Arabidopsis thaliana FLOWERING LOCUS D Is Required for Systemic Acquired Resistance

Vijayata Singh,¹ Shweta Roy,¹ Mrunmay Kumar Giri,¹ Ratnesh Chaturvedi,² Zulkarnain Chowdhury,² Jyoti Shah,² and Ashis Kumar Nandi¹

¹School of Life Sciences, Jawaharlal Nehru University, New Delhi -110067, India; ²Department of Biological Sciences and Signaling Mechanisms in Plants Cluster, University of North Texas, Denton 76203, U.S.A.

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Localized infection in plants often induces systemic acquired resistance (SAR), which provides long-term protection against subsequent infections. A signal originating in the SAR-inducing organ is transported to the distal organs, where it stimulates salicylic acid (SA) accumulation and priming, a mechanism that results in more robust activation of defenses in response to subsequent pathogen infection. In recent years, several metabolites that promote long-distance SAR signaling have been identified. However, the mechanism or mechanisms by which plants perceive and respond to the SAR signals are largely obscure. Here, we show that, in *Arabidopsis thaliana*, the *FLOWERING LOCUS D* (*FLD*) is required for responding to the SAR signals leading to the systemic accumulation of SA and enhancement of disease resistance. Although the *fld* mutant was competent in accumulating the SAR-inducing signal, it was unable to respond to the SAR signal that accumulates in petiole exudates of wild-type leaves inoculated with a SAR-inducing pathogen. Supporting *FLD*'s role in systemic SAR signaling, we observed that dehydroabietinal and azelaic acid, two metabolites that, in wild-type plants, promote SAR-associated systemic accumulation of SA and priming, respectively, were unable to promote SAR in the *fld* mutant. *FLD* also participates in flowering, where it functions to repress expression of the flowering repressor *FLOWERING LOCUS C* (*FLC*). However, epistasis analysis indicates that *FLD*'s function in SAR is independent of *FLC*.

Recognition by plants of molecular patterns that are conserved among various microbes results in the activation of pathogen-associated molecular pattern-triggered immunity (PTI), which contributes to basal resistance against pathogens, thus limiting the extent of pathogen growth (Spoel and Dong 2012). However, the impact of PTI on curtailing pathogen growth is weak compared with that of effector-triggered immunity (ETI), which is induced upon recognition by the plant of race-specific effectors produced by avirulent (Avr) pathogens. In addition to PTI and ETI, which locally control pathogen growth, plants also have the ability to enhance resis-

tance status of the distal pathogen-free organs in response to a prior infection elsewhere on the foliage. This mechanism, which is termed systemic acquired resistance (SAR), confers long-term protection throughout the foliage against subsequent infections by a variety of pathogens (Chaturvedi and Shah 2007; Durrant and Dong 2004; Sticher et al. 1997). In plants exhibiting SAR, defense responses are primed for stronger activation in response to subsequent infections (Shah and Zeier 2013). Indeed, under conditions of high disease pressure, SAR confers a fitness advantage (Traw et al. 2007). Studies in *Arabidopsis thaliana* have indicated that the protective effect of SAR can be epigenetically transmitted to the next generation (Luna et al. 2012).

SAR is accompanied by an increase in salicylic acid (SA) content and elevated expression of the SA-inducible *PATHOGENESIS-RELATED 1* (*PR1*) gene. This increase in expression of *PR1* has been used as a molecular marker for monitoring SAR activation (Chaturvedi and Shah 2007; Durrant and Dong 2004). SA signaling is critical for the manifestation of SAR-conferred enhanced disease resistance. In *Arabidopsis*, limiting SA accumulation, either due to a block in its synthesis in the *isochorismate synthase 1* (*ics1*) mutant or by expressing the bacterial *nahG* gene-encoded salicylate hydroxylase that converts SA to catechol, resulted in the attenuation of SAR-conferred resistance (Lawton et al. 1995; Wildermuth et al. 2001). Similarly, SAR was also attenuated in NahG tobacco (Vernooij et al. 1994). Also required for SAR is NPR1, a transcription co-regulator of SA-responsive genes, which itself is activated by SA binding (Spoel and Dong 2012; Wu et al. 2012).

Activation of SAR is dependent on the ability of the primary pathogen-infected organs to efficiently communicate with the rest of the foliage. Girdling experiments in tobacco and cucumber suggested that the phloem is the likely route for transport of long-distance signals that mediate this communication (Guedes et al. 1980; Tuzun and Kuc 1985). Indeed, a SAR-inducing activity can be recovered in vascular sap-enriched petiole exudates (Pex) collected from *Arabidopsis* leaves inoculated with a SAR-inducing Avr pathogen (Chaturvedi et al. 2008, 2012; Jung et al. 2009; Maldonado et al. 2002). When locally applied to lower leaves, Avr-Pex collected from *Arabidopsis* leaves was effective in promoting SAR in the distal leaves. Although SA is present in vascular sap, it was previously shown not to be the long-distance translocated SAR signal (Pallas et al. 1996; Rasmussen et al. 1991; Vernooij et al. 1994).

