ALD1 Regulates Basal Immune Components and Early Inducible Defense Responses in Arabidopsis

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Robust immunity requires basal defense machinery to mediate timely responses and feedback cycles to amplify defenses against potentially spreading infections. AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) is needed for the accumulation of the plant defense signal salicylic acid (SA) during the first hours after infection with the pathogen Pseudomonas syringae and is also upregulated by infection and SA. ALD1 is an aminotransferase with multiple substrates and products in vitro. Pipoenic acid (Pip) is an ALD1-dependent bioactive product induced by P. syringae. Here, we addressed roles of ALD1 in mediating defense amplification as well as the levels and responses of basal defense machinery. ALD1 needs immune components PAD4 and ICS1 (an SA synthesis enzyme) to confer disease resistance, possibly through a transcriptional amplification loop between them. Furthermore, ALD1 affects basal defense by controlling microbial-associated molecular pattern (MAMP) receptor levels and responsiveness. Vascular exudates from uninfected ALD1-overexpressing plants confer local immunity to the wild type and ald1 mutants yet are not enriched for Pip. We infer, in addition to affecting Pip accumulation, ALD1 produces non-Pip metabolites that play roles in immunity. Thus, distinct metabolite signals controlled by the same enzyme affect basal and early defenses versus later defense responses, respectively.

Plant innate immunity has several mechanisms that enable the recognition of microorganisms through different types of immune receptors. Pattern recognition receptor (PRR) complexes sense conserved microbial or self-derived molecules called pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs, respectively) (Boller and Felix 2009; Conrath et al. 2006; Kohler et al. 2002; Sato et al. 2010; Vlot et al. 2009; Yi et al. 2014), while cytoplasmic or plasma membrane plant resistance (R) proteins recognize, directly or indirectly, specific “avirulence” effectors injected into plants from microbes (Dangl and Jones 2001). One of the best-characterized recognition events in plant immunity is the perception of the bacteria flagellum-derived MAMP peptide flg22 by the PRR FLAGELLIN-SENSITIVE 2 (FLS2) and its coreceptor, BR11-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Chinchilla et al. 2007; Gómez-Gómez and Boller 2000; Heese et al. 2007). flg22-FLS2 binding triggers very rapid responses that include calcium influx, NADPH oxidase dependent oxidative burst (ROS), and mitogen-activated protein kinase (MAPK) or calcium-dependent protein kinase activation during the first approximately 10 min (Boller and Felix 2009). Later responses include callose deposition to strengthen the cell wall against pathogen attack (Boller and Felix 2009; Gómez-Gómez et al. 1999) and transcriptional reprogramming to activate defense-related genes (Zippel et al. 2004). These responses are collectively called PAMP-triggered immunity (PTI).

A central player necessary for defense induction against many pathogens is the plant hormone salicylic acid (SA) (Robert-Seilaniantz et al. 2007). SA increases after microorganisms’ recognition (Mishina and Zeier 2007; Nawrath et al. 2002; Song et al. 2004a; Tsuda et al. 2008) and is needed for the orchestration of downstream resistance responses (Delaney et al. 1994; Nawrath and Métrax 1999). Moreover, SA is also a key factor for systemic defense programs such as the systemic acquired resistance (SAR) (Cao et al. 1994; Mauch-Mani and Métrax 1998; Nawrath and Métrax 1999). SA can potentiate important basal defense responses, such as flg22-triggered oxidative burst and callose deposition or MAPK cascades, induce a large number of defense genes, such as PATHOGENESIS RELATED GENE 1 (PR1), and defense metabolite production (Beckers et al. 2009; Conrath et al. 2006; Kohler et al. 2002; Sato et al. 2010; Vlot et al. 2009; Yi et al. 2014). SA is synthesized through the action of ISOCORRISAMTE SYNTHASE 1 enzyme (ICS1/SSID2) in chloroplasts, the source organelle of many phytohormones and defense-related metabolites (Fragnière et al. 2011; Padmanabhan and Dinesh-Kumar 2010; Strawn et al. 2007; Wildermuth et al. 2001).

Correlative data indicate that the timely accumulation of SA determines how susceptible to infections plants are. An early defect in SA accumulation (during the first 24 h after infection) defines the output of the plant–pathogen interaction (Feyts et al. 2001; Glazebrook et al. 1996, 1997, 2003; Gupta et al. 2000; Shapiro and Zhang 2001; Zhou et al. 1998). Several Arabidopsis defense component mutants that show early defects in kinetics of SA production also have increased susceptibility to different pathogens (Glazebrook et al. 1997; Lee et al. 2007; Lu et al. 2003; Nawrath and Metraux 1999; Rietz et al. 2011; Song et al. 2004b; Tsuda et al. 2008). These components form part of the so-called type II regulators of SA, affecting accumulation of SA and SA-dependent compounds, but are not directly implicated.
in SA synthesis (Lu et al. 2009). Examples of those regulators include AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1), and PHYTOALEXIN DEFICIENT 4 (PAD4) (Jirage et al. 1999; Lu et al. 2003; Song et al. 2004a). Although these regulators are very important for defense induction, how these proteins act to affect the timely production of SA is not known. It is conceivable that they affect early steps after pathogen recognition, before accumulation of SA. They may produce intermediates that maintain basal levels of defense components necessary to respond effectively to pathogens, signals, or signaling intermediates that quickly stimulate SA production.

ALD1 is one particularly interesting example of an SA regulator that is very important for resistance to Pseudomonas syringae strains (Song et al. 2004a). ALD1 transcripts are induced within 6 to 12 h during P. syringae infection or by an SA agonist treatment (Song et al. 2004b). ALD1 transcript upregulation depends on another SA regulator, PAD4 (Song et al. 2004a,b). In systemic tissues, during SAR establishment, ALD1 transcript induction also depends on the FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1), a component necessary for SAR (Návarová et al. 2012). It was suggested that ALD1 participates together with PAD4 and FMO1 in defense signal potentiation to regulate SA-dependent and -independent defenses (Návarová et al. 2012; Song et al. 2004b). In agreement with this idea, ald1 mutant plants show diminished SA accumulation, PRI induction, and camalexin production (Song et al. 2004a,b). Interestingly, although these defects in ald1 mainly exist at early times and decrease or disappear approximately 24 h after infection, the mutant plants are more susceptible to P. syringae infections.

