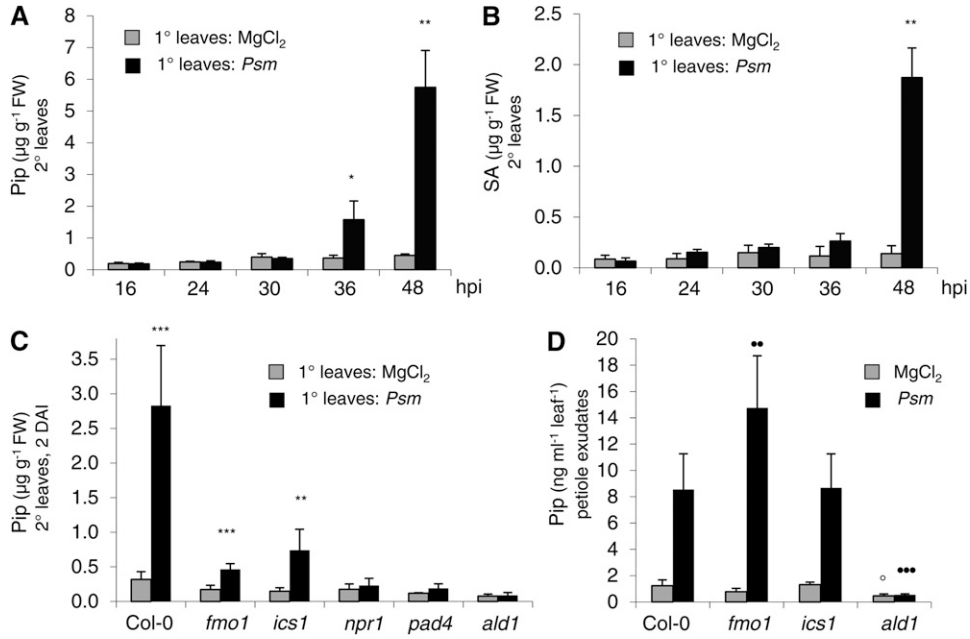

Figure 3. Pip and SA Accumulation in Distal (2°) Leaves and Pip Levels in Leaf Petiole Exudates.

(A) and **(B)** Time course of Pip **(A)** and free SA **(B)** accumulation in upper (2°) leaves following inoculation of lower (1°) leaves with *Psm*. FW, fresh weight; hpi, hours postinoculation.

(C) Systemic Pip accumulation at 2 DAI in Col-0 and different defense mutant plants.

(D) Pip levels in petiole exudates of leaves collected between 6 to 48 h after *Psm* or $MgCl_2$ treatment.

Bars represent the mean ± SD of at least four replicate samples. In **(A)** to **(C)**, asterisks denote statistically significant differences between *P. syringae* and $MgCl_2$ samples. In **(D)**, open (closed) circles indicate statistically significant differences between an $MgCl_2$ (*Psm*) mutant and the $MgCl_2$ (*Psm*) wild-type sample.

the effect, but 100 nmol showing no effect (see Supplemental Figure 8B online). The L- but not the D-enantiomer chemically complemented the *ald1* resistance defect, suggesting that L-Pip is the active metabolite in *Arabidopsis* (see Supplemental Figure 8C online). This is consistent with the exclusive detection of the naturally occurring L-enantiomer in plants (Morrison, 1953; Zacharius et al., 1954; Fujioka et al., 1987). Importantly, in the presence of Pip, *Psm* or *Psm avrRpm1* inoculation of *ald1* plants in lower leaves also rendered upper leaves more resistant to subsequent infection, demonstrating that exogenous Pip is able to restore SAR in *ald1* (Figure 6A; see Supplemental Figure 8D online).

The amino acid BABA is not produced in plants but effectively enhances plant disease resistance via a SAR-like mechanism when exogenously supplied through the soil (Zimmerli et al., 2000). Similar to exogenous Pip treatment, feeding with 10 µmol BABA via the root system resulted in an uptake of the amino acid and translocation into the shoot (see Supplemental Figure 7D online). BABA feeding also led to a moderate augmentation of Lys and Aad levels and, more importantly, to a strong increase of Pip levels in leaves. This augmentation was quantitatively comparable to the systemic accumulation of Pip during SAR (see Supplemental Figure 7D online; Figures 3B and 3C). BABA application increased the resistance of Col-0 plants toward *Psm* to an extent comparable to exogenous Pip treatment, and simultaneous application of both BABA and Pip had no synergistic effect. In the *ald1* mutant, however, BABA-induced resistance was fully blocked, indicating that induced biosynthesis of Pip following BABA application causes the enhanced resistance to *P. syringae* (Figure 6B). Similar to SAR (Mishina and Zeier, 2006), BABA-induced resistance was also fully blocked in *fmo1*, but exogenous Pip was neither able to restore SAR nor BABA-induced resistance in this mutant (Figures 6A and 6B). This is consistent with a possible function of FMO1 downstream of Pip in resistance induction. Exogenous Pip was also not able to abolish the severe defects in basal resistance of *pad4* and *npr1*, although a small Pip-mediated resistance effect in these mutants was detected (Figure 6C). In comparison, Pip more pronouncedly induced resistance in the SA-deficient *ics1* mutant, rendering this mutant approximately as resistant as the nontreated but less resistant than the Pip-treated wild type (Figure 6C). These findings indicate that Pip is capable of inducing resistance in the absence of SA but that SA is required for full Pip-induced resistance. Similar to FMO1, PAD4 and NPR1 seem to have a role downstream of Pip in resistance induction.

We furthermore observed that exogenous Pip application via the root not only resulted in increased Pip levels in Col-0 or *ald1* leaves (see Supplemental Figure 7A online) but also to a moderate but significant increase in Aad levels (see Supplemental Figure 7B online). This indicates that plants can convert some of the exogenous Pip to Aad. Exogenous Aad application, by contrast, did not heighten the levels of Pip in plants (see Supplemental Figure 7C online). Aad application also resulted in a moderate resistance increase in plants, but this effect was quantitatively not comparable to the strong Pip-induced resistance effect (see Supplemental Figure 8E online).

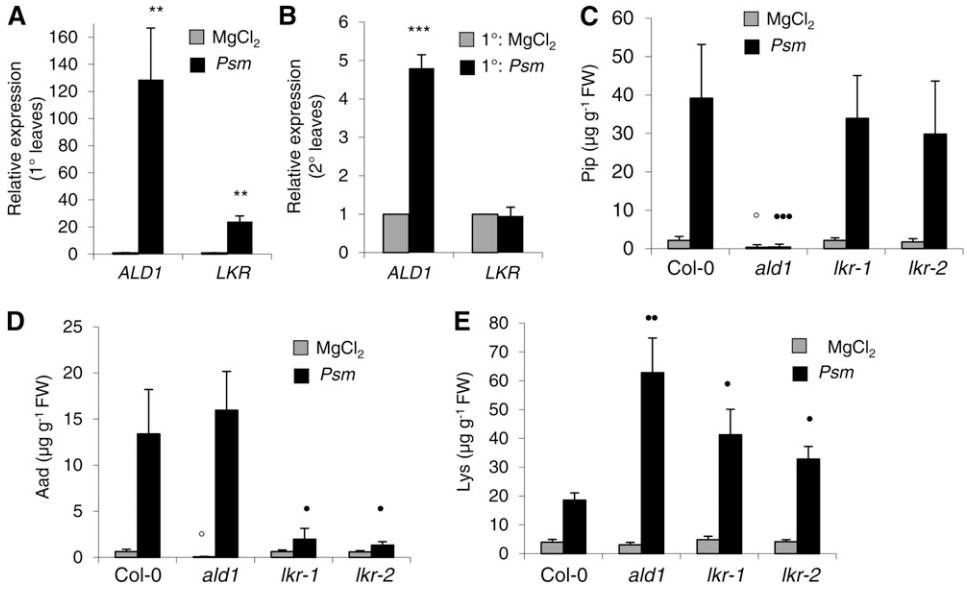

Figure 4. Pathogen-Inducible Pip and Aad Accumulation Is Dependent on *ALD1* and *LKR*, Respectively.

(A) and **(B)** *Psm*-induced *ALD1* and *LKR* expression in Col-0 plants. Transcript levels were assessed by quantitative real-time PCR analysis, are given as means ± SD of three replicate samples, and are expressed relative to the respective mock control value. Asterisks denote statistically significant differences between *Psm* and $MgCl_2$ samples.

(A) Relative expression in *Psm*-inoculated leaves (1 DAI).

(B) Relative expression in upper (2°) leaves upon *Psm* inoculation of lower (1°) leaves (2 DAI).