In recent years, several metabolites potentially involved in long-distance signaling in SAR have been described (Dempsey and Klessig 2012). Methyl salicylate (MeSA) was reported as

Corresponding author: A. K. Nandi; Telephone: +91-11-26704152; E-mail: ashis_nandi@mail.jnu.ac.in, ashis_nandi@yahoo.com

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a long-distance SAR signal in tobacco plants infected with *Tobacco mosaic virus* (Park et al. 2007). It was suggested that MeSA produced from SA in the virus-inoculated leaves was transported to the distal leaves, where it was hydrolyzed by the MeSA esterase activity of SA-BINDING PROTEIN 2 (SABP2) to release SA; which, in turn, could promote downstream defenses (Park et al. 2007). Similarly, MeSA has also been suggested to function as a long-distance SAR signal in potato and *Arabidopsis* (Manosalva et al. 2010; Vlot et al. 2008). However, other studies have seen no role, or a weak function, for MeSA in SAR in *Arabidopsis* (Attaran et al. 2009; Chaturvedi et al. 2012). It has been suggested that light is a likely factor that influences the overall contribution of MeSA as a long-distance signal in SAR, thus explaining some of the variability in the requirement of MeSA in SAR (Liu et al. 2011). Dehydroabietinal (DA), an abietane diterpenoid, has been suggested as another long-distance signal in SAR (Chaturvedi et al. 2012). DA was purified as a SAR-inducing metabolite from *Arabidopsis* Avr-Pex. DA applied to lower leaves of *Arabidopsis* is rapidly translocated to the distal leaves, where it promotes SA accumulation and enhanced disease resistance. Compared with MeSA and DA, which promote systemic disease resistance by promoting systemic SA accumulation, azelaic acid (Aza), a 9-C dicarboxylic acid, and the lysine metabolite pipecolic acid are proposed to promote priming of defenses during SAR (Jung et al. 2009; Navarova et al. 2012). Aza and DA, when applied together, had a synergistic effect on the activation of SAR in *Arabidopsis* (Chaturvedi et al. 2012). Glycerol-3-phosphate (G3P) is another metabolite that has been suggested to participate in long-distance SAR signaling (Chanda et al. 2011). G3P levels were found to increase in Avr-Pex; and G3P, when applied with Pex, was capable of systemically activating disease resistance. The involvement of a G3P-dependent factor in long-distance signaling is also supported by earlier observations that the G3P-synthesizing dihydroxyacetone phosphate reductase, encoded by *SUPPRESSOR OF FATTY ACID DESATURASE-DEFICIENCY 1* (*SFD1*), was required for the accumulation or translocation of the SAR signal (Chaturvedi et al. 2008; Lorenc-Kukula et al. 2012; Nandi et al. 2004). The G3P-dependent factor has been suggested to facilitate long-distance movement of *DEFECTIVE IN INDUCED-RESISTANCE 1* (*DIR1*) (Chanda et al. 2011), a putative lipid-transfer protein that is required for the activation of SAR (Maldonado et al. 2002). Like the *sfd1* mutant, Avr-Pex collected from the *dir1* mutant were unable to induce SAR when applied to wild-type (WT) plants (Chaturvedi et al. 2008; Maldonado et al. 2002). However, when co-infiltrated into lower leaves of WT plants, *sfd1* plus *dir1* Avr-Pex were competent in inducing SAR (Chaturvedi et al. 2008). Presence of *SFD1* also enhances the effectiveness of DA, and *DIR1* is required for DA-induced systemic disease resistance, thus suggesting interaction between *DIR1*-, *SFD1*-, and DA-mediated signaling (Chaturvedi et al. 2012).

Here, we show that the *FLOWERING LOCUS D* (*FLD*) is required for *Arabidopsis* to respond to the SAR signals leading to the systemic accumulation of SA and priming the robust activation of SA signaling in response to subsequent exposure to a virulent pathogen. Although *FLD* is also involved in flowering as a negative regulator, *FLOWERING LOCUS C* (*FLC*) expression, we provide evidence that *FLD* function in SAR does not involve *FLC*.

RESULTS

The *reduced systemic immunity 1* mutant is defective in SAR.

To identify genes involved in SAR signaling, a genetic screen was set up for mutants that were unable to activate

SAR-conferred enhanced disease resistance against the virulent pathogen *Pseudomonas syringae* pv. *maculicola* ES4326. To induce SAR, three fully expanded rosette leaves were infiltrated (primary treatment) with *P. syringae* pv. *tomato* DC3000 that carried the *AvrRpt2* gene (Avr-Pst). Plants that received a primary treatment consisting of 10 mM MgCl₂ (mock), which was used to prepare the bacterial suspension, provided the negative control for SAR. Three days after the primary treatment, the distal leaves of these plants were challenged with *P. syringae* pv. *maculicola*. Bacterial numbers in these leaves were determined 3 days postinfiltration (dpi). The SAR-deficient mutant *reduced systemic immunity 1* (*rsi1*) mutant was uncovered after screening about 1,800 M₂ plants. As expected, SAR was induced in the WT plants, thus resulting in lower numbers of *P. syringae* pv. *maculicola* in plants that received a primary treatment consisting of the SAR-inducing Avr-Pst, than in plants that received a first mock treatment (Fig. 1A; Supplementary Fig. S1). Compared with the WT, SAR-conferred resistance was compromised in the *rsi1* mutant (Fig. 1A).

P. syringae pv. *maculicola* numbers were comparable in the distal leaves of WT and *rsi1* plants that received a first mock treatment, suggesting that the mutation in *rsi1* does not compromise basal resistance against *P. syringae* pv. *maculicola* (Fig. 1A). Additional experiments in which the virulent pathogen *P. syringae* pv. *tomato* DC3000 was infiltrated into leaves of WT and *rsi1* plants that did not receive the primary treatment also indicated comparable population size in the WT and *rsi1* mutant, thus confirming that basal resistance against these virulent pathogens was not compromised in the *rsi1* mutant (Fig. 1B). Similarly, resistance against Avr-Pst was not compromised in the *rsi1* mutant (Fig. 1C). Thus, *RSI1* is required for SAR but not for local resistance.

Mutant *rsi1* is a missense allele of *FLD*.