ALD1 encodes an aminotransferase with multiple substrates and products in vitro (Song et al. 2004a). One proposed ALD1-dependent product is piperolic acid (Pip), a nonprotein amino acid (Návarová et al. 2012). Pip increases in petiole exudates after infection and its accumulation is dependent on ALD1. When applied to the plants, Pip has only a small effect (≤40% increase) on basal SA in the absence of pathogen infection. However, Pip treatment significantly primes pathogen-induced SA accumulation, similar to the effect of azelaic acid (AZA), another plant-produced priming agent (Jung et al. 2009). Because of ALD1’s multiple substrates (Song et al. 2004a) and the dynamic regulation of the ALD1 gene, it is probable that ALD1 produces different biologically active products that may vary depending on the in vivo conditions. ALD1-dependent metabolites in noninfection conditions (basal state products or metabolites) could differ from those during induction of defenses after infection (defense induced products or metabolites). The identity of those metabolites and their targets are of main importance to understand the key ALD1-dependent early regulation of defenses induction.

Here, we further investigated the effect of ALD1 on early defense responses, other defense regulatory factors, and SA accumulation. Based on studying plants that overexpress ALD1 and ald1 mutants, we show several roles for ALD1 in regulating (i) basal defense component levels, (ii) a rapid response (in minutes) to flg22, and (iii) slower responses (hours) to pathogen infection, including the transcription of other defense regulators and SA accumulation. A number of effects of ALD1 appear to be independent of Pip accumulation, suggesting the possibility that ALD1 makes or regulates other metabolites with defense-regulatory effects.

RESULTS

ALD1 overexpression is sufficient to confer disease resistance.

Plants lacking ALD1 are more susceptible to P. syringae (Song et al. 2004a,b). To test whether ALD1 overexpression is sufficient to confer disease resistance, we produced Arabidopsis with constitutively high expression of ALD1 under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. Independent transgenic plant lines (ALD1ox, number 3 and number 8) strongly expressed ALD1 mRNA compared with wild-type (WT) Col-0 plants in pathogen-free conditions (Fig. 1B). To assess the infection phenotype of these lines, we infiltrated leaves with a virulent bacteria and analyzed pathogen growth 3 days after inoculation. ALD1ox plants were overtly indistinguishable from control (not shown) but were more resistant than the WT to virulent P. syringae pv. maculicola (PmaDG3) (Fig. 1A). The results support the view that ALD1 is important in determining the level of resistance to P. syringae.

ALD1 modulates MAMP-triggered immunity.

Previously, signaling responses regulated by ALD1 have been characterized as occurring in the order of hours (usually six or more) to days after infection (Song et al. 2004a,b). Moreover, ALD1 transcript levels are upregulated within 6 to 12 h during virulent P. syringae infection. However, an open question is whether a basal state or infection-induced ALD1-dependent products are involved in regulating other defenses. We sought to test a response on a short time scale to determine whether preexisting ALD1 products might have defense-regulatory roles. To do this, we used plants with different ALD1 levels (ald1-T2, WT, and ALD1ox). We monitored a very rapid defense response to the PAMP flg22, the reactive oxygen species (ROS) burst, which happens within minutes of flg22 application (Chinchilla et al. 2007) and can be sensitively measured with a luminol-based assay (Schwessinger et al. 2011). Relative to the WT, ald1-T2 showed reduced ROS whereas ALD1ox plants showed increased ROS after 100 nM flg22 treatment (Fig. 2A and B). Using aniline blue staining of treated leaves, we also observed that callose deposition was similarly affected (Fig. 2C). These data show that, during basal state conditions, ALD1-dependent products affects the responsiveness to flg22 regulating ROS production.

We also monitored levels of FLS2 and its coreceptor BAK1 in plants with different levels of ALD1 by Western blot analysis of total proteins. Interestingly, ald1-T2 had reduced levels of both proteins, consistent with ALD1-dependent products regulating receptor levels (Fig. 2D). In contrast, ALD1ox did not have higher receptor levels than the WT. Possibly, a critical
threshold level of ALD1-dependent products is needed to achieve normal receptor levels. Together, the results suggest that ALD1-dependent products regulate receptor levels and function during noninfection conditions.

**Responsiveness to flg22 is affected by more than one ALD1-dependent product.**

Upon infection, vascular exudates of WT but not ald1-T2 plants accumulate Pip (Návarová et al. 2012). We confirmed these findings (Supplementary Fig. S1). However, in uninfected plants, there was no statistically significant difference in Pip levels in WT, ald1-T2, or ALD1ox plants (Fig. 3A). One day after watering plants with 1 mM Pip, the levels of FLS2 and BAK1 in WT or ald1-T2 plants were not affected (Fig. 3B and C). This suggests that an ALD1 basal metabolite regulates receptor levels in a Pip-independent manner.

Interestingly, Pip treatment rescued the defect in flg22-induced ROS and callose accumulation in ald1-T2 (Fig. 3D and E). However, Pip did not confer increased flg22 responses to WT plants. Possibly, a metabolite made by ALD1 competes with exogenous Pip for affecting receptor-dependent events. Because ALD1ox shows enhanced flg22 responses and ALD1ox did not constitutively accumulate increased Pip levels, we infer that a non-Pip, ALD1-dependent metabolite also can modulate receptor signaling.

**Overexpression of ALD1 primes defenses to more rapidly induce defenses upon P. syringae infection.**

We have shown that ALD1ox affected the output of flg22 signaling and ALD1ox plants were more resistant to P. syringae. Therefore, we tested whether ALD1ox plants also had altered signaling responses to P. syringae. High-performance liquid chromatography and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of leaf tissue samples were used to analyze the kinetics of SA accumulation and the abundance of two defense gene transcripts, PR1 and ICS1/SID2 (SA biosynthetic enzyme), respectively. Overexpression of ALD1 did not alter the basal SA level in Arabidopsis leaves before infection (Fig. 4A, 0 h after inoculation). After infection with virulent PmuDG3, SA accumulated faster and to higher levels in leaves of ALD1ox plants than in WT plants (Fig. 4A). Transcript levels of PR1 and ICS1/SID2 were induced to higher levels earlier after PmuDG3 infection in leaves of ALD1ox plants than in the WT (Fig. 4B). Small increases of PR1 and ICS1 transcript levels (1.4- and 3.4-fold, respectively, versus 228- and 38-fold 18 h after infection) in ALD1ox were observed in the basal, uninoculated state. The effect of ALD1ox on defense activation was observed at 9 and 15 h postinfection but not at later times. Thus, the early robust activation of defense responses against pathogens was correlated with increased disease resistance.