(C) to **(E)** Accumulation of Pip **(C)**, Aad **(D)**, and Lys **(E)** in *Psm*-inoculated leaves of Col-0, *ald1*, and *lkr* plants at 2 DAI. Data represent the mean ± SD of at least four replicate samples. Open (closed) circles indicate statistically significant differences between an $MgCl_2$ (*Psm*) mutant and the $MgCl_2$ (*Psm*) wild-type sample. FW, fresh weight.

Biologically Induced SAR Confers Priming of Defense Metabolite Accumulation and Defense Gene Expression in an *ALD1*-Dependent Manner

Our results indicate that L-Pip is a critical mediator of several forms of inducible plant immunity, including SAR, basal, specific, and BABA-induced resistance. BABA application is known to promote plants to a primed state in which they are able to more quickly and vigorously mobilize defense responses after pathogen attack (Zimmerli et al., 2000). Although defense priming induced by resistance-activating chemicals, such as BABA or *S*-methyl-1,2,3-benzothiadiazole-7-carbothioate (BTH) is a well-studied phenomenon, evidence for priming in plants in which SAR has been biologically induced is rare (Beckers et al., 2009; Jung et al., 2009). To examine defense priming during biological SAR, we conducted a double inoculation experiment. *Arabidopsis* plants were first treated in lower (1°) leaves with *Psm* to induce SAR. Two days later, a challenge infection with *Psm* in upper (2°) leaves was performed that was scored for early defense response activation at 10 h after inoculation. Mock and *Psm* inoculations in 1° and 2° leaves were combined to yield four different cases: a control situation (1° mock/2° mock), a systemic pathogen trigger (1° *Psm*/2° mock), a local pathogen trigger (1° mock/2° *Psm*), and a combination of both the systemic and the local stimulus (1° *Psm*/2° *Psm*).

When free SA levels were monitored as a defense output, we observed that *Psm*-triggered SA accumulation at 10 h after inoculation was similar in control plants and in SAR preinduced plants, suggesting that SA production is not subject to priming during biological SAR (Figure 7A). However, since SA can be converted to different derivatives upon infection, we reasoned that the determination of total SA would be a more relevant parameter to assess SA production over time. For total SA, we observed that the increases detected for the combined pathogen treatments approximately represented the sum of the systemic and the local SA enhancements (Figure 7B), indicating an additive contribution of the local and systemic pathogen trigger for SA accumulation during SAR. By contrast, *ald1* mutants lacked the systemic response and exhibited an attenuated local response with respect to SA production, resulting in a comparatively low and similar accumulation of (total) SA after the mock/*Psm* and *Psm*/*Psm* treatments (Figures 7A and 7B). In the Col-0 wild type, Pip levels increased in 2° leaves due to the systemic pathogen trigger, resulting from 1° leaf infection, but not yet due to the local *Psm* trigger at 10 h after inoculation. However, a substantial increase of Pip biosynthesis was observed 10 h after the challenge infection in SAR-induced plants, indicating a strong potentiation of Pip biosynthesis during SAR (Figure 7C). By contrast, the *Arabidopsis* phytoalexin camalexin accumulated only as a consequence of the local but not the systemic pathogen trigger in Col-0, and local camalexin production was again strongly potentiated when SAR had been biologically activated (Figure 7D). This priming effect was completely absent in the Pip-deficient *ald1* mutant (Figure 7D).

Next, we monitored defense gene expression as an output and detected reproducible priming responses for the transcript

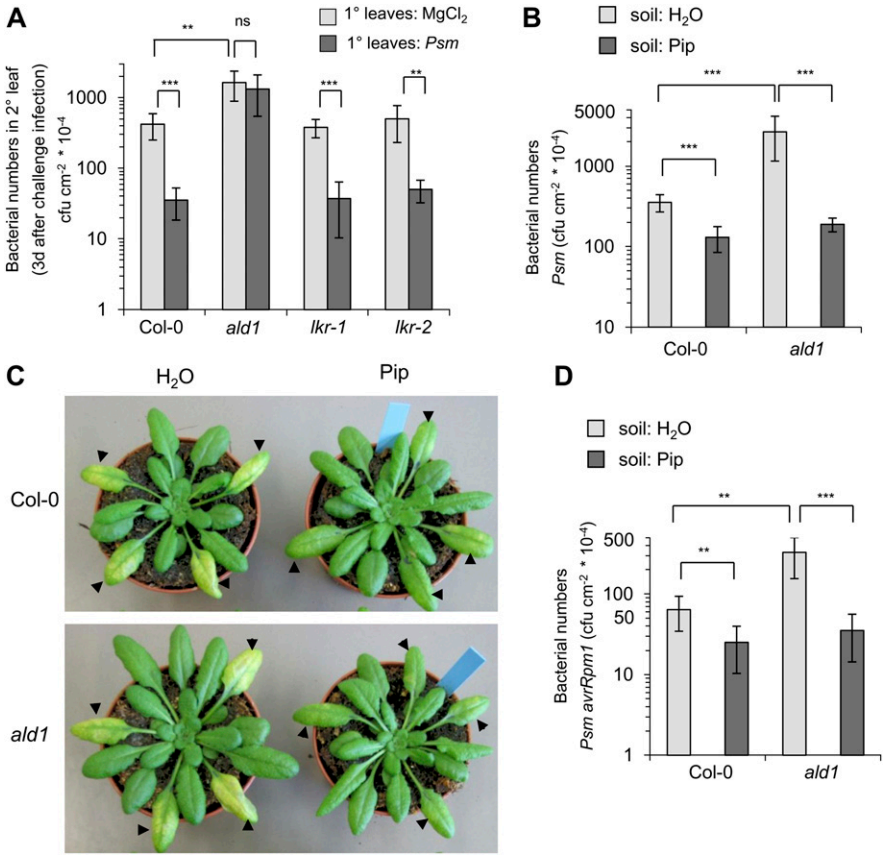

Figure 5. Exogenous Pip Enhances Disease Resistance of Wild-Type *Arabidopsis* and Overrides *ald1* Defects in PTI and ETI.

(A) SAR assay in Col-0, *ald1*, and *lkr* plants. Lower (1°) leaves were infiltrated with either 10 mM $MgCl_2$ or *Psm* (OD 0.005), and 2 d later, three upper leaves (2°) were challenge infected with *Psm* (OD 0.001). Bacterial growth in upper leaves was assessed 3 d after 2° leaf inoculation.
(B) and **(D)** Bacterial numbers of compatible *Psm* (applied in titers of OD 0.001) **(B)** and incompatible *Psm avrRpm1* (applied in titers of OD 0.002) **(D)** in *Arabidopsis* Col-0 and *ald1* leaves at 3 DAI. Plant pots were supplied with 10 mL of water or 10 mL of 1 mM (≡ 10 μmol) Pip 1 d prior to inoculation.
(C) Disease symptoms of *Psm*-infected Col-0 and *ald1* plants in the absence and presence of exogenous Pip. Arrowheads denote inoculated leaves.
Bars represent the mean ± SD of at least seven replicate samples. Asterisks denote statistically significant differences between indicated samples (***$P < 0.001$ and **$P < 0.01$; ns, not significant; two-tailed *t* test).

accumulation of *ALD1*, *FMO1*, and the SA-inducible *PR-1* gene following biological SAR induction in Col-0. This demonstrates that plants exhibiting biological SAR are promoted into an alarmed state that enables fortified defense gene expression. The *ald1* mutant essentially lacked this priming capacity (Figures 7E to 7G). Together, the SAR-induced state provides moderate, additive priming for SA accumulation and strong, multiplicative priming for both Pip and camalexin production as well as expression of the defense-related genes *ALD1*, *FMO1*, and *PR-1*. These priming responses are absent in *ald1*, indicating the importance of endogenous Pip generation for defense priming during SAR (Figures 7A to 7G).

Exogenous Pip Promotes Plants to a Primed, SAR-Like State

We next tested whether plant defense responses would be potentiated by exogenous Pip in early stages of the *Arabidopsis*–*Psm* interaction. Accumulation of total SA in leaves at 10 h after *Psm* infection was significantly higher in Col-0 than in *ald1*, and exogenous Pip boosted induction of SA biosynthesis in both genotypes to 2.5-fold to threefold higher levels than those detected in *Psm*-infected leaves of unfed Col-0 plants (Figure 8A). Pip feeding also restored the capacity of *ald1* plants to systemically enhance SA levels upon localized pathogen inoculation, indicating the importance of Pip for systemic SA accumulation during SAR (Figure 8B). Moreover, exogenously applied Pip influenced SA levels of untreated leaves only slightly but markedly enhanced local and systemic SA levels in plants pressure infiltrated with 10 mM $MgCl_2$, a treatment that routinely serves as a mock control for *P. syringae* inoculation (Figures 8A and 8B). These findings suggest that Pip alone is not sufficient to efficiently stimulate SA biosynthesis but that it positively regulates local and systemic SA production in the presence of pathogen-derived, SAR-related, or mechanical stimuli.