During the vegetative phase of growth, the *rsi1* mutant was morphologically indistinguishable from the WT plant (Supplementary Fig. S2A). However, compared with the WT, *rsi1* plants exhibited a delay in transition from vegetative to the reproductive stage. The delayed flowering phenotype of *rsi1* segregated as a single locus that was recessive to the WT *RSI1* allele in F₂ progenies derived from a cross between *rsi1* and WT accession Columbia (Col-0), as well as from a *rsi1* × WT accession Wassilewskija (Ws) cross (Supplementary Table S1). The F₃ progeny of F₂ plants showing a delayed flowering phenotype showed a SAR-deficient phenotype, suggesting that the delayed flowering and SAR-deficient phenotypes are likely due to the same mutation. Analysis of 131 F₂ progeny plants derived from the *rsi1* × WT Ws cross mapped *rsi1* to the upper arm of chromosome 3, between the ChiB and LugSSLP467 molecular markers, 2.3 centimorgans from LugSSLP467. No recombinants were identified in the *rsi1*-ChiB interval. A scan of the region between ChiB and LugSSLP467 for genes known to be associated with the transition from vegetative to reproductive stage identified *FLD* (At3g10390), which is located close to ChiB. Sequencing of the *FLD* locus from WT Col-0 and the *rsi1* mutant identified a guanine to adenine (G→A) transition in the first exon of *FLD* in the *rsi1* mutant (Fig. 2A). Polymerase chain reaction (PCR) amplification with a set of modified primers that introduce an *HpaII* restriction site in the amplicon derived from WT Col-0 identified distinct restriction patterns in the WT compared with plants containing the *rsi1* allele, thus confirming the presence of the G→A transition mutation in the *FLD* gene in the *rsi1* mutant (Supplementary Fig. S3A). *RSI1/FLD* expression was upregulated in the Avr-Pst-treated and distal leaves, further supporting *FLD*'s involvement in SAR (Fig. 2B and C).

FLD encodes a homolog of the human LYSINE-SPECIFIC DEMETHYLASE 1 and contains a Swi3p, Rsc8p, and Moira domain and a large polyamineoxidase domain (He et al. 2003). The *rsi1* mutation results in a glycine to arginine substitution (Gly₅₁₂→Arg₅₁₂) in the polyamineoxidase domain of *FLD* (Fig. 2D). Gly₅₁₂ is highly conserved among *FLD* homologs from a variety of organisms (Fig. 2D), suggesting that this is an important residue in these proteins. To further confirm that the SAR defect of *rsi1* is, indeed, due to a mutation in *FLD*, SAR-conferred resistance against *P. syringae* pv. *maculicola* was characterized in additional loss-of-function alleles of *FLD*. Indeed, similar to *rsi1*, compared with the WT, the SAR-conferred curtailment of *P. syringae* pv. *maculicola* population was weaker in the *fld-3*, *fld-4*, and *fld-5* mutants (Fig. 2E and F). These results confirm an important role for *FLD* in SAR.

SAR-associated systemic SA accumulation is compromised in *rsi1*.

SAR is accompanied by a systemic increase in SA content in the pathogen-free leaves of plants that were locally treated with a SAR-inducing pathogen (Durrant and Dong 2004; Shah 2009). Furthermore, functional SA signaling is required for the activation of SAR-conferred resistance in the distal leaves. The distal leaves of WT plants, which were inoculated on their lower leaves with Avr-Pst, accumulated a higher level of SA (Fig. 3A and B). A comparable increase in systemic SA content was not observed in the *rsi1* mutant (Fig. 3A and B). To determine whether loss of *FLD* function results in a general loss of ability to accumulate SA in response to biotic stress, SA content and expression of the SA-responsive *PR1* gene were determined in the primary pathogen-inoculated leaves. The *rsi1* mutant retained the ability to accumulate elevated levels of SA and *PR1* transcript in the primary pathogen-inoculated leaves (Supplementary Figs. S4 and S5A), thus confirming that it does not have a general defect in SA accumulation. The *rsi1* mutant also was not defective in SA perception or signaling because, like the WT plant, SA application enhanced resistance against *P. syringae* pv. *maculicola* in the *rsi1* mutant (Fig. 3C). Taken together, these results suggest that the SA accumulation defect in *rsi1* is limited to SAR.

Unlike the defect in SAR-associated systemic enhancement of SA content, the *rsi1* mutant retained the SAR-associated upregulation of *PR1* in the distal pathogen-free tissues of plants that were inoculated on their lower leaves with Avr-Pst (Fig. 4A). In fact, the systemic induction of *PR1* in the pathogen-free leaves was stronger in the *rsi1* mutant compared with the WT plant. These results suggest that the SAR signal is being translocated from the pathogen-treated leaves to the distal leaves in *rsi1*, resulting in the systemic increase in *PR1* transcript accumulation. Thus, the inability of *rsi1* to express SAR-conferred enhanced disease resistance is due to defects in signaling affecting some of the responses to the SAR signal. Similarly, systemic induction of the ethylene- and jasmonic-acid-responsive defense marker *PDF1.2* was also stronger in the *rsi1* mutant than the WT plant (Fig. 4B). The elevated expression of *PR1* in the pathogen-free distal tissue of *rsi1* was unexpected and was in striking contrast to the lack of systemic SA accumulation and attenuated systemic resistance phenotype of the *rsi1* mutant. Because SAR-expressing plants exhibit a more robust induction of SA signaling in response to challenge with pathogen, we compared *PR1* expression between the distal leaves of *rsi1* and WT plants after challenge inoculation with *P. syringae* pv. *maculicola*. As expected, in WT plants, a stronger induction of *PR1* expression was observed in the distal *P. syringae* pv. *maculicola*-challenged (secondary inoculation) leaves that were previously treated with Avr-Pst (primary