The faster increase relative to the WT of SA accumulation and PR1 and ICS1 transcript levels after infection in ALD1ox plants was reminiscent of the effects of treating plants with Aza, a systemic priming signal that confers increased resistance to P. syringae (Chaturvedi et al. 2012; Jung et al. 2009; Yu et al. 2013). However, unlike its effect on WT plants, Aza treatment did not make ALD1ox plants even more resistant to P. syringae (Fig. 4C). The nonadditive effect of ALD1ox and Aza is probably due to a common priming pathway or component being targeted by an ALD1-dependent metabolite and Aza.

**Pathogen resistance conferred by ALD1 requires PAD4 and ICS1/SID2, genes that are regulated by ALD1 during infection.**

ALD1 transcript levels are positively regulated by SA agonist treatment and by defense components that affect SA accumulation during infection (Návarová et al. 2012; Song et al. 2013). ALD1ox transcript levels are increased 15 h postinfection in ALD1ox plants compared to WT plants (Fig. 4A, 0 h after inoculation) . After infection with virulent PmuDG3, SA accumulated faster and to higher levels in leaves of ALD1ox plants than in the WT (Fig. 4A). Transcript levels of PR1 and ICS1/SID2 transcripts were induced to higher levels earlier after PmuDG3 infection in leaves of ALD1ox plants than in the WT (Fig. 4B). Small increases of PR1 and ICS1 transcript levels (1.4- and 3.4-fold, respectively, versus 228- and 38-fold 18 h after infection) in ALD1ox were observed in the basal, uninoculated state. The effect of ALD1ox on defense activation was observed at 9 and 15 h postinfection but not at later times. Thus, the early robust activation of defense responses against pathogens was correlated with increased disease resistance.

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transgene by crossing ALD1ox with sid2, pad4, and fmo1 mutants, and infected these plants with the virulent strain *PmaDG3* to analyze bacteria growth 3 days after inoculation. Overexpression of *ALD1* failed to confer disease resistance against *PmaDG3* infection in pad4 and sid2 plants, whereas it was still effective in fmo1 plants (Fig. 5A). We validated that overexpression of *ALD1* occurred in the WT, pad4, sid2, and fmo1 plants (Supplementary Fig. S2). Therefore, the increased resistance conferred by ALD1ox was dependent on PAD4 and ICS1/SID2 but not FMO1.

We also tested whether ALD1 normally regulates PAD4, ICS1/SID2, or FMO1 during infection. To address this, we compared the accumulation of these transcripts by quantitative RT-PCR in WT, *ald1-T2*, and ALD1ox plants at different times postinfection with the avirulent strain *PmaDG6*. Indeed, *ald1-T2* showed a transient reduction (at 5 h) in PAD4 and ICS1/SID2 transcript levels early after infection (Fig. 5B and C). This defect did not extend to *FMO1* (Fig. 5D), indicating that there was some specificity to the effect of the loss of ALD1. In agreement, PAD4 and ICS1/SID2 induction were enhanced in ALD1ox compared with WT plants (Fig. 5E and F). However, *FMO1* also showed a faster induction in ALD1ox, suggesting that ALD1 may also have a local stimulatory role on this gene (Fig. 5G). Together these results indicate that ALD1 is limiting for the proper regulation of PAD4 and SID2 during infection with *P. syringae*.

**Exudates of plants overexpressing ALD1 confer disease resistance.**

The defense-related phenotypes of ALD1ox plants could be due to preexisting or accumulated ALD1-dependent basal compounds capable of conferring disease resistance. As discussed above (and see Figure 3A), although exudates from uninfected independent transgenic lines of ALD1ox plants did not have increased Pip (or higher SA) (Supplementary Fig. S3), they nevertheless conferred increased disease resistance when applied by leaf infiltration to WT plants (Fig. 6A). Local application of ALD1ox exudates did not confer increased resistance to distal leaves (Fig. 6B), suggesting the active exudate components are not highly mobile in intact plants. Alternatively, some active mobile components could be degraded before or during movement. Importantly, the ALD1ox exudate could suppress the pathogen-susceptible phenotype of the *ald1-T2* mutant (Fig. 6C). It also suppressed pathogen susceptibility in *fmo1* but not in *pad4* or *sid2*. This suggests that PAD4 and ICS1/SID2 are needed for responding to the ALD1ox exudate. Together with the need for PAD4 and ICS1/SID2 to establish ALD1ox-mediated increased resistance (Fig. 5A), these results suggest that the ALD1 products generated during basal noninfection conditions are transduced into resistance by these two key factors.

**ALD1 localizes to chloroplast.**

According to the TargetP (Emanuelsson et al. 2007) subcellular prediction algorithm, *ALD1*, like its homolog AGD2 (Song et al. 2004a), has a predicted transit peptide for targeting to chloroplast. To gain insight further into how ALD1 might contribute to defense, we studied its localization in *Arabidopsis*. We transformed *ald1-T2 Arabidopsis* plants with a construct harboring *ALD1* fused to green fluorescent protein (GFP) controlled by a dexamethasone (dex)-inducible promoter. The fusion protein was functional and complemented *ald1-T2* mutation. This was shown by transgenic plants pretreated for 21 h with dex supporting reduced growth of two strains of *P. syringae* (Fig. 7A), to which the *ald1-T2* mutant has higher susceptibility (Song et al. 2004b). ALD1-GFP predominantly colocalized with the autofluorescence of epidermal cell chloroplasts in confocal micrographs (Fig. 7B) and cofractionated with the chloroplast marker ATPaseβ in chloroplast-enriched

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**Fig. 3.** Pipolic acid (Pip) content of ALD1- overexpressor plants and pathogen-associated molecular pattern-triggered immune responses in wild-type (wt) Col-0 and *ald1-T2* mutant plants 24 h after watering with water (−Pip) or 1 mM Pip (+Pip). A, Pip accumulation in petiole exudates of untreated wt Col-0, *ald1* mutant, and ALD1ox plants in the absence of pathogen infection. Results are average, with standard error from at least two biological replicates (*n* = 3 for each replicate); wt = three replicates, *ald1* = two replicates, and ALD1ox = four replicates. B, FLS2 and BAK1 levels in total extracts in wt Col-0 and *ald1* mutant plants. Immunoblots were analyzed using FLS2 and BAK1 antibodies. Blots stained with Coomassie blue (CB) are presented to show loading. Similar results were observed in four independent experiments. * indicates the correct BAK1 band. C, Levels of FLS2 and BAK1 relative to the total proteins (minus Rubisco) content in each CB membrane lane as quantified by densitometry. Averages with standard error from four biological replicates are shown. D, Average of maximum reactive oxygen species (ROS) production of wt Col-0 and *ald1* mutant plants leaf discs treated with 100 nM flg22 measured in relative light units (RLUs). Bars show averages, with standard error from three independent experiments, each of which used 12 replicates. E, Callose deposition quantified in indicated plants treated with mock or 1 μM flg22. Number of callose deposits is shown as the average, with standard error from three biological replicates. In A, C, D, and E, statistically significant differences (*P* < 0.01, analysis of variance, Tukey test) are shown using different lowercase letters.
extracts (Fig. 7C). The presence of the cytoplasmic marker protein cytHsc70 only in total fraction indicated the purity of our fractionation. Together, these results show that ALD1 amino transferase localizes to chloroplasts, where it likely generates amino or o xo acid-derived defense signals.