At 10 h after inoculation, accumulation of the phytoalexin camalexin was just detectable in *Psm*-infected Col-0 leaves, but camalexin biosynthesis was strongly potentiated in plants exogenously

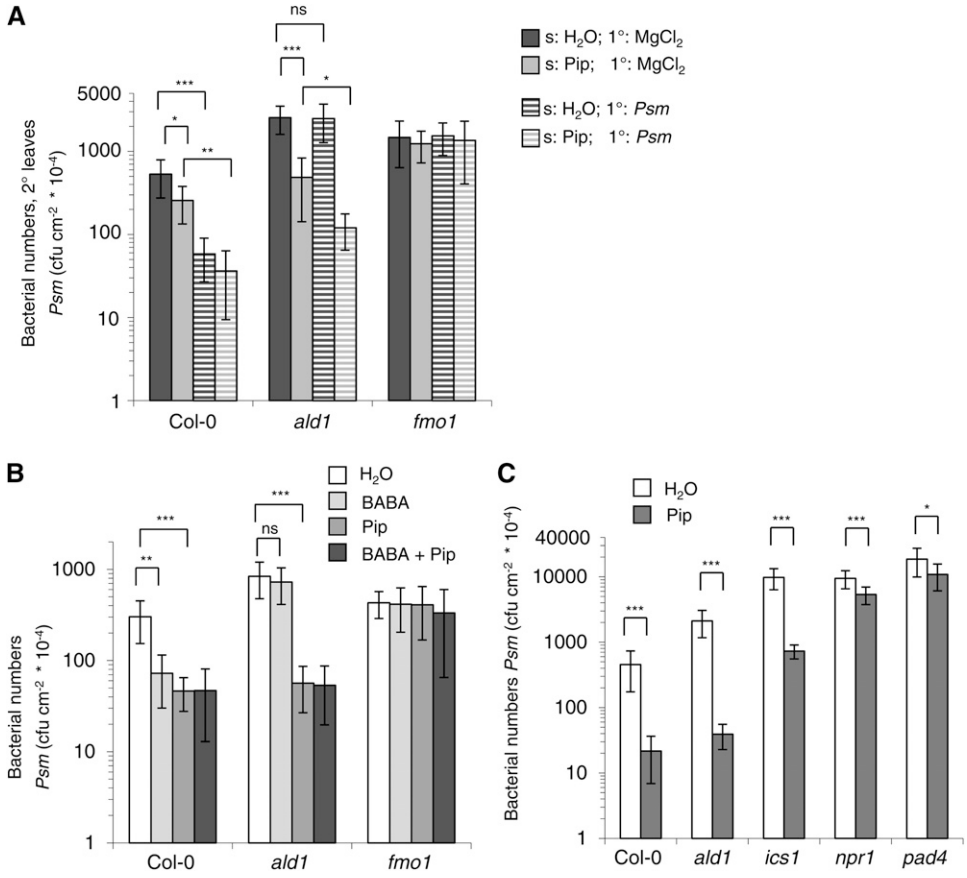

Figure 6. Exogenous Pip Overrides *ald1* Defects in SAR and BABA-Induced Resistance.

(A) SAR assay in Col-0, *ald1*, and *fmo1*. Water or 10 µmol Pip was applied through the soil (s) of each plant pot, and 1 d later a 1° leaf infiltration with either 10 mM $MgCl_2$ or *Psm* (OD 0.005) in three lower leaves was performed. Another 2 d later, three upper leaves (2°) were challenge infected with *Psm* (OD 0.001). Bacterial growth in upper leaves was assessed 3 d after 2° leaf inoculation. ns, not significant.

(B) BABA-induced resistance assay in Col-0, *ald1*, and *fmo1*. Water, BABA (10 µmol), Pip (10 µmol), or a combination of both BABA and Pip (10 µmol each) were applied through the soil, and resistance toward *Psm* was assessed. Bars represent mean values (± SD) of colony-forming units (cfu) per cm^2 from at least seven replicate samples, each consisting of three leaf disks. Asterisks denote statistically significant differences between indicated samples. No significant differences in initial bacterial numbers (1 h after inoculation) were detected.

(C) Pip-induced resistance in wild-type Col-0 and different mutant lines to *Psm* infection. Colony-forming units of *Psm* (applied in titers of OD 0.001) at 3 DAI. Plant pots were supplied with 10 mL of water or 10 mL of 1 mM (=10 µmol) Pip 1 d prior to inoculation.

fed with Pip (Figure 8C). Thus, similar to the priming response characteristic for biological SAR, exogenous Pip positively regulates SA biosynthesis and strongly potentiates camalexin production. Pip feeding also led to an early, *Psm*-triggered camalexin accumulation in *ald1* that exceeded the respective wild-type value in the absence of exogenous Pip but was lower than the wild-type levels in the presence of exogenous Pip (Figure 8C). This suggests that defense priming mediated by exogenous Pip is still reinforced by the capacity of endogenous Pip biosynthesis present in the wild type. In addition, Pip application to Col-0 plants significantly increased *ALD1* transcript levels and strongly primed plants for pathogen-triggered expression of *ALD1*, indicating a positive regulatory role for Pip on its own biosynthesis (Figure 8D). Similarly, Pip feeding led to strong priming of *Psm*-triggered *FMO1* and *PR-1* expression (Figures 8E and 8F). Together, these findings indicate that Pip accumulation is sufficient to promote plants into a state that enables SAR-like priming. Furthermore, the observation that Pip-deficient *ald1* is defective in SAR priming suggests that endogenously accumulating Pip mediates priming during SAR (Figure 7). The facts that the biosynthesis of the regulatory metabolite itself is boosted during SAR (Figure 7C) and that Pip promotes enhanced induction of *ALD1* expression (Figure 8D) indicate the existence of a positive defense amplification loop within SAR in which Pip acts as a central player. This amplification mechanism also seems to include *FMO1*, since the SAR-defective *fmo1* mutant is unable to establish Pip-induced resistance (Figure 6), and expression of *FMO1* is primed during SAR and upon Pip treatment (Figures 7F and 8E).

As described above, Pip positively regulates SA biosynthesis. A converse positive feedback of SA on Pip production seems to exist in systemic rather than in inoculated tissue because functional ICS1 is required for normal pathogen-induced Pip accumulation in distal but not in inoculated leaves (Figures 2C, 2D, and