inoculation) compared with the plants that received a primary inoculation consisting of 10 mM MgCl₂ (Fig. 4C). However, a similar enhancement of *PR1* expression was not observed in the *P. syringae* pv. *maculicola*-challenged distal leaves of the *rsi1* mutant that were previously (primary inoculation) inoculated on their lower leaves with Avr-Pst compared with 10 mM MgCl₂ (Fig. 4C). Even though the level of *PR1* expression in the pathogen-free distal tissue of *rsi1* was higher compared with comparably treated WT plants (Fig. 4A), this level is quantitatively much lower than in WT tissues exhibiting SAR-conferred enhanced disease resistance (Fig. 4C). The above results indicate that *FLD* is not required for the expression of the *PR1* gene in the distal tissue prior to secondary inoculation

Fig. 1. Systemic acquired resistance (SAR) but not local resistance is compromised in the *rsi1* mutant. **A**, SAR-conferred resistance in the wild type (WT) and the *rsi1* mutant. A suspension (10⁷ CFU/ml) of *P. syringae* pv. *tomato* DC3000 carrying AvrRpt2 (Avr-Pst) in 10 mM MgCl₂ was infiltrated into the abaxial surface of three lower leaves with a needleless syringe. Plants that were similarly treated with 10 mM MgCl₂ provided the mock controls for SAR. Three days later, four distal leaves of each plant received a challenge inoculation consisting of *Pseudomonas syringae* pv. *maculicola* ES4326 (5 × 10⁵ CFU/ml). *P. syringae* pv. *maculicola* numbers in the distal leaves were determined 3 days postinfiltration (dpi). Bars: mv, primary treatment = 10 mM MgCl₂ and secondary treatment = *P. syringae* pv. *maculicola*; av, primary treatment = Avr-Pst and secondary treatment = *P. syringae* pv. *maculicola*. **B**, Local defense against *P. syringae* pv. *tomato* DC3000 in the WT and the *rsi1* mutant. A suspension of virulent pathogen *P. syringae* pv. *tomato* (5 × 10⁵ CFU/ml) was infiltrated into the abaxial surface of leaves with a needleless syringe. Bacterial numbers were determined at 3 dpi. **C**, Local defense against Avr-Pst in the WT and the *rsi1* mutant. A suspension of the avirulent pathogen Avr-Pst (5 × 10⁵ CFU/ml) was infiltrated into the abaxial surface of leaves with a needleless syringe. Bacterial numbers were determined at 3 dpi. Each bar represents the mean ± standard deviation of four samples, each carrying five leaf discs of 5 mm in diameter randomly taken from different plants. Letters above the bars indicate values that are significantly different (P < 0.05) from each other as analyzed by one-way analysis of variance (post-hoc Holm-Sidak method). Disease resistance studies were repeated three times, with similar results.

Fig. 2. *rsi1* is a loss-of-function allele of *FLD*. **A**, *RSI1/FLD* gene structure with the relative position of mutation in each allele. Boxes and lines indicate exons and introns, respectively. Start and stop codons, position of point mutations associated with *rsi1* (missense mutation) and *fld-5* (non-sense mutation), and sites of T-DNA insertions in *fld-3* and *fld-4* are indicated. **B**, Relative abundance of *FLD* mRNA in leaves of wild-type (WT) plants, 12 h postinoculation with *P. syringae* pv. *tomato* DC3000 carrying AvrRpt2 (Avr-Pst) or 10 mM MgCl₂. **C**, Relative abundance of *FLD* mRNA in the distal leaves of WT plants that were previously (3 days earlier) treated on their lower leaves with Avr-Pst (Avr) or 10 mM MgCl₂ (mock). **D**, Amino acid sequences surrounding Gly₅₁₂ of *FLD* and close homologues in other eukaryotes. AtL1, AtL2, and AtL3 = *Arabidopsis* *LSD1* like (*LDL*) genes; Al = *A. lyrata*; Zm = *Zea mays*; Rc = *Ricinus communis*; Os = *Oryza sativa*; Pp = *Physcomitrella patens*; Vv = *Vitis vinifera*; Hs = *Homo sapiens* *LSD1*. **E**, *Pseudomonas syringae* pv. *maculicola* numbers in the distal leaves of the WT (Col-0), *fld-3*, and *fld-4* mutant plants that were previously treated on their lower leaves with Avr-Pst or 10 mM MgCl₂. **F**, *P. syringae* pv. *maculicola* numbers in the distal leaves of the WT (Ws) and *fld-5* plants that were previously treated on their lower leaves with Avr-Pst or 10 mM MgCl₂. **B** and **C**, Real-time polymerase chain reaction was used to determine the abundance of the *FLD* transcript relative to the abundance of *ACTIN 2* transcript. Each bar represents mean $\pm$ standard deviation (SD) of three biological samples with two technical replications of each. The experiment was repeated two times, with similar results. **E** and **F**, Challenge inoculation with *P. syringae* pv. *maculicola* was initiated by infiltrating the distal leaves with a suspension (5×10^5 CFU/ml) of *P. syringae* pv. *maculicola*. Bacterial numbers were determined at 3 days postinfiltration. Each bar represents the mean $\pm$ SD of four samples each carrying five leaf discs of 5 mm in diameter. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other as analyzed by one-way analysis of variance (post-hoc Holm-Sidak method). Bars: mv, primary treatment = 10 mM MgCl₂ and secondary treatment = *P. syringae* pv. *maculicola*; av, primary treatment = Avr-Pst and secondary treatment = *P. syringae* pv. *maculicola*. Disease resistance studies were repeated three times, with similar results.

but, rather, invokes stronger expression of *PR1* during the manifestation of SAR-associated enhanced disease resistance.