**ALD1-like gene of Nicotiana benthamiana is important for local and systemic resistance.**

To determine whether ALD1 is important for disease resistance in plants other than Arabidopsis, we studied its regulation and role in Nicotiana benthamiana, which has a homolog (NbALD1) with 81% similarity and 67% identity to Arabidopsis ALD1. N. benthamiana leaves infected with a virulent and avirulent derivatives of P. syringae pv. tabaci PTBR2004 robustly induced transcripts of NbALD1 (Fig. 8A). We employed virus-induced gene silencing (VIGS) to reduce NbALD1 expression, and infected mock-treated and NbALD1-silenced plants with P. syringae pv. syringae B728a, a strain that grows both endophytically and epiphytically when spray inoculated on N. benthamiana (Lee et al. 2012; Vinatzer et al. 2006). VIGS worked well to significantly reduce NbALD1 levels in infected local and systemic leaves of N. benthamiana (Fig. 8C and E). P. syringae pv. syringae B728a grew better both on leaf surfaces (epiphytic) and in extracellular spaces (endophytic) of NbALD1-silenced plants than in nonsilenced control plants (Fig. 8B). Infection of lower leaves with P. syringae pv. tabaci or avrRpt2 successfully activated SAR against B728a infection in distal leaves of nonsilenced plants (Fig. 8D). However, silencing of NbALD1 abolished SAR.

These results show that NbALD1’s role during both local and systemic defenses is similar and comparable with Arabidopsis ALD1 (Song et al. 2004b) and indicate a key conserved function of these aminotransferases for pathogen defenses.

**DISCUSSION.**

Metabolites are increasingly appreciated for their roles as modulators of diverse signaling processes in plants. ALD1 is an enzyme that can produce multiple metabolite products in vitro (Song et al. 2004a). The enzyme’s broad substrate specificity combined with the gene’s dynamic regulation raised the possibility that ALD1-dependent, biologically active products and their targets may differ depending on the in vivo conditions. Previously, increased Pip accumulation has been shown to be ALD1-dependent during infection, and exogenous Pip treatment boosted disease resistance. Here, we present data that argue for additional basal state products, generated by ALD1 during noninfection conditions, that are also biologically active in defense regulation. First, the level of flg22-induced ROS accumulation increased in proportion to the level of ALD1 in plants, although plants with different amounts of ALD1 did not differ in their basal Pip levels. The ROS response was much faster than the time needed for Pip to accumulate (Navarova et al. 2012), implying that a basal, non-Pip metabolite affected the ROS response. Second, ALD1ox plants conferred disease resistance without producing additional Pip and exudates of ALD1ox were sufficient to confer disease resistance to WT plants. Together, our results indicate that ALD1 makes more than one biologically active product and, furthermore, one or more of them acts in a very early defense response.

During infections, plants reprogram their metabolism and increase the production of many compounds and antimicrobial molecules as well as defense signals. In agreement with the idea that in vivo ALD1 could produce more than one defense metabolite, our ALD1ox petiole exudate mass spectrometry (MS) analyses showed novel metabolites whose levels are increased relative to the WT but whose identities are unknown due to their

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**Fig. 4.** Salicylic acid (SA)-dependent inducible defense of ALD1ox Arabidopsis plants. A, Increased free or total SA levels in leaves of ALD1ox (line 8) plants during mock inoculation (1 mM MgSO4) or infection with Pseudomonas syringae pv. maculicola ES4326 carrying empty vector (PmaDG3) (OD600 = 0.01); wt = wild type. Error bars indicate standard deviation from three biological replications (n = 3). B, PR1 and ICS1/SID2 mRNA levels in leaves of ALD1ox (line 8) during PmaDG3 infection. Leaves of the wild type (white) or ALD1ox (black) were taken at the indicated times after mock treatment or PmaDG3 infection. Error bars indicate standard deviation from three technical replicates (n = 3). Asterisks indicate statistically differences in expression level between wild type and ALD1ox plants at given time points (** and *** indicate P < 0.01 and 0.001, respectively; Student’s t test). Experiments shown in A and B were repeated four times with similar results. C, Pretreatment of azelaic acid (AZA), a defense-priming inducing signal, could not render ALD1ox plants hyper-resistant against PmaDG3 infection. At 2 days prior to PmaDG3 inoculation (OD600 = 0.0001), 5 mM morpholineethanesulfonic acid (MES) (white) and 1 mM AZA in 5 mM MES (black) were sprayed on the plants. Different letters indicates statistically significant differences (P < 0.01, ANOVA, Tukey test). Error bars indicate standard error (n = 6 or 8). The experiment was repeated twice with similar results.
absence from mass spectral libraries. Their purification will be necessary for their structures to be determined. It is possible that an ALD1-unique product could be used downstream to generate different defense metabolites depending on conditions (e.g., whether or not a pathogen is present). ALD1 may produce metabolites with different targets such as PTI or later infection responses. Defense-related metabolic pathways producing different products are known. An example is the shikimate pathway, where the shikimic acid product is used to generate an array of different defense-related metabolites and signals, including SA (Verberne et al. 2007; Vogt 2010).

If it is not Pip, what is the ALD1 metabolite acting during basal noninfection conditions? As an aminotransferase, we expect ALD1 to generate amino-acid-derived compounds. Amino acids, aminotransferases, and amino-acid-derived molecules are involved in the synthesis of secondary metabolites known to be important as antimicrobials (Namwat et al. 2002; Sønderby et al. 2010) and defense signals (Adio et al. 2011; Cecchini et al. 2011; Leric et al. 2012; Liu et al. 2010; Návarová et al. 2012; Stuttmann et al. 2011; Taler et al. 2004; van Damme et al. 2009). Moreover, amino acid conjugates, particularly to hormones, play essential roles in physiologic processes, including plant defenses (Ljung et al. 2002; Okrent and Wildermuth 2011; Piotrowska and Bajguz 2011; von Saint Paul et al. 2011). Additionally, ALD1’s chloroplastic localization could also have great impact in defense metabolic pathways, considering that this organelle is a factory of amino acids, secondary metabolites, and phytohormones such as SA (Bryan 1990; Wildermuth et al. 2001).