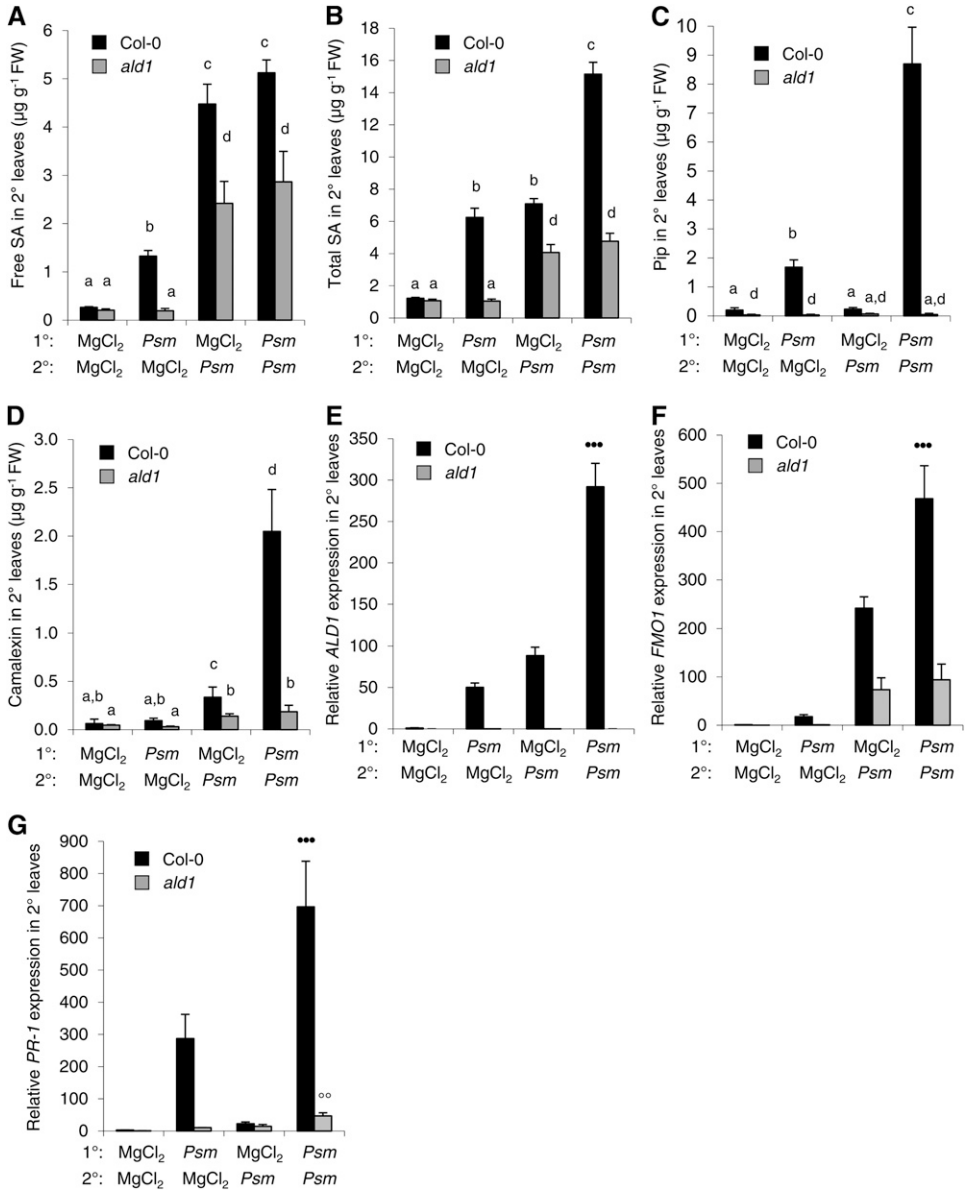

Figure 7. Biological SAR Confers Defense Priming on the Metabolite and Gene Expression Levels in an *ALD1*-Dependent Manner.

Double inoculation experiment to assess defense priming during SAR in Col-0 and *ald1*. Plants were treated in lower (1°) leaves with $MgCl_2$ or *Psm* (OD 0.005), and 2 d later, upper (2°) leaves were infiltrated with $MgCl_2$ or *Psm*. Upper leaves were then scored for defense metabolite accumulation or defense gene expression at 10 h after inoculation. This yielded four distinguishable cases corresponding to a control situation (1° $MgCl_2$/2° $MgCl_2$), a systemic pathogen stimulus (1° *Psm*/2° $MgCl_2$), a local pathogen stimulus (1° $MgCl_2$/2° *Psm*), and a combination of both the systemic and the local stimuli (1° *Psm*/2° *Psm*). FW, fresh weight.

(A) to **(D)** SAR priming of defense metabolite accumulation. Free SA **(A)**, total SA (sum of free SA and conjugated SA) **(B)**, Pip **(C)**, and camalexin **(D)** accumulation at 10 h after 2° treatment. Bars represent the mean ± SD of at least four replicate samples. Different letters above the bars denote statistically significant differences between pairwise compared samples ($P < 0.05$, two-tailed *t* test).

(E) to **(G)** SAR priming of defense gene expression. Relative *ALD1* **(E)**, *FMO1* **(F)**, and *PR-1* **(G)** expression at 10 h after 2° treatment. Transcript levels were assessed by quantitative real-time PCR analysis, are given as means ± SD of three replicate samples, and are expressed relative to the respective mock control value. Statistical differences in transcript abundance upon 2° *Psm* infection of SAR-noninduced versus SAR-induced plants were assessed. Closed (open) circles indicate whether statistically significant differences in Col-0 (*ald1*) between the 1° $MgCl_2$/2° *Psm* and the 1° *Psm*/2° *Psm* treatments exist (two-tailed *t* test).

3C; see Supplemental Figure 4A online). To further investigate the relationship of Pip and SA, we tested whether SA application alone was sufficient to increase Pip levels and *ALD1* expression in plants. Indeed, 24 h after infiltration of 0.5 mM SA, we detected a significant increase of Pip in Col-0 leaves. However, this response was small compared with pathogen-induced Pip accumulation (see Supplemental Figure 9A online). *ALD1* transcript levels also rose slightly, to ~1.5-fold, upon SA treatment, but again, this effect was small compared with the strong, more than 3000-fold SA-induced increase of *PR-1* transcript levels in the same experiment (see Supplemental Figure 9B online).

DISCUSSION

The widespread occurrence of the non-protein amino acid L-Pip in plants, animals, fungi, and microorganisms and its biosynthetic origin from Lys in plants and animals was realized in the 1950s (Morrison, 1953; Zacharius et al., 1954; Broquist, 1991). The pipecolate pathway represents the major Lys catabolic route in rat brain (Chang, 1976), and Pip has been recognized as a weak inhibitory neurotransmitter and GABA agonist (Charles, 1986). In plants, enhanced Pip levels have been measured following growth-affecting treatments, such as application of the plant growth regulator maleic hydrazide and osmotic stress (Yatsu and Boynton, 1959; Moulin et al., 2006). The physiological function of Pip in plants has remained elusive, although it was described as a flower-inducing substance in the aquatic plant *Lemna gibba* and as an indicator of abnormal protein metabolism in diseased plants (Pálfi and Dézsi, 1968; Fujioka et al., 1987).

Here, we demonstrate that Pip is a critical metabolic mediator of several forms of inducible resistance in *Arabidopsis*. These include PTI, ETI, and BABA-induced resistance directed against hemibiotrophic bacterial pathogens, and, in particular, SAR. The reported neurotransmitter function of Pip in the brain (Charles, 1986) and our findings indicate that Pip can have regulatory roles in both animals and plants. Evidence for a critical function of Pip in inducible plant immunity is provided by the findings that *ald1* mutant plants, which fail to establish SAR (Figures 5A and 6A; Song et al., 2004a), BABA-induced resistance to *P. syringae* (Figure 6B), and exhibit enhanced disease susceptibility against incompatible and compatible *P. syringae* strains (Figures 5B to 5D; Song et al., 2004a), are completely defective in induced Pip biosynthesis (Figures 3C, 3D, and 4C). Moreover, exogenous Pip is able to complement the resistance defects of *ald1* plants and increases PTI and ETI of wild-type plants toward *P. syringae* infection (Figures 5B to 5D). Thus, our study uncovers a plant resistance pathway that is activated in an SA-independent manner in inoculated leaf tissue. The occurrence of Pip is widespread in the plant kingdom, and pathogen-induced Pip accumulation has been reported to take place in leaves of rice (*Oryza sativa*), soybean (*Glycine max*), potato (*Solanum tuberosum*), and tobacco (*Nicotiana tabacum*) (Yatsu and Boynton, 1959; Pálfi and Dézsi, 1968; Moulin et al., 2006). It is therefore likely that Pip also orchestrates immune responses in plant species other than *Arabidopsis*.

Along with another Lys catabolite, α-aminoadipic acid, its biosynthetic precursor Lys, and several aromatic and branched-chain amino acids, Pip strongly accumulates in pathogen-inoculated leaves (Figures 1, 2A, and 9). In addition, Pip production is triggered by exogenous treatment of leaves with PAMPs (Figure 2F). The biosynthesis of Pip is positively influenced by the defense regulator PAD4 (Figures 2C and 2D) and proceeds via the ALD1 aminotransferase (Figure 4C). Expression of *ALD1* is upregulated at sites of pathogen attack and in distal, non-inoculated leaves (Figures 4A and 4B; Song et al., 2004a). The partial dependency of Pip biosynthesis on PAD4 is consistent with an attenuated expression of *ALD1* in *pad4* mutants after *P. syringae* infection (Song et al., 2004a). A second pathogen-inducible Lys catabolic pathway produces Aad via LKR/SDH and the saccharopine pathway. Our data indicate that pathogen-induced Lys catabolism leading to Aad has no major relevance for induced resistance to hemibiotrophic bacteria and the establishment of SAR (Figure 5A), although exogenous Aad resulted in modestly enhanced *P. syringae* resistance. Via Aad and acetyl-CoA, the saccharopine pathway provides a possibility for plants to convert Lys to Glu, which can serve as a precursor for several stress-related metabolites (Arruda et al., 2000; Galili et al., 2001).

Our mutant analyses suggest that in pathogen-infected *Arabidopsis* plants, Pip and Aad are essentially produced independently of each other via separate ALD1- and LKR/SDH-dependent pathways, respectively (Figures 4C and 4D; see Supplemental Figure 3 online). However, Pip can be converted in vitro to the Aad precursor Δ1-piperideine-6-carboxylic acid by recombinant *Arabidopsis* sarcosine oxidase (Goyer et al., 2004). Decreased amounts of basal Aad in Pip-deficient *ald1* mutants and modestly increased Aad levels in plants upon Pip feeding indicate that in planta, a pathway enabling direct Pip to Aad conversion might exist. Both Aad feeding and *lkr* mutant analyses suggest that a reverse Aad-to-Pip transition does not take place in *Arabidopsis* (Figures 4B and 4C; see Supplemental Figures 3, 7A and 7B online).