***RSII/FLD* function is required for response to the long-distance SAR signal in the distal tissue.**

The long-distance SAR signal can be recovered in Avr-Pex collected from Avr-Pst-inoculated leaves of WT plants (Chaturvedi et al. 2008, 2012; Jung et al. 2009; Maldonado et al. 2002). When applied to a few leaves of the WT plant, Avr-Pex is capable of protecting the distal leaves from subsequent infections. To determine whether *rsi1* contains this SAR-activating factor, Avr-Pex collected from the *rsi1* mutant was infiltrated into lower leaves of a WT plant. Three days later, the distal leaves were challenged with *P. syringae* pv. *maculicola*. Avr-Pex collected from WT plants provided the positive control. Avr-Pex from the WT and *rsi1* mutant were equally effective in enhancing resistance against *P. syringae* pv. *maculicola* in the distal leaves of WT plants (Fig. 5A), suggesting that the *rsi1* mutant produces the long-distance SAR signal. Next, we tested whether the *rsi1* mutant was responsive to the SAR signal by applying Avr-Pex from WT plants to the lower leaves of the *rsi1* mutant and challenging the distal leaves with *P. syringae* pv. *maculicola*. Avr-Pex collected from WT plants was unable to enhance resistance against *P. syringae* pv. *maculicola* in the distal leaves of the *rsi1* mutant (Fig. 5A). DA and Aza applied to the lower leaves were also unable to enhance resistance against *P. syringae* pv. *maculicola* in the distal leaves of *rsi1* compared with the WT plant (Fig. 5B). These results suggest that, in the *rsi1* mutant, one or more processes

that are required for SAR-conferred enhanced disease resistance are unable to respond to the SAR signals.

***FLD/RSII*-mediated activation of SAR is independent of *FLC* function.**

The *FLC* gene, which is a target of the *FLD*-containing repressor complex, encodes a MADS box protein that negatively controls transition from vegetative growth to flowering by suppressing the transcription of genes that promote flowering (Michaels and Amasino 1999). In agreement with *FLD* negatively regulating *FLC* expression, *FLC* transcripts accumulated at higher levels in the *rsi1* mutant than the WT plant (Fig. 6A) and correlated with the delayed flowering phenotype of *rsi1*, which was suppressed in the *rsi1 flc* double mutant (Fig. 6B). To determine whether the *rsi1*-mediated SAR defect is linked to elevated *FLC* gene activity, SAR-conferred resistance to *P. syringae* pv. *maculicola* was monitored in the *flc* and *rsi1 flc* double mutants. Like the WT, the *flc* mutant was SAR competent (Fig. 6C). By contrast, the *rsi1 flc* double mutant retained the SAR-deficient phenotype of the *rsi1* single mutant (Fig. 6D; Supplementary Fig. S6). These results confirm that the *FLD*-mediated suppression of *FLC* expression is critical for the transition to flowering but not for *FLD*'s function during SAR.

DISCUSSION

We have shown that *FLD* is a critical player in SAR that is involved in signaling subsequent to the perception of the SAR

Fig. 3. Salicylic acid (SA) accumulation and response to SA application in the wild type (WT) and the *rsi1* mutant. **A**, Total SA (Free SA + SA-glucoside) content in the distal leaves of WT and *rsi1* plants 2 days after infiltration of *P. syringae* pv. *tomato* DC3000 carrying AvrRpt2 (Avr-Pst) (10^7 CFU/ml) (a) or 10 mM MgCl₂ (m) into the lower leaves. **B**, Free SA content in the distal leaves of WT and *rsi1* plants 2 days after infiltration of Avr-Pst (10^7 CFU/ml) (a) or 10 mM MgCl₂ (m) into the lower leaves. **C**, Effect of exogenous SA application on *Pseudomonas syringae* pv. *maculicola* population in WT and *rsi1* plants. Plants were either sprayed with 500 μM SA dissolved in water or as a control with water (w). One day later, leaves were infiltrated with *P. syringae* pv. *maculicola* (5×10^5 CFU/ml). *P. syringae* pv. *maculicola* numbers in these leaves were determined at 3 days postinfiltration. **A** and **B**, Each bar represent mean $\pm$ standard deviation (SD) of four samples, with each sample consisting 100 mg of freshly harvested leaf samples. **C**, Each bar represents the mean $\pm$ SD of four samples, each carrying five leaf discs of 5 mm in diameter from different leaves. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other as analyzed by one-way analysis of variance (post-hoc Holm-Sidak method). Both SA accumulation and SA response studies were repeated two times, with similar results.

signal. *FLD* expression was upregulated and *FLD* function was required for promoting disease resistance in the distal organs of plants which were previously inoculated on the lower leaves with a SAR-inducing microbe. Furthermore, Avr-Pex from WT leaves, as well as DA and Aza, were unable to systemically promote disease resistance in the *rsi1* mutant, which contains a missense mutation in *FLD*. By contrast, comparable with WT Avr-Pex, local application of *rsi1* Avr-Pex enhanced resistance against subsequent infections in the distal leaves of WT plants, thus confirming that the *rsi1* mutant is not deficient in the synthesis or accumulation of the mobile SAR signals. Our results further suggest that a long-distance signal is delivered and perceived by the distal tissues of *rsi1*, because *PR1* and *PDF1.2* transcript levels increased in the distal pathogen-free leaves of the *rsi1* mutant in response to inoculation of the lower leaves with Avr-Pst. However, perception of this SAR signal was not sufficient to systemically promote disease resistance in mutant plants lacking *FLD* function. Absence of *FLD* function in *rsi1* resulted in the attenuation of the SAR-associated systemic accumulation of SA and priming of the *PR1* gene for stronger induction when subsequently challenged with the pathogen. However, the *rsi1* mutation did not significantly impact SA accumulation in the Avr-Pst-treated leaves or basal resistance against virulent and avirulent pathogens, thus confirming that *FLD*'s involvement in defense is limited to SAR. Exogenously applied SA was an effective inducer of disease resistance in *rsi1*, therefore indicating that the *rsi1* mutant is responsive to SA. We suggest that *FLD* function in SAR is required subse-

quent to perception of the long-distance SAR signal and upstream of SA accumulation and priming of *PR1* genes in the pathogen-free distal leaves.