We show here that ALD1 is necessary for normal production of the FLS2 receptor and its coreceptor BAK1 but may work only up to some threshold level, such that producing even more ALD1 does not confer higher receptor levels but induces increased PTI responses. Although increasing FLS2 levels was shown to be a way to increase flg22 responsiveness (Gómez-Gómez and Boller 2000), our results show that other modes of altering responsiveness exist. It is possible that more than one ALD1 product is affecting different components of the FLS2 receptor complex. A basal state metabolite could be acting to maintain receptor levels and another metabolite may regulate responsiveness. Alternatively, the same metabolite could be regulating different components of the FLS2 complex. Interestingly, it has recently been shown that the responses to the endogenous DAMP Pep2 are reduced in ald1-T2 mutant (Ross et al. 2014). Considering this, an attractive possibility is that ALD1 products may be affecting shared PRR complexes’ components such as SERKs, BIK1s, or NADPH oxidases (Kadota et al. 2014; Li et al. 2014; Macho and Zipfel 2014; Roux et al. 2011). If this is true, ALD1 may regulate more than one PRR complex and their responses.

Pip treatment can increase resistance against virulent and avirulent bacterial infections in ald1-T2 and WT plants (Návarová et al. 2012). However, it is striking that Pip does not similarly affect flg22-triggered responses, and only compensates for them when ALD1 is missing. This suggests that Pip’s effect on PTI is due to a different mechanism than the one acting during defenses induced after infections. In any case, why does Pip only compensate PTI fast responses in the ald1-T2 mutant without affecting them in WT plants? One idea to explain this is that an inhibition or competition exists between ALD1 basal state metabolites and Pip. An alternative explanation could be that an excess of exogenously added Pip could be metabolically redirected to new active metabolites only when ALD1 is not present. These possibilities show the complexity of resistance program networks. Defense-related enzymes with many substrates or products, such as ALD1, could provide the necessary fine control to tune defense programs.

**Fig. 5.** Relationship between ALD1 and other salicylic-acid-related defense regulators. A, ALD1 required PAD4 and ICS1/SID2 but not FMO1 to confer disease resistance. The number of bacteria in wild-type Col-0 or indicated single mutants (white) or the indicated single mutants crossed with ALD1ox (line 8) plants (black) were measured on day 3 after infection with a virulent strain *Pseudomonas syringae* pv. *maculicola* ES4326 carrying empty vector (*PmaDG3*) (OD600 = 0.0001). Error bars indicate standard error (*n* = 6 or 8). The experiment was repeated at least two times for each background with similar results. Asterisks indicate statistically significant differences between ALD1ox crossed or noncrossed plant (** *P* < 0.01, *t* test). Bacterial growth for each background should be compared with those of the same genotype, because different genotypes were grown separately (dotted divisor lines). B to G, Defense gene expression dependent on ALD1 during infection. Relative expression of B to E, PAD4, C to F, SID2; and D and G, FMO1 in the wild type; B to D, the ald1-T2 mutant and E and G, wild-type and ALD1ox plants. Infected local leaves were taken at the indicated times after infection of an avirulent derivative of *P. syringae* pv. *maculicola* ES4326 carrying *avrRpt2* (*PmaDG6*) (OD600 = 0.01). Error bars in B to G indicate standard deviation from three technical replicates. Asterisks indicate statistical differences in expression level between wild-type Col-0 and ald1-T2 mutant at given time points (*P* < 0.01, Student’s *t* test). All experiments were repeated at least twice with similar expression pattern. H, Noninoculated healthy plants.
Upon infection, ALD1ox showed an early trigger of free SA accumulation that was correlated with SA signaling, as indicated by PR1 induction. Conversely, ald1-T2 mutant plants showed slower induction of SA and PR1 (Song et al. 2004b). The higher resistance to infection in ALD1ox supports the idea that early defense responses drive the output of plant pathogen interactions (Fey et al. 2001; Glazebrook et al. 1996, 1997, 2003; Gupta et al. 2000; Shapiro and Zhang 2001; Zhou et al. 1998). This ALD1-dependent primed state may reflect the action of ALD1-produced compounds that sensitize defense components to produce stronger responses upon stimulus. Interestingly, two well-characterized priming signals, AZA and β-aminobutyric acid (Jung et al. 2009; Zimmerli et al. 2000), are not effective on ald1 mutant plants (Jung et al. 2009; Návarová et al. 2012). Additionally, the effects of ALD1ox and AZA are nonadditive, suggesting a common priming pathway. In agreement, it was shown that AZA primes SA accumulation and PR1 induction (Jung et al. 2009; Yu et al. 2013). Furthermore, it was recently reported that AZA production depends on ROS increases (Wang et al. 2014). Because ALD1 has a positive effect on ROS generation during PTI, it is also possible that ALD1 indirectly affects AZA accumulation or priming during this defense program. Together, these observations support an important role for ALD1 in the priming establishment mechanisms.

It is probable that one of the ALD1 metabolites capable of priming defenses is Pip. However, because ALD1 can also produce active exudates that do not have elevated Pip levels, another product could transduce signals into primed defenses. In agreement, treatments with ALD1ox petiole exudates can induce resistance. In this case, and contrary to what is proposed for Pip, the effect of ALD1-exudate metabolites is only local; it cannot induce resistance in systemic leaves. An interesting idea is that the ALD1ox effect is partially mimicking a distal tissue during the SAR-primed state (i.e., some non-Pip metabolite might accumulate).

PAD4 and ICS1/SID2 but not FMO1 are needed to establish increased resistance in ALD1ox. Importantly, the same defense components are needed in recipient plants to respond to

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**Fig. 6.** Biological activity of petiole exudates from untreated ALD1ox plants. A, Petiole exudates from ALD1 overexpressor line 3 and 8 (ALD1ox) plants inhibited the growth of a virulent Pseudomonas syringae pv. maculicola ES4326 carrying empty vector (PmaDG3) (OD600 = 0.0001) in the leaves of wild-type (wt) plants in which they were applied prior to pathogen infection. The inoculation studies were repeated six times, with similar results in five of the six trials. B, Exudates from ALD1ox could not induce systemic resistance against PmaDG3 in the distal leaves of recipient wt plants locally pretreated with ALD1ox-exudates (line 8). Similar results were found in three biological replicates. Error bars indicate standard error (A and B). Different letters indicate statistical differences among the different samples (P < 0.01, analysis of variance, Tukey test). C, ALD1ox exudates rescued hypersusceptibility phenotype of ald1 and fmo1 but not sid2 or pad4 mutants. Petiole exudates from wt Col-0 leaves (white) and from ALD1ox (black) were pretreated into leaves of indicated mutant plants 2 days prior to pathogen infection. The number of bacteria was measured on day 3 after infection with a virulent strain of PmaDG3 (OD600 = 0.0001). This experiment was repeated three times with similar results. Error bars indicate standard error. Different numbers of asterisks indicate statistically significant differences between wt or ALD1ox-exudates treatments (* and ** indicate P < 0.05 and 0.01, respectively; t test). Bacterial growth for each background should be compared with those of the same genotype, because different genotypes were grown separately (dotted divisor lines).