ALD1 functions as an aminotransferase with high in vitro activity for Lys (Song et al., 2004b). Possible products of Lys transamination are ε-amino-α-ketocaproic acid or α-amino adipic semialdehyde, depending on the abstraction of the α- or ε-amino group from Lys, respectively. Feeding of bean (*Phaseolus vulgaris*) with isotope-labeled Lys suggests that conversion of Lys to Pip in plants proceeds via ε-amino-α-ketocaproic acid and/or the cyclized form Δ1-piperideine-2-carboxylic acid (Gupta and Spenser, 1969; see Supplemental Figure 3 online). Whether the Lys aminotransferase ALD1 produces these intermediates and whether a subsequent reductase exists to generate Pip from these intermediates remains to be determined. It is noteworthy in this context that bacteria use a cyclodeaminase to convert Lys to Pip in a reductive one-step reaction with a concomitant loss of ammonia (Gatto et al., 2006).

In this study, we also addressed mechanistic aspects that underlie Pip-mediated pathogen resistance. The Pip induction–deficient *ald1* mutant shows attenuated SA accumulation, camalexin production, and defense gene expression at sites of bacterial inoculation (Figures 7A, 7B, 7D, 7F, and 7G; Song et al., 2004a), suggesting that endogenously generated Pip contributes to full PTI and ETI by amplification of defense responses (Figure 9). This is corroborated by the fact that exogenous Pip boosts SA

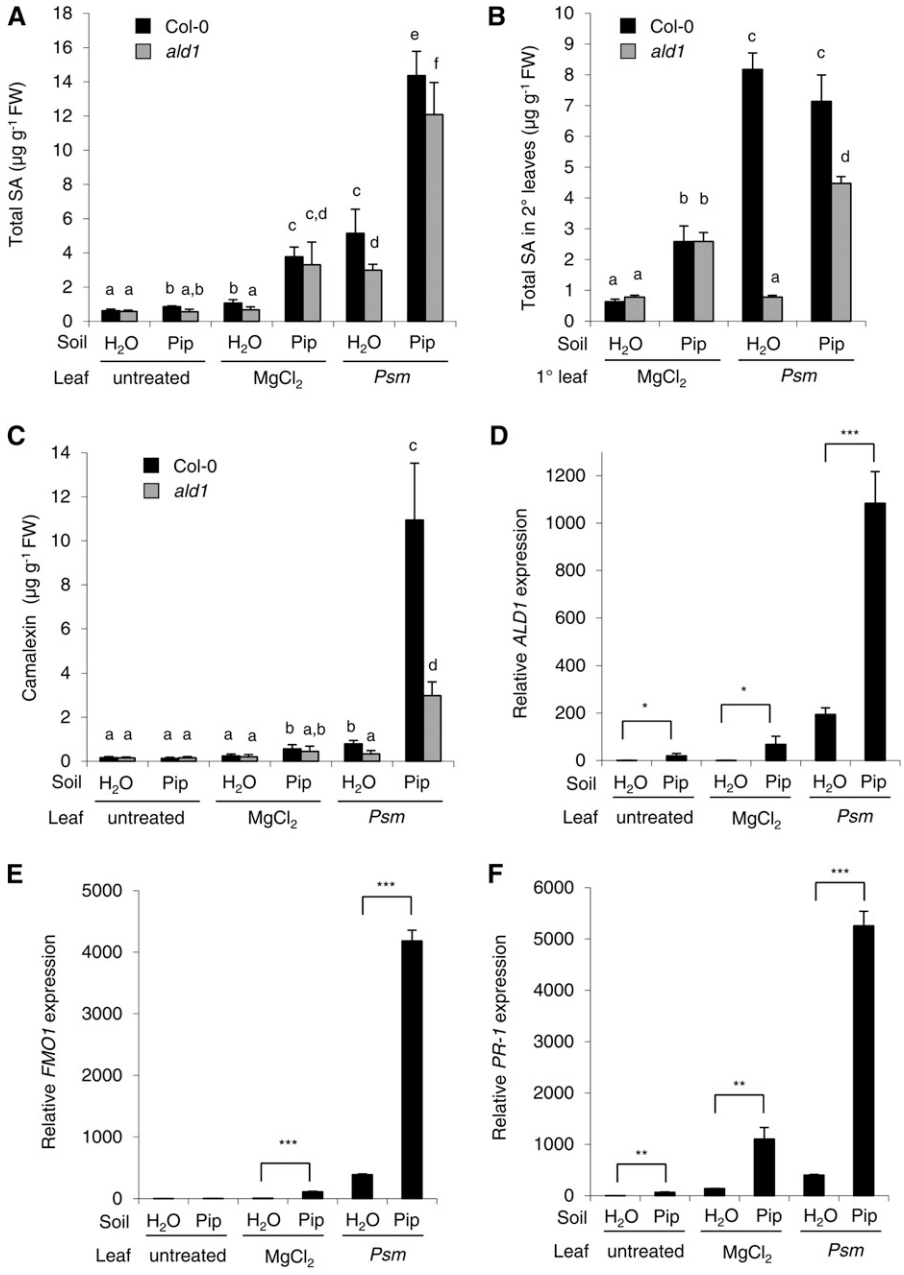

Figure 8. Exogenous Pip Confers SAR-Related Defense Priming on the Metabolite and Gene Expression Level and Restores Systemic SA Accumulation in *ald1*.

Water or 10 µmol Pip were applied to plants through the soil. Leaves were infiltrated 1 d later with *Psm* or $MgCl_2$. FW, fresh weight.
(A) and **(C)** to **(F)** Local defense responses were scored 10 h after infiltration. At the same time, a set of samples consisting of untreated leaves was assessed.
(B) Total SA accumulation in upper (2°) leaves upon *Psm* or $MgCl_2$ infiltration of lower (1°) leaves was assessed at 2 DAI for Col-0 and *ald1*.
(A) and **(C)** Accumulation of total SA **(A)** and camalexin **(C)** in Col-0 and *ald1* plants. Bars represent the mean ± SD of at least four replicate samples. Different letters above the bars denote statistically significant differences between pairwise compared samples ($P < 0.05$, two-tailed *t* test).
(D) to **(F)** Relative expression of *ALD1* **(D)**, *FMO1* **(E)**, and *PR-1* **(F)**. Transcript levels were assessed by quantitative real-time PCR analysis, are given as means ± SD of three replicate samples, and are expressed relative to the respective mock control value. Asterisks denote statistically significant differences between indicated samples (***$P < 0.001$; two-tailed *t* test).

biosynthesis and early camalexin production in the wild type and leads to an overcompensation of *ald1* defects in these defense responses (Figures 8A and 8C). The action of Pip in amplification of defense responses explains why exogenous Pip is able to enhance resistance in the wild type and restore full PTI and ETI in *ald1* (Figures 5B to 5D and 6C).

Defense amplification is important for effective PTI and ETI, local forms of induced resistance that are activated upon direct pathogen contact via recognition of preexisting PAMPs and pathogen effectors, respectively (Jones and Dangl, 2006). By contrast, induction of systemic immunity is indirect, relying on the recognition of mobile, endogenous signals in leaves distal from pathogen contact that are generated in inoculated leaves (Shah, 2009). The overall direct defense eliciting capacity of numerous PAMPs and/or pathogen effectors at infection sites is presumably higher than the elicitor strength of SAR signals in leaves distal from inoculation, and signal amplification should thus be of particular importance for SAR. This is exemplified by the fact that genetic defects in defense regulators such as NON-RACE SPECIFIC DISEASE RESISTANCE1, ALD1, or FMO1 do not generally entail a complete suppression of local defense responses but do imply a total loss of the SAR response (Shapiro and Zhang, 2001; Song et al., 2004a; Bartsch et al., 2006; Mishina and Zeier, 2006; Figure 7).