Increased accumulation of the *PR1* transcript has been extensively utilized as a molecular marker for the activation of SA signaling and SAR. However, despite the defect in systemic SA accumulation, *PR1* transcript accumulated at elevated levels in the pathogen-free distal leaves of the *rsi1* mutant that were inoculated on their lower leaves with Avr-Pst. Similar nonassociation between SAR-conferred enhanced disease resistance and systemic accumulation of *PR1* transcript have been reported for the *mpk3* and *hsfb1* mutants (Beckers et al. 2009; Pick et al. 2012). Thus, although a good marker for studying activation of the SAR process, accumulation of the *PR1* transcript in the distal tissue prior to secondary inoculation is not sufficient for SAR-conferred enhanced disease resistance. It is possible that a factor that negatively regulates *PR1* expression or promotes turnover of *PR1* transcript is lacking in the *rsi1* mutant. In addition to positive regulatory elements, the *PR1* promoter also contains negative regulatory elements which, when bound by the TGA2 transcription factor in association with NPR1, negatively regulate *PR1* expression (Despres et al. 2000; Kesarwani et al. 2007). Indeed, activation of SA signaling resulted in higher levels of *PR1* transcript in the *tga2* mutant than the WT plant (Rochon et al. 2006). However, despite the elevated *PR1* transcript accumulation, loss of *tga2* was not sufficient to increase disease resistance (Zhang et al. 2003). It has also been suggested that induced expression of

Fig. 4. Relative abundance of *PR1* transcript in wild-type (WT) and *rsi1* plants. **A**, Relative abundance of *PR1* transcript in the distal tissues of WT and *rsi1* plants 3 days after infiltration of lower leaves with *P. syringae* pv. *tomato* DC3000 carrying AvrRpt2 (Avr-Pst) (10^7 CFU/ml) (a) or 10 mM MgCl₂ (m). **B**, Relative abundance of *PDF1.2* transcript in the distal tissues of WT and *rsi1* plants 3 days after infiltration of lower leaves with Avr-Pst (10^7 CFU/ml) (a) or 10 mM MgCl₂ (m). **C**, Relative abundance of *PR1* transcript during systemic acquired resistance (SAR) exhibition in WT and *rsi1* plants. Plants were inoculated on their lower leaves (primary treatment) with Avr-Pst (10^7 CFU/ml) or 10 mM MgCl₂. Three days later, the distal leaves of these plants were challenged with *Pseudomonas syringae* pv. *maculicola* (5×10^5 CFU/ml) (secondary treatment). The *P. syringae* pv. *maculicola*-inoculated leaves were harvested 24 h later for monitoring gene expression by real-time polymerase chain reaction (PCR). Bars: mv, primary treatment = 10 mM MgCl₂ and secondary treatment = *P. syringae* pv. *maculicola*; av, primary treatment = Avr-Pst and secondary treatment = *P. syringae* pv. *maculicola*. In **A**, **B** and **C** real-time PCR was used to determine the abundance of the *PR1* and *PDF1.2* transcripts relative to the abundance of *ACTIN 2* transcript. Each bar represents the mean $\pm$ standard deviation of three biological samples with two technical replications of each. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other as determined by one-way analysis of variance (post-hoc Holm-Sidak method). **A** and **B**, Experiments were repeated two times by taking *rsi1* mutant, with similar results. **C**, Experiment was repeated two times with *rsi1* and one time with *fld-3* mutant, with similar results.

PR genes may not be sufficient for SAR because genes involved in protein folding, modification, and secretion also have to be coordinately upregulated for resistance (Wang et al. 2005).

FLD promotes flowering by downregulating expression of the flowering repressor *FLC* (Chou and Yang 1998; He et al. 2003; Kim and Sung 2012; Ruiqiang et al. 2005). Indeed, the delayed flowering phenotype of the *rsi1* mutant was suppressed in the *rsi1 flc* double mutant, thus confirming that *flc* is epistatic to *rsi1* during flowering. However, the SAR-deficient phenotype of *rsi1* was retained in the *rsi1 flc* double mutant, thus indicating that FLD's involvement in SAR is independent of its role in regulation of *FLC*. Interestingly, SA has also been shown to promote flowering in *Arabidopsis* in an *FLC*-independent manner (Martinez et al. 2004). A delayed flowering phenotype was displayed by *Arabidopsis* accession Col-0 plants expressing the *nahG*-encoded SA-degrading salicylate hydroxylase, and by the *ics1* (*sid2*) and *eds5* (*sid1*) mutants (Martinez et al. 2004). The

nahG-determined delayed flowering phenotype was retained in the *nahG flc* plants, thus suggesting that the effect of SA on promoting flowering was independent of *FLC* function (Martinez et al. 2004). In *Arabidopsis*, a few other defense-associated genes have been identified that influence flowering, as well. For example, flowering time is influenced by the *ENHANCED DOWNY MILDEW 2* (*EDM2*) gene, which is required for *RPP7*-mediated race-specific resistance to *Hyaloperonospora arabidopsidis* (Tsuchiya and Eulgem 2010). Flowering time was delayed in the *edm2* mutant compared with the WT plant (Tsuchiya and Eulgem 2010). In contrast, mutations in the *HOPW1-1-INTERACTING3* (*WIN3*) gene (also known as *PBS3*, *GDI1*, and *GH3.12*) that result in enhanced susceptibility to *P. syringae* and *Botrytis cinerea* and a modestly reduced SAR phenotype also result in early flowering under long-day conditions (Lee et al. 2007; Wang et al. 2011). *WIN3*, which encodes a protein in the firefly luciferase family, is required for promoting SA accumulation. However, whether the early flowering phenotype of the