**Fig. 7.** AtALD1: green fluorescent protein (GFP) fusion protein is targeted to chloroplast of Arabidopsis leaves. A, Complementation of the ald1-T2 mutant (ald1) disease susceptibility with AtALD1:GFP protein controlled by dexamethasone (dex)-inducible promoter (ALD1:GFP [ald1]) used to study ALD1 localization. This experiment was repeated three times with similar results. Error bars indicate standard error (n = 8). Letters show statistically significant differences between the indicated plants infected with virulent Pseudomonas syringae pv. maculicola ES4326 carrying empty vector (PmaDG3) and avirulent P. syringae pv. maculicola carrying avrRpt2 (PmaDG6) (P < 0.01, analysis of variance, Tukey test). B, Laser-scanning confocal microscopy micrographs showing localization of ALD1:GFP controlled by dex-inducible promoter in transgenic Arabidopsis seedlings. GFP fluorescence is shown in green and chlorophyll autofluorescence is shown in blue. Bars = 10 µm. C, Western blots of total (T) and chloroplast (Ch) fractions proteins from ALD1:GFP transgenic Arabidopsis seedlings. Bands were revealed using anti-GFP, ATPaseβ (chloroplast marker), and cyHsc70 (cytosolic marker) antibodies. Adult or seedlings plants were sprayed with dex 30 µM solution 21 h before analysis or infection (A to C).
...P. syringae with silenced (white) and NbALD1 (OD600 P. syringae secondary done as in D; avr = NbALD1-endophytic (En) bacterial population were higher on day 5 after inoculation inpression of NbALD1 were immunized by syringe infiltration of 10 mM MgSO4 (white) or NbALD1 virus [TRV]-based virus-induced gene silencing [VIGS] empty vector) and systemic acquired resistance defective. Nonsilenced (TRV:00;pression of B, harvested at the indicated times after treatments.

Secondary challenge inoculation. Error bars indicate standard error in B and D significant differences as determined by Student's t-test. All experiments were replicated two or three times with similar results. The panels show one representative result among them.

Fig. 8. NbALD1 gene of Nicotiana benthamiana is involved in both local and systemic resistance against Pseudomonas syringae infection. A, Transcription of NbALD1 gene was strongly induced in the leaves by infection with P. syringae pv. tabaci PTBR2004. Leaves syringe inoculated with mock (10 mM MgSO4) or infected with virulent P. syringae pv. tabaci (Vir) or avirulent derivative P. syringae pv. tabaci carrying avrRpt2 (Avr) (OD600 = 0.01) were harvested at the indicated times after treatments. B, NbALD1-silenced plants showed enhanced disease susceptibility to P. syringae in local leaves. Non-silenced (white) and NbALD1-silenced (black) plants were spray inoculated with P. syringae pv. syringae B728a (OD600 = 0.01). Both epiphytic (Ep) and endophytic (En) bacterial population were higher on day 5 after inoculation in NbALD1-silenced plants compared with those in nonsilenced plants. C, Expression of NbALD1 was suppressed in nonALD1-silenced plants. Inset: expression of ALD1 is plotted on a log scale. D, NbALD1-silenced plant is systemic acquired resistance defective. Nonsilenced (TRV:00; Tobacco rattle virus [TRV]-based virus-induced gene silencing [VIGS] empty vector) and silenced plants (TRV/NbALD1; VIGS-vector carrying NbALD1 fragment) were immunized by syringe infiltration of 10 mM MgSO4 (white) or P. syringae pv. tabaci carrying avrRpt2 (OD600 = 0.01) (black) 3 days prior to secondary P. syringae pv. syringae B728a infection by spray inoculation (OD600 = 0.01). The number of bacteria was examined 5 days after inoculation.

E, Transcript levels of NbALD1 were increased in systemic leaves of non-silenced plants (white) immunized with avrRpt2 but not in NbALD1-silenced plants (black). Primary immunization (1*) and secondary challenge (2*) were done as in D; avr = P. syringae pv. tabaci carrying avrRpt2, mo = mock (10 mM MgSO4), and vir = B728a. Samples were collected 3 days after secondary challenge inoculation. Error bars indicate standard error in B and D (n = 18 or 24) and standard deviation in A, C, and E (n = 3). Asterisks in B indicate statistically significant differences relative to nonsilenced plants (P < 0.01, Student’s t test). Different letters in other panels indicate statistically significant differences as determined by P < 0.01, analysis of variance. Tukey test. All experiments were repeated two or three times with similar results. The panels show one representative result among them.

ALD1ox exudate. This suggests that ALD1 function requires the action and regulation of major genes already known to be important for P. syringae resistance, PAD4 and ICS1/SID2 (Glazebrook et al. 1996; Wildermuth et al. 2001). It also suggests that a basal state ALD1 product is transduced into resistance by these factors. In contrast, PAD4 and FMO1 are needed for Pip to fully increase resistance, whereas ICS1/SID2 is needed only partially (Návarová et al. 2012). Moreover, Pip accumulation after local infection depends on PAD4 but not on ICS1/SID2 or FMO1. Again, these differences between ALD1 basal state metabolites and Pip support the idea of ALD1 producing more than one defense metabolite.