We previously proposed the existence of an FMO1-dependent signal amplification loop operating in noninoculated, distal leaves that is critical for SAR establishment (Mishina and Zeier, 2006). Similar to *ald1* (Figures 7A and 7B; Song et al., 2004a), *fmo1* plants do allow significant rises of SA in inoculated leaves, whereas SA accumulation is fully absent in systemic leaves. Since SA is not transported from locally infected to systemic leaves (Vernooij et al., 1994; Attaran et al., 2009), the systemic accumulation of SA necessary for SAR induction must occur by de novo biosynthesis of SA in distal tissue, and this proceeds via isochorismate synthase (Wildermuth et al., 2001). Our findings that exogenous Pip does restore the capacity of *ald1* to systemically enhance SA levels and establish SAR demonstrate that Pip is a critical SAR player that is required for systemic SA accumulation. A role of Pip upstream of SA in SAR (Figure 9) is consistent with our findings that Pip accumulation precedes SA accumulation at the systemic level (Figures 3A and 3B). The observation that FMO1 is required for SAR and Pip-mediated resistance induction (Figure 6) suggests that FMO1 acts downstream of Pip to realize SAR (Figure 9). Flavin-dependent monooxygenases from plants, animals, or fungi oxidize either nitrogen- or sulfur-containing substrates (Schlaich, 2007). It is thus tempting to speculate that FMO1 could convert Pip or a Pip derivative into an oxidized form and thereby transduce the Pip signal. This hypothesis would also

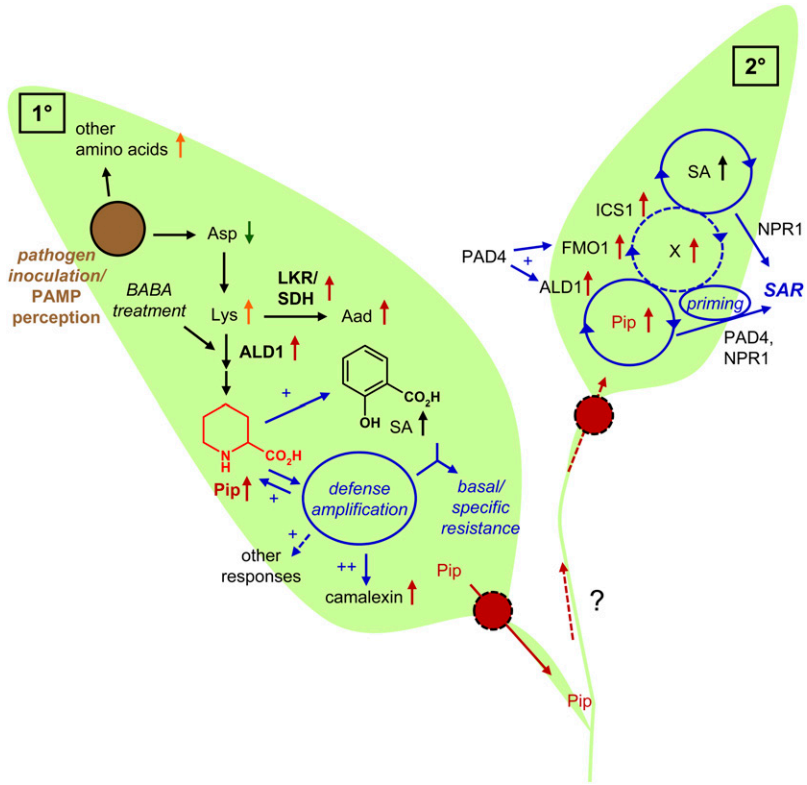

Figure 9. Proposed Model for the Role of Pip during Activation of Local Resistance, SAR, and Defense Priming.

1°, pathogen-inoculated leaf; 2°, distal leaf. Pathogen-induced changes in free amino acids, activation of Lys catabolism, including Pip formation, the roles for Pip in defense amplification and priming, and the proposed feedback amplification cycle in 2° leaves enabling SAR establishment that involves ALD1, Pip, FMO1, ICS1, and SA are illustrated. Dotted lines represent still hypothetical events. A possible role for Pip in long-distance signaling from 1° to 2° leaves remains to be clarified.

explain our observation that *fmo1* overaccumulates Pip in inoculated tissue at later stages of *Psm* infection (Figures 2D and 3D). Future experiments are necessary to clarify the exact biochemical role of FMO1 in Pip-mediated resistance induction.

To extend our previous working model for SAR, we propose that early Pip accumulation in systemic leaves drives further Pip production via upregulation of *ALD1* and subsequent FMO1-mediated activation of SA biosynthesis. Since systemic Pip accumulation is markedly attenuated in both the *fmo1* and the *ics1* mutant (Figure 3C), we assume a feedback amplification mechanism that requires SA to activate ALD1-mediated increases in Pip. This can, via the action of FMO1, lead to higher SA levels that will further fuel feedback amplification. Further repetition of this amplification cycle would then ultimately lead to SAR establishment (Figure 9). Modest Pip increases upon SA feeding also support this feedback scenario (see Supplemental Figure 9 online). A more stringent requirement of the proposed amplification loop for the activation of systemic compared with local resistance responses would explain why Pip accumulation, which does occur independently of SA and FMO1 at inoculation sites (Figures 2C, 2D, and 3D), essentially becomes SA- and FMO1-dependent at the systemic defense level (Figure 3C).

Two other regulatory proteins involved in the Pip-mediated amplification cycle are PAD4 and NPR1 (Figure 8). PAD4 positively regulates pathogen-induced Pip production (Figures 2C and 2D; see Supplemental Figure 4A online), presumably because it potentiates *ALD1* transcription (Song et al., 2004a). Transcriptional control of downstream components, such as FMO1 (Bartsch et al., 2006), could be causative for our finding that PAD4 is also required to transduce the Pip signal (Figure 6C). The requirement of NPR1 to mediate Pip-induced resistance (Figure 6C) might partly rely on its function as a downstream mediator of SA signaling (Durrant and Dong, 2004). However, because of the significant Pip-induced resistance effect in SA-deficient *ics1* (Figure 6C), an additional, SA-independent function of NPR1 in Pip signal transduction is probable.

As discussed above, our working model implies small initial systemic rises of Pip at the onset of SAR (Figure 9). Two scenarios are imaginable to realize this. A first possibility is that systemic Pip levels, after the perception of SAR long-distance signals, increase exclusively by de novo synthesis via systemic upregulation of *ALD1*. Alternatively, the initial small rises of Pip in distal leaves might be a consequence of long-distance transport of Pip from inoculated leaves, which accumulate high levels of Pip (Figure 2). Movement of Pip out of inoculated leaves is conceivable because of its strong and selective enrichment in collected petiole exudates (Figure 1; see Supplemental Figure 4 online). Furthermore, small systemic rises of Pip in *fmo1* mutant plants that completely lack systemic upregulation of *ALD1* might indicate long-distance transport of Pip (see Supplemental Figure 5C online). However, the possible mobile character of Pip and its putative function in long-distance transport are hypothetical at this stage. Grafting experiments and tracer studies using isotope-labeled Pip may be helpful to clarify this issue in the future.

In recent years, SAR long-distance signaling has become a matter of intense debate. It appears that several cross-interacting signals activate SAR and that some signals vary in their contribution to resistance induction under different environmental conditions (Liu et al., 2011; Dempsey and Klessig, 2012). Proposed candidates for mobile SAR long-distance signals include the putative lipid transfer protein DIR1 (Maldonado et al., 2002), glycerol-3-phosphate (Chanda et al., 2011), the abietane diterpenoid dehydroabietinal (Chaturvedi et al., 2012), the C9-dicarboxylic acid AZA (Jung et al., 2009), and, depending on experimental conditions, methyl salicylate (Park et al., 2007; Attaran et al., 2009; Liu et al., 2011). Light availability and activated phytochrome signaling are essential factors for SAR establishment (Zeier et al., 2004; Griebel and Zeier, 2008), and the length of a light period before pathogen infection can influence the significance of individual long-distance signals (Liu et al., 2011). Recent experiments from Liu et al. (2011) suggest that FMO1 represents a central SAR node required for systemic resistance induction irrespective of the light environment applied. It was also shown that FMO1 is required for dehydroabietinal- and AZA-induced resistance (Jung et al., 2009; Chaturvedi et al., 2012). Moreover, AZA enhances resistance in an *ALD1*-dependent manner (Jung et al., 2009). These findings indicate that the proposed Pip-/FMO1-dependent amplification loop (Figure 9) is necessary to mediate SAR signal transduction of at least a subset of mobile SAR signals. An important future task will be to systematically clarify the interrelationship between Pip and other metabolic SAR activators.

SAR can be activated by localized inoculation with avirulent and virulent bacterial pathogens. Both kinds of pathogens elicit highly similar systemic response patterns that include SA accumulation, Pip accumulation, and enhanced expression of a variety of classical SAR marker genes, such as *PR-1*, *PR-2*, and *PR-5* (Mishina and Zeier, 2006; see Supplemental Figure 4B online). Furthermore, various resistance assays with SAR-defective and SAR-competent *Arabidopsis* mutants demonstrate that establishment of the SAR response following avirulent and virulent pathogen inoculation proceeds via a closely related mechanistic network (Mishina and Zeier, 2006, 2007; Zhang et al., 2010; Liu et al., 2011). This network involves SA biosynthesis and downstream signaling (Mishina and Zeier, 2007; Zhang et al., 2010) and an intact Pip pathway that includes functional *ALD1* and *FMO1* (Mishina and Zeier, 2006; Figure 5A; see Supplemental Figure 8D online). This does not rule out that discrete differences in the contributions of particular signals might exist for SAR elicited by different pathogen types or by the same pathogen type under different experimental conditions, as discussed below for the oxylipin derivatives JA and AZA, which have both been implicated with SAR long-distance signaling (Truman et al., 2007; Jung et al., 2009).