Fig. 5. Systemic acquired resistance (SAR) defect of the *rsi1* mutant is due to its inability to respond to the SAR signal. **A**, Effect of avirulence–petiole exudate (Avr-Pex) on SAR induction in the wild type (WT) and *rsi1* plants. Leaves of WT and *rsi1* plants were treated with *P. syringae* pv. *tomato* DC3000 carrying AvrPpt2 (Avr-Pst) (10^7 CFU/ml) or as a mock-treatment with 10 mM MgCl₂. These Avr-Pst- and mock-treated leaves were detached and Pex collected from the cut ends of petioles. These Pex were infiltrated into lower leaves of naïve WT and *rsi1* plants. Two days later, the distal leaves were challenged with *Pseudomonas syringae* pv. *maculicola* (5×10^5 CFU/ml). *P. syringae* pv. *maculicola* numbers in these leaves were determined 3 days postinfiltration (dpi). The experiment was repeated two times with *rsi1* and two times with *fld-3* mutant, with similar results. **B**, Effect of azelaic acid (Aza) and dehydroabietinal (DA) on SAR in WT and *rsi1* plants. Three lower leaves of WT and *rsi1* plants were infiltrated with Aza (1 mM) dissolved in 5 mM morpholinoethanesulfonic acid (MES) buffer (pH 5.6) or DA (10 pM) dissolved in 0.1% dimethyl sulfoxide (DMSO) or Avr-Pst (10^7 CFU/ml) suspended in 10 mM MgCl₂. The control sets received 5 mM MES (pH 5.6), 0.1% DMSO, or 10 mM MgCl₂, respectively. After 2 days, the distal leaves were challenged with *P. syringae* pv. *maculicola* (5×10^5 CFU/ml). *P. syringae* pv. *maculicola* numbers in these leaves were determined 3 dpi. Each bar represents the mean $\pm$ standard deviation of four samples, each carrying five leaf discs of 5 mm in diameter. The experiment was repeated two times, with similar results. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other as determined by one-way analysis of variance (post-hoc Holm-Sidak method).

win3 mutant is related to its function in SA signaling is not known.

Recently, it was shown that SAR activation is accompanied by changes in chromatin at the promoter of some *WRKY* genes and priming of their expression by SA (Conrath 2011; Jaskiewicz et al. 2011; Luna et al. 2012). *FLD* is known to associate with histone demethylases and histone deacetylases to influence chromatin modification (He et al. 2003; Liu et al. 2007; Yu et al. 2011). Further work is needed to determine whether *FLD* is similarly involved in promoting chromatin alterations that accompany SAR.

MATERIALS AND METHODS

Plant growth conditions.

Plants were cultivated as previously described (Swain et al. 2011) with the following modifications. *Arabidopsis* was cultivated in a soil mixture containing soilrite and vermiculite (5:1) in a growth room that was set at 21 to 22°C and a regime of 12 h of light (80 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 12 h of darkness with 65% relative humidity, unless otherwise indicated. During transplantation and every 15 days thereafter, garden fertilizer mix (N-P-K, 20:20:20) was supplied along with irrigation water at 1 g/liter.

SAR and local defense assay.

SAR assays were performed as previously described (Maldonado et al. 2002; Nandi et al. 2004). In brief, overnight-

grown Avr-Pst was infiltrated in lower three leaves of 5-week-old plants at 10^7 CFU/ml. Plants that were simultaneously treated with 10 mM MgCl_2 were used as a mock control. Three days later, the distal leaves were challenged with *P. syringae* pv. *maculicola* (10^5 CFU/ml). *P. syringae* pv. *maculicola* counts were determined at 3 dpi by plating appropriate dilution of ground leaf extracts on media containing streptomycin. For determining the bacterial load in a given genotypic plant, four samples were prepared, with each sample containing five discs (5 mm in diameter) from different leaves. CFU numbers were directly used to determine the mean and standard deviations. To compare between the lines, the CFU values were $\log_{10}$ transformed and one-way analysis of variance was carried out by using Sigma-plot 11.0 software (post-hoc Holm-Sidak method). The local resistance studies were similar to that of SAR studies except that the plants were given no treatment prior to the pathogen inoculation.

Mutagenesis and generation of double mutants.