Previously, we have shown that ALD1 induction depends mainly on PAD4 and, under certain conditions (in acd6-1 plants that constitutively produce SA and high transcript levels of ALD1 and PAD4) (Lu et al. 2003), ALD1 weakly affects PAD4 transcript levels (Song et al. 2004b). Here, we show that, at early times (5 h) after infection, PAD4 and ICS1/SID2 induction strongly depend on ALD1. In addition, ALD1 overexpression enhances the induction of both transcripts. Altogether, the transcriptional interdependence between these components suggests that an amplification loop exists between ALD1-PAD4-ICS1/SID2. In agreement with this, SA and its agonist benzo (1, 2, and 3) thiadiazole-7-carboxylic acid (BTH) can induce ALD1 (Návarová et al. 2012; Song et al. 2004b). FMO1 is also stimulated by ALD1 overexpression. However, because FMO1 is not necessary for ALD1ox-conferred increased resistance, it does not appear to have a role in local resistance. Supporting this, a similar loop between ALD1-ICS1 and SID2-FMO1 was suggested to work in systemic leaves during SAR establishment (Návarová et al. 2012). Here, we propose that an ALD1-PAD4-ICS1/SID2 loop accelerates SA-related defenses locally and at early times postinfection. Considering the effect of ALD1 on flg22 responses, it is possible that PTI components are also part of the ALD1-dependent defense amplification loop. In support of this idea, it is known that SA or BTH can potentiate PTI (Kohler et al. 2002; Sato et al. 2010; Yi et al. 2014) and increase MAMP receptor levels at the plasma membrane (Zhang et al. 2014). It is also known that ICS1/SID2 and PAD4 affect and are part of a network necessary for triggering a robust PTI (Tsuda et al. 2009, 2008). Thus, it is probable that, during early defense responses, there exists an SA-pathway amplification loop involving PTI components.

In summary, increased or decreased ALD1 affects earlier defense events than were previously described (Song et al. 2004a,b), as exemplified by altered ROS production after flg22 treatment. We speculate that the ALD1 effect could be due to a direct or indirect modulation of PTI and SA early-pathway components by chloroplastic ALD1-produced amino-acid-derived compounds. These compounds are likely to be conserved in other plants, because ALD1 homologs in Lotus japonicus (Chen et al. 2014) and N. benthamiana are also important in defense pathways.

MATERIALS AND METHODS

Plants.

All Arabidopsis plants were in the Columbia-0 background. Arabidopsis plants were grown under 12-h day (8:00 AM to 8:00 PM) and 12-h night conditions at 20°C, as described (Greenberg et al. 2000). Arabidopsis aldl-T2, fmo1, npr1-1, pad4-1, and sid2-1 were previously described (Cao et al. 1994; Glazebrook et al. 1996; Jung et al. 2009; Mishina and Zeier 2006; Nawrath and Métraux 1999; Song et al. 2004a).

The coding region of Arabidopsis ALD1 was amplified with PCR primers linked to specific sequences compatible with the GATEWAY cloning procedure (Supplementary Table S1) and...
introduced into plant expression vectors pGWB20, kindly provided by Dr. Jeff Dangl (University of North Carolina), and pBAV150 (Vinatzer et al. 2006). The resulting plasmids allow the expression of \(\text{ALD1}\) with the C-terminal myc epitope tag controlled by the CaMV 35S promoter (pGWB20) or with the C-terminal GFP tag controlled by the dexamethasone-inducible promoter (pBAV150). Transgenic plants were established by dippling WT or \(\text{ald1}\) mutant flowers into suspensions of \text{Agrobacterium tumefaciens} strain GV3101 harboring the pGWB20::\text{ALD1} or pBAV150::\text{ALD1} (Clough and Bent 1998), then selected in Murashige and Skoog media (Sigma-Aldrich) supplemented with kanamycin (50 \(\mu\)g/ml) and hygromycin (50 \(\mu\)g/ml) or Basta (10 \(\mu\)g/ml), respectively. To generate the mutant plants overexpressing \(\text{ALD1}\), a homozygous transgenic \(\text{ALD1}\)ox plant (pGWB20::\text{ALD1}/line 8) was crossed with \text{pad4}, \text{sid2}, and \text{fmo1}. Homozygous plants were selected in the F2 generation.

VIGS.

A 447-bp fragment that corresponds to nucleotides 855 to 1,301 of \text{N. benthamiana ALD1} mRNA (\text{NhALD1}; TC23014, The Gene Indices) was amplified by cDNA and cloned into the pTRV2 vector (Liu et al. 2002). The VIGS assay was performed as described by Liu and associates (2002).

Pathogen infection.

To analyze bacterial growth in \text{Arabidopsis}, leaves of 23- to 25-day-old plants were infected by syringe infiltration with a virulent derivative of \(P. syringae pv. maculicola\) ES4326 carrying an empty vector (\text{PmaDG3}) (optical density at 600 nm \([\text{OD}_{600}] = 0.0001\)) (Guttman and Greenberg 2001). Growth was determined 3 days after inoculation. For local gene expression studies in \text{ald1} and SA-dependent defense mutants, leaves were infected with an avirulent derivative of \(P. syringae pv. maculicola\) ES4326 carrying \text{avrRpt2} (\text{PmaDG6}) (\text{OD}_{600} = 0.01) (Guttman and Greenberg 2001). The biological activity of petiole exudates (see Petiole Exudate Collection, below) to confer disease resistance was examined by syringe infiltration of exudates into leaves of the WT or mutants 2 days prior to subsequent pathogen infection (Jung et al. 2009). Transgenic \text{Arabidopsis/pBAV150::ALD1} plants were sprayed with 30 \(\mu\)M dexamethasone plus 0.1\% Tween 20 solution 21 h before \text{PmaDG3} and \text{PmaDG6} syringe inoculations.

For \text{N. benthamiana} infections, plants were grown under 12-h day (8:00 A.M. to 8:00 P.M.) and 12-h night conditions at 24°C (Vinatzer et al. 2006). To examine expression of \text{NhALD1}, 5-week-old tobacco plants were syringe inoculated with a virulent strain of \(P. syringae pv. tabaci\) PTB2004 or an avirulent derivative of \(P. syringae pv. tabaci\) carrying \text{avrRpt2} (\text{OD}_{600} = 0.01) (Vinatzer et al. 2006). The bacterial strain of \(P. syringae pv. syringae\) B728a (\text{OD}_{600} = 0.01) (Vinatzer et al. 2006) was sprayed on \text{N. benthamiana} leaves to evaluate bacterial epiphytic and endophytic growth after 5 days, as described previously (Lee et al. 2012). For SAR experiments, lower leaves of non silenced or \text{NhALD1}-silenced plants were immunized with \(P. syringae pv. tabaci\) carrying \text{avrRpt2} (\text{OD}_{600} = 0.01) 3 days prior to secondary B728a infection (\text{OD}_{600} = 0.01).

Exogenous application of Pip and AZA.

These treatments were done as previously described (Jung et al. 2009; Návarová et al. 2012).

Petiole exudate collection.

Leaves of 25-day-old WT, \text{ald1-T2}, or \text{ALD1ox} plants were excised and petioles submerged in 1 mM EDTA solution supplemented with carbencillin at 50 \(\mu\)g/ml and streptomycin for 72 h. To eliminate residual bacteria, the petiole exudates were centrifuged three times at 12,000 rpm for 10 min, and the cleared exudates were plated onto Luria-Bertani media to verify the lack of bacteria. Bacteria-free petiole exudates were immediately frozen and stored at −75°C until application to plants. Mock induced exudates and pathogen-induced exudates from leaves of WT and \text{ald1} plants were infected and harvested as described previously (Jung et al. 2009).

mRNA analysis.