It is well documented that the degree of formation of the oxylipin derivatives JA and AZA after bacterial inoculation is positively correlated with the inoculum density and the concomitant extent of tissue necrosis formation (Mishina and Zeier, 2007; Zoeller et al., 2012). Leaf necrosis also occurs much earlier and is more pronounced in response to HR-inducing avirulent bacterial infection than in response to virulent bacteria. This can explain observed differences in exudate composition in our experiments and those of other groups (Truman et al., 2007; Jung et al., 2009). Truman et al. (2007) have reported accumulation of JA in petiole exudates collected from leaves treated with high doses (OD_{600} 0.2) of avirulent *Pst* harboring *avrRpm1*, and Jung et al. (2009)

have detected elevated AZA levels in exudates following *Psm avrRpt2* inoculation (OD_{600} 0.01). By contrast, JA or AZA levels in exudates collected from leaves inoculated with lower densities (OD_{600} = 0.005) of virulent *Psm* are not elevated (see Supplemental Figure 5 online). AZA application has been reported to confer local and systemic plant resistance (Jung et al., 2009), but, according to our analyses, its accumulation in exudates is not a requirement for SAR. Thus, AZA might add an additional resistance contribution to the overall SAR effect without being essential to establish the response per se. Genetic and physiological evidence suggests that the previously proposed role of JA as a mobile SAR signal (Truman et al., 2007) is strongly debatable (reviewed in Shah, 2009).

Our study reveals that biologically induced SAR promotes plants into an alarmed state that accelerates the reaction to subsequent pathogen attack on several levels. The phenomenon of defense priming has been most extensively studied in context with chemical priming (i.e., plant defense activation with unnatural compounds, such as BTH or BABA). Compelling evidence that defense priming also occurs during biological SAR is still rare (Beckers et al., 2009; Jung et al., 2009; Conrath, 2011). We show that, on the metabolite level, SAR priming is characterized by a strongly potentiated induction of both camalexin and Pip biosynthesis after pathogen inoculation (Figures 7C and 7D), which can be explained by a synergistic interplay between signaling events triggered by the 1° inducing and the 2° challenge infection. SAR priming has been previously reported also for SA accumulation (Jung et al., 2009). Our results confirm that SAR allows a more pronounced accumulation of SA in the early *Psm–Arabidopsis* interaction. However, the difference in SA levels between challenged SAR-induced and control plants essentially results from a nonsynergistic additive contribution of the systemic SA increase resulting from the 1° inoculation on top of the rises resulting from the challenge infection in 2° leaves (Figure 6B). We also show that biological SAR enables plants to more pronouncedly increase expression levels of the two essential SAR regulatory genes *ALD1* and *FMO1*, and, as previously reported by Jung et al. (2009), of the SA-inducible *PR-1* gene (Figures 7E to 7G). Importantly, the Pip-deficient *ald1* plants are defective in these SAR-associated priming responses (Figure 7), providing genetic evidence for a critical action of Pip in SAR priming. Moreover, the facts that exogenous Pip promotes plants into a primed state highly similar to SAR and partially restores defense priming in *ald1* corroborates the role of Pip as an endogenous mediator of defense priming. As discussed in the context of SAR establishment (see above), feedback mechanisms within the proposed Pip resistance pathway also underlie defense priming: Pip not only triggers enhanced induction of *ALD1* to enforce its own biosynthesis (Figures 7C, 7E, and 8C), but it also amplifies expression of the downstream mediator FMO1 (Figures 7F and 8D).

The individual responses that are conditioned upon SAR establishment might add different contributions to the enhanced resistance effect toward subsequent infection. For a *P. syringae* challenge, priming of the SA- and Pip-associated pathways most likely provide significant protection because these pathways positively regulate resistance toward this bacterial pathogen. By contrast, camalexin priming might not contribute to SAR directed against *P. syringae*, since camalexin-deficient *pad3* mutants display normal basal resistance to this pathogen (Glazebrook and Ausubel, 1994). However, SAR confers broad-spectrum resistance against different pathogen types, and the potentiated production of the antimicrobial phytoalexin might protect against other microbes, such as fungal or oomycete pathogens (Thomma et al., 1999; Schlaeppi et al., 2010).

Defense priming is considered as the central mechanistic aspect of BABA-induced resistance against *P. syringae* or *Hyaloperonospora arabidopsidis* infection (Zimmerli et al., 2000; Ton et al., 2005). We found that BABA application entails Pip accumulation and induces resistance to *P. syringae* in an *ALD1*-dependent manner, indicating that Pip also orchestrates BABA-induced resistance against bacterial pathogen infection (Figure 6B; see Supplemental Figure 7B online). Interestingly, the plant activator BTH triggers *ALD1* expression in *Arabidopsis* (Song et al., 2004a), suggesting a contribution of Pip defense signaling also to BTH-triggered resistance. BTH is an example of a synthetic plant activator that is commercially applied (Du et al., 2012). In this context, our finding that the endogenous plant natural product Pip activates priming and resistance when exogenously applied to plants might gain applied relevance in modern plant protection.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown in individual pots containing a mixture of soil (Klasmann-Deilmann, Substrat BP3), vermiculite, and sand (8:1:1) inside a controlled environmental chamber with a 9-h day (9 AM to 6 PM; photon flux density 100 μmol m^{-2} s^{-1})/15-h night cycle and a relative humidity of 70%. Growth temperatures during the day and night period were 21 and 18°C, respectively. Experiments were performed with 5- to 6-week-old, naïve, and unstressed plants exhibiting a uniform appearance.

The *ald1* (*ald1_T2*; Song et al., 2004a), *lkr-1*, and *lkr-2* mutants correspond to the SALK T-DNA insertion lines SALK_007673, SALK_068769, and SALK_127160, respectively, which are all in the Col-0 background. To identify homozygous T-DNA insertion lines by PCR, the method described by Alonso et al. (2003) was applied, using gene-specific primers (see Supplemental Table 1 online).

Other lines used in this study are *fmo1* (Mishina and Zeier, 2006), *npr1-2* (*npr1*, NASC ID N3801), *pad4-1* (*pad4*; Jirage et al., 1999), *cpr5* (Bowling et al., 1997), and *sid2-1* (*ics1*; Nawrath and Métraux, 1999). Wild-type plants in the Col-0 background were used as control plants.

Cultivation of Bacteria

Pseudomonas syringae pv *maculicola* strain ES4326 (*Psm*) and *Psm* carrying the *avrRpm1* avirulence gene (*Psm avrRpm1*) were grown in King's B medium containing the appropriate antibiotics at 28°C (Zeier et al., 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to different final optical densities (OD) at 600 nm for leaf inoculations.

Assessment of SAR, Defense Priming during SAR, and Local Plant Resistance

To induce SAR, plants were infiltrated between 10 and 12 AM into three lower (1°) leaves with a suspension of *Psm* (±avrRpm1) at OD 0.005. Infiltration with 10 mM $MgCl_2$ served as a control treatment. Upper (2°) leaves were routinely harvested 2 d after the primary treatment for the determination of systemic responses (except for the time-course analyses depicted in Figures 3A and 3B).

For SAR growth assays, 2° leaves were inoculated with *Psm* (OD 0.001) 2 d after the 1° treatment. Growth of *Psm* in 2° leaves was scored another 3 d later by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d.

For the assessment of defense priming during SAR, 2° leaves were infiltrated with either 10 mM $MgCl_2$ or *Psm* (OD = 0.005) 2 d after the 1° treatment. The 2° leaves were collected 10 h after they had been treated.

For the determination of local defense responses, bacterial suspensions of OD 0.005 (determination of gene expression and metabolite levels), OD 0.001 (*Psm* growth assays), or OD 0.002 (*Psm avrRpm1* growth assays) were infiltrated into three full-grown leaves per plant. Bacterial growth was assessed 3 d after infiltration as described above.

flg22, LPS, and SA Treatments

The flg22 peptide and purified LPS from *Escherichia coli* were diluted in 10 mM $MgCl_2$ to final concentrations of 200 nM (flg22) and 100 μg mL^{-1} (LPS) and infiltrated into leaves. The flg22 peptide was synthesized by Mimotopes. The chromatographically purified LPS preparation was acquired from Sigma-Aldrich (L3024). Control infiltrations were performed with 10 mM $MgCl_2$. SA was infiltrated into leaves in a concentration of 0.5 mM.