Total RNA isolation and reverse transcription were done using Trizol reagent and Superscript II reverse transcription, respectively, according to the manufacturer’s procedures (Invitrogen). Quantitative real-time RT-PCR was performed as described previously (Jung et al. 2009). Internal standards used for data normalization were \text{EFLa} and \text{ACTI} for \text{Arabidopsis} and \text{N. benthamiana}, respectively. Oligonucleotide sequences used as primers are presented in Supplementary Table S1.

Determination of SA and AZA treatment.

These experiments were done as described previously (Jung et al. 2009; Seskar et al. 1998).

Determination of Pip.

Exudates were trimethylsilyl (TMS) derivatized and analyzed by gas chromatography-MS, as described previously (Jung et al. 2009), with some modifications. Sorbitol (15 \(\mu\)l of aqueous solution at 1 mg/ml) was added as an internal standard to 250 to 400 \(\mu\)l of thawed exudate, with samples then dried in a nitrogen stream. The internal standard was added to correct for differences in derivatization efficiency and changes in sample volume during heating. Dried exudates were dissolved in 500 \(\mu\)l of silylation-grade acetone/toluene followed by the addition of 500 \(\mu\)l of N-methyl trimethylsilyl trifluoroacetamide with 1% triethylchlorosilane (Thermo Scientific, Bellefonte, PA, U.S.A.), and samples were then heated for 1 h at 70°C to generate TMS derivatives. After 1 to 3 days, 1- to 2-\(\mu\)l aliquots were injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, U.S.A.) coupled to an Agilent 5975C inert XL mass spectrometer (Agilent Technologies), fitted with an Rtx-5MS (crosslinked 5% PH ME Siloxane) capillary column (30 m by 0.25 mm by 0.25 \(\mu\)m film thickness) (Restek, Bellefonte, PA, U.S.A.). The standard quadrupole gas chromatograph mass spectrometer was operated in electron impact (70 eV) mode, with 2.5 full-spectrum (50 to 650 Da) scans per second. Carrier gas (helium) flow was set at 1.3 ml/min with an injection port configured in the splitless mode. The injection port, MS source, and MS quadrupole temperatures were set to 250, 230, and 150°C, respectively. The initial oven temperature was held at 50°C for 2 min and programmed to increase at 20°C/min to 325°C and held for another 11 min, before cycling back to the initial conditions. Peaks were quantified by area integration using a key selected ion (characteristic m/z fragment) rather than the total ion chromatogram to minimize integrating coeluting metabolites, and the relative concentrations were determined based on the quantity of the internal standard.

Subcellular localization of \text{ALD1}.

For localization studies in transgenic \text{Arabidopsis thaliana/pBAV150::ALD1}, homozygous seedlings were sprayed with 30 \(\mu\)M dexamethasone plus 0.1\% Tween 20 solution and analyzed by confocal microscopy 21 h later. A Zeiss LSM710 laser-scanning confocal microscope (Zeiss, Germany) was used to visualize GFP fluorescence (excitation: 488 nm; emission: 505 to 530 nm) and chlorophyll autofluorescence (excitation: 633, emission: 650 to 750 nm). Images were taken using an LD C-Apochromat 40×/1.1 W Korr objective. Images for GFP and plant autofluorescence were acquired for the same field using a sequential acquisition mode.
chloroplasts were isolated from 1 g of A. thaliana/pBAV150: ALD1 seedlings sprayed with 30 µM dextrex 0.1% Tween 20 solution and fractionated 21 h later. Intact chloroplasts were purified using Percoll gradients as described (Pattanayak et al. 2012). Organellar purity was assessed by Western blot using organellar-specific marker antibodies (see Western blot analysis).

Isolation of proteins for Western blot analysis.

For immunoblot studies of GFP, cytHsc70, and chloroplast ATPase β, total (extract before fractionation) and chloroplastic fractions were mixed with sodium dodecyl sulfate (SDS) sample buffer. For analysis of FLS1 and BAK1, leaves (1 g) of 24- to 26-day-old plants were excised and collected. Total extracts were isolated in extraction buffer (Chinchilla et al. 2007): 50 mM Tris-HCL (pH8.0), 10% glycerol, 0.5% sodium deoxycholate, 1% Igepal CA-630 from Sigma, and complete protease inhibitor cocktail from Roche. Cellular debris was removed by centrifugation, and supernatant was mixed with SDS sample buffer.

Western blot analysis.

Equal amounts of solubilized total proteins were separated by SDS polyacrylamide gel electrophoresis. Primary antibodies used for Western blots were as follows: FLS2 antibody (Agrisera AS12 1858, 1:3000), GFP antibody (Covance MMS-118P, monoclonal, 1:3000), cytosolic Hsc70 antibody (cytHsc70, Stressgen SPA-817, monoclonal, 1:3000), and ATPase β antibody (Draper et al. 1992) (1:3000). Secondary horseradish peroxidase conjugated antimouse and antirabbit antibodies (Thermo Scientific) were used for Western blots were as follows: FLS2 antibody (Chinchilla et al. 2007) (1:500), BAK1 antibody (Agrisera AS12 1858, 1:3000), cytosolic Hsc70 antibody (cytHsc70, Stressgen SPA-817, monoclonal, 1:3000), and ATPase β antibody (Draper et al. 1992) (1:3000). Secondary horseradish peroxidase conjugated antimouse and antirabbit antibodies (Thermo Scientific) were used at 1:1000. SuperSignal West Pico/Femto Stable Peroxidase (Thermo Scientific) was used to detect the signals. Gel-Pro analyzer software was used to quantify bands and Coomassie blue on Western blots by densitometry.

Callose quantitation.

Callose deposits were stained with aniline blue as described by Kim and Mackey (2008), except that chlorophyll was cleared with ethanol. Leaves from at least six independent plants for each genotype/treatment were used for measurements. Callose was quantified by counting deposits in images of plants for each genotype/treatment were used for measurements. Callose was quantified by counting deposits in images of plants for each genotype/treatment were used for measurements. Callose was quantified by counting deposits in images of plants for each genotype/treatment were used for measurements. Callose was quantified by counting deposits in images of plants for each genotype/treatment were used for measurements.


AUTHOR-RECOMMENDED INTERNET RESOURCE

The Gene Indices: compbio.dfci.harvard.edu/tgi/