Exogenous Application of Pip, BABA, and Aad

One day prior to bacterial inoculation or 1° treatments in the case of SAR experiments, 10 mL of a 1 mM (10 μmol) D,L-Pip solution (S47167; Sigma-Aldrich), 10 mL of 0.5 mM (5 μmol) L-Pip (P1404; TCI Europe) solution, 10 mL of 0.5 mM (5 μmol) D-Pip (P1830; TCI Europe) solution, 10 mL of 1 mM (10 μmol) BABA (A44207; Sigma-Aldrich) solution, or 10 mL of 1 mM (10 μmol) Aad (A7275; Sigma-Aldrich) solution was pipetted onto the soil substrate of individually cultivated plants. Control plants were supplemented in the same manner with 10 mL of water.

Determination of Defense Metabolites

Determination of the levels of free SA, conjugated SA, camalexin, methyl salicylate, JA, and AZA in leaves or petiole exudates was performed using vapor-phase extraction and subsequent GC-MS analysis as described by Mishina and Zeier (2006) and Attaran et al. (2009) with minor modifications. The vapor-phase extraction procedure was performed with Porapak-Q absorbent (VCT-1/4X3-POR-Q; Analytical Research Systems). The injected sample mixture (4 μL) was separated on a gas chromatograph (GC 7890A; Agilent Technologies) equipped with a fused silica capillary column (ZB-5MS 30 m × 0.25 mm; Zebron, Phenomenex), and mass spectra were recorded with a combined 5975C mass spectrometric detector (Agilent Technologies) in the electron ionization mode. For GC separation, the injector temperature was set to 250°C. A constant flow of helium (1.2 mL/min) and the following temperature program were used: 50°C/3 min with 8°C/min to 240°C, with 20°C/min to 320°C/3 min.

Determination of Amino Acids

Amino acid levels were determined by the EZ:faast free amino acid analysis kit for GC-MS (Phenomenex), which is based on the separation and mass spectrometric identification of propyl chloroformate–derivatized amino acids (Kugler et al., 2006; see Supplemental Figure 1 online). Fifty milligrams of homogenized leaf material was extracted with 200 μL of buffer (25% acetonitrile in 0.01 N HCl). The sample was shaken thoroughly for 15 min at room temperature and centrifuged at 14,000 rpm for 4 min. An aliquot (100 μL) of the supernatant was extracted following the protocol of the EZ:faast user's manual. The sample was finally dissolved in 70 μL of dichloromethane and subjected to GC-MS analysis. Three microliters of the sample mixture was separated on a silica capillary column (ZB-AAA 10 m × 0.25 mm; Zebron, Phenomenex). The injector temperature was set to 250°C, and a constant flow of helium (1.2 mL/min) and the following temperature program were used: 70°C/3 min with 8°C/min to 240°C, with 20°C/min to 320°C/2 min. For quantitative determination of individual amino acids, peaks originating from selected ion chromatograms were integrated: Gly (mass-to-charge ratio [*m/z*] 116), Ala (*m/z* 130), Val (*m/z* 158), β-Ala (*m/z* 116), Leu (*m/z* 172), Ile (*m/z* 172), GABA (*m/z* 130), Ser (*m/z* 146), Thr (*m/z* 101), Pro (*m/z* 156), Pip (*m/z* 170), Aad (*m/z* 244), Asp (*m/z* 216), Glu (*m/z* 84), Asn (*m/z* 69), Gln (*m/z* 84), Cys (*m/z* 248), Orn (*m/z* 156), Lys (*m/z* 170), His (*m/z* 282), Phe (*m/z* 148), Tyr (*m/z* 107), and Trp (*m/z* 130). The area of a substance peak was related to the peak area of norvaline (*m/z* 158), which served as an internal standard. Experimentally determined correction factors for each amino acid were considered. Arg and Met could not be analyzed with the applied method.

Quantitative Real-Time PCR Analysis

Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (PeqLab) following the manufacturer's instructions. One micrograms of RNA was treated with DNase I (Fermentas) for 30 min at 37°C to remove genomic DNA, and the DNase was inactivated by incubation at 70°C for 10 min in the presence of 2.5 mM EDTA. mRNA was converted to cDNA with the oligo(dT) primers and reverse transcriptase (Omniscript RT kit, Qiagen) according to the manufacturer's instructions. cDNA (2.5 μL) was amplified in 10 μL of reaction volume with 5 μL of SenziMix SYBR Green (Bioline) and 0.75 μM gene-specific primers (see Supplemental Table 1 online). The gene encoding a polypyrimidine tract binding (*PTB*) protein 1 (At3g01150) which is nonresponsive to *P. syringae* inoculation was used as a reference gene (Czechowski et al., 2005). The quantitative PCR reaction was performed in triplicate in a Rotor-Gene Q apparatus (Qiagen) using the cycling program: 95°C for 7 min, followed by 45 cycles at 95°C for 10 s, 60°C for 30 s, and finally 72°C for 3 min. The data were analyzed using the Rotor-Gene Q 2.0.2 software, setting the threshold of the normalized fluorescence to 0.1, which corresponded to the exponential phase of the fluorescence signal. The resulting cycle threshold (C_T) and E values were used to calculate the relative mRNA abundance according to the $\Delta\Delta C_T$ method. The values were normalized to those of the reference gene and expressed relative to the $MgCl_2$-treated wild-type control sample.

Petiole Exudate Collection and Analyses

Petiole exudates were collected essentially as described (Maldonado et al., 2002; Chaturvedi et al., 2012). Plant leaves were either infiltrated with a suspension of *Psm* (OD 0.005) or 10 mM $MgCl_2$ as a control treatment. Six hours after infiltration, leaves were cut at the base of their petioles and the cut surface sterilized by successive dipping in 50% ethanol and 0.0005% bleach for 10 s. After rinsing with sterile 1 mM EDTA, pH 8.0, the petioles were submerged in fresh EDTA solution for exudate collection. Twelve-well tissue culture plates were used for this purpose, whereas each well was filled with 2.5 mL of collection solution and equipped with 10 harvested leaves. Exudates were continuously collected in the period from 6 to 48 h after inoculation. For the determination of amino acid (defense metabolite) contents, 100 μL (1 mL) of collected exudates was analyzed as described above.

Reproducibility of Experiments and Statistical Analyses

The presented data generally resulted from a single biological experiment. Unless otherwise stated, the results were similar in three biologically independent experiments. Statistical analyses were performed using Student's *t* test.

Accession Numbers

Sequence data from this article for the major genes discussed in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ALD1* (At2g13810), *LKR* (At4g33150), *FMO1* (At1g19250), *ICS1* (At1g74710), *PR-1* (At2g14610), *PAD4* (At3g52430), *NPR1* (At1g64280), and *PTB* (At3g01150).

Supplemental Data

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

Supplemental Figure 1. Mass Spectral Identification of the Initially Unknown Substance Detected in Extracts of *P. syringae*–Inoculated Plants as Pipecolic Acid.

Supplemental Figure 2. The Plant-Derived Substance Identified as Pip and Authentic Pip Have Identical GC Retention Times.

Supplemental Figure 3. Proposed Scheme for Pathogen-Inducible Pip and Aad Biosynthesis in Plants via Lys Catabolism.

Supplemental Figure 4. *Psm avrRpm1*–Induced Pip Accumulation in Inoculated and Distal Leaves.

Supplemental Figure 5. Metabolite Levels in Petiole Exudates of Leaves Collected between 6 and 48 h Post *Psm* or $MgCl_2$ Treatment.

Supplemental Figure 6. Pathogen-Induced *ALD1* and *LKR* Expression in Wild-Type Col-0 and Different Mutant Plants.

Supplemental Figure 7. Metabolite Levels in Leaves Following Pip, Aad, and β-Amino Butyric Acid Application via the Root in Col-0 and *ald1* Plants.

Supplemental Figure 8. Concentration Dependency of Resistance Induction by Exogenous Pipecolic Acid, Resistance-Enhancing Activity of L- but Not D-Pip, and Chemical Complementation of *ald1* Defects in *Psm avrRpm1*–Induced SAR by Exogenous Pip.

Supplemental Figure 9. Effect of Exogenous SA on Leaf Pip Levels, *ALD1* Transcript Levels, and *PR-1* Transcript Levels.

Supplemental Table 1. Primers Used in This Study.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation (Grant 3100A-125374 to J.Z.) and by a grant from the German Research Foundation (Deutsche Forschungsgemeinschaft International Research Training Group 1525). We thank Volker Lipka for critically reading the article.

AUTHOR CONTRIBUTIONS

H.N., F.B., A.-C.D., and J.Z. performed the experiments. H.N. and J.Z. contributed to article preparation. J.Z. designed the research and wrote the article.

Received July 31, 2012; revised November 8, 2012; accepted November 15, 2012; published December 7, 2012.

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