M_2 population of ethylmethanesulfonate-treated *Arabidopsis* accession Col-0 plants was screened for the inability to activate SAR, as described previously (Nandi et al. 2003). The *flc* mutant (Salk_003346) was obtained from the *Arabidopsis* Biological Resource Centre at the Ohio State University (Columbus, OH, U.S.A.). Presence of the T-DNA was confirmed by PCR, with primers (Supplementary Table S2) designed with the T-DNA primer-designing tool available from the Salk Insti-

Fig. 6. *FLC* function is required for the *rsi1*-conferred delayed flowering phenotype but not for systemic acquired resistance (SAR) deficiency. **A**, Relative abundance of the *FLC* transcript in leaves of the wild type (WT) and *rsi1* plants. Real-time polymerase chain reaction was used to determine the abundance of the *FLC* transcript relative to the transcript abundance for *ACTIN 2*. Each bar represents the mean $\pm$ standard deviation (SD) of three biological samples with two technical replications of each. **B**, Number of rosette leaves at the flowering stage in WT, *rsi1*, and *rsi1 flc* plants that were grown under a cycle of 12 h of light and 12 h of darkness. **C**, *Pseudomonas syringae* pv. *maculicola* numbers in WT, *rsi1*, and *flc* plants that were previously treated on their lower leaves with *P. syringae* pv. *tomato* DC3000 carrying AvrRpt2 (Avr-Pst) (10^7 CFU/ml) or 10 mM MgCl_2 . **D**, *P. syringae* pv. *maculicola* numbers in WT and *rsi1 flc* double-mutant plants that were previously treated on their lower leaves with Avr-Pst (10^7 CFU/ml) or 10 mM MgCl_2 . **C** and **D**, Challenge inoculation with *P. syringae* pv. *maculicola* was initiated by infiltrating the distal leaves with a suspension (5×10^5 CFU/ml) of *P. syringae* pv. *maculicola*. Bacterial numbers were determined at 3 days postinfiltration (dpi). Each bar represents the mean $\pm$ SD of four samples, each carrying five leaf discs of 5 mm in diameter. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other as analyzed by one-way analysis of variance (post-hoc Holm-Sidak method). Bars: mv, primary treatment = 10 mM MgCl_2 and secondary treatment = *P. syringae* pv. *maculicola*; av, primary treatment = Avr-Pst and secondary treatment = *P. syringae* pv. *maculicola*. Experiments were repeated two (A and B) or three (C and D) times, with similar results.

tute of Genome Analysis Laboratory. The *rsil* mutant was crossed with the *flc* mutant and a segregating F₂ population was screened for double mutants by PCR with primers that could distinguish each mutant allele from the corresponding WT allele. Out of a total of 16 F₂ plants tested, plants number 10 and 18 were double mutants because they showed only *rsil*-specific bands after *Hpa*II digestion, showed presence of T-DNA, and failed to generate WT *FLC*-specific bands, indicating that they were homozygous for the mutant *flc* allele (Supplementary Fig. S7). The *rsil flc* double mutant phenotype was further confirmed by monitoring the early flowering phenotype conferred by the *rsil* allele.

Collection of Pex and induction of SAR.

Pex were collected using a modified version of our published protocol (Chaturvedi et al. 2008). WT and *rsil* plants were infiltrated with *P. syringae* pv. *tomato* DC3000 carrying *AvrRpt2* (10⁷ CFU/ml) or 10 mM MgCl₂ (mock). Plants were kept in a growth room covered with a plastic dome for 6 h. The infiltrated leaves were cut at the base of the petioles with a sharp scissor and immediately surface sterilized by dipping in 50% ethanol and 0.0006% bleach for 10 s. The Pex were collected by dipping the cut end of the petioles in 1.5 ml of 1 mM EDTA (pH 8.0) solution contained in 24-well tissue culture plates. Five harvested leaves were placed in each well and a batch of 50 to 70 leaves was processed each time. After 24 h of collection, exudates were filtered with a 0.2-μm syringe filter (MDI, Ambala Cantt, India) and diluted twofold with sterile water. The collected Avr-Pex and, as a negative control, the Mock-Pex were infiltrated with a needleless-syringe into the lower three leaves of naive plants to induce SAR. Secondary challenge inoculations, when required, involved infiltration of the virulent pathogen *P. syringae* pv. *maculicola* ES4326.

SA estimation.

SA extraction from ground *Arabidopsis* leaves (100 mg) was carried out twice with 0.8 ml of 80% methanol and once with 0.8 ml of 100% methanol in 1.5-ml centrifuge tubes as previously described (Nandi et al. 2004). To the extracts, 20 μl of 0.2 M NaOH was added to minimize sublimation of SA during drying (Verberne et al. 2002). The extracts were dried under speed vac by keeping 30 to 40 μl of liquid in the tubes and resuspended in 500 μl of sodium-acetate buffer (0.1 M, pH 5.2) by vortexing thoroughly and by sonicating in the water bath sonicator for 16 min. The content was divided into two halves, one for free SA and the other for total SA. For estimating total SA, 50 μl of β-galactosidase (40-mg/ml stock prepared in 0.1 M NaOAc, pH 5.2; Sigma catalog number G0395) was added to each tube and incubated for 2 h at 37°C (Defraia et al. 2008). The free-SA tubes were left at room temperature without any treatment. Trichloroacetic acid (final concentration 5%) was added to all the tubes and precipitates were removed by centrifugation. SA was extracted by partitioning with a mixture of 0.8 ml of cyclohexane and ethylacetate (1:1) two times. To the extracts, 60 μl of mobile phase (0.2 M NaOAc [pH 5.2] and 10% methanol) was added and dried under speed vac by keeping 30 to 40 μl of liquid in the tubes (Verberne et al. 2002). The samples were dissolved in 200 μl of mobile phase and 100 μl was analyzed through high-performance liquid chromatography (HPLC Agilent 1220 LC) by passing over a 5-μm C18 reverse-phase column (4.6 by 150 mm) at the flow rate of 0.8 ml/min and detection by UV at 310 nm. For generating SA standard curve, pure SA (Sigma catalog number S7401) dissolved in methanol at different concentrations (three replicates for each concentration) was followed by the method described above.

Chemical treatment.

DA was prepared as a 10-pM solution in 0.1% dimethyl sulfoxide (Chaturvedi et al. 2012) and Aza was prepared as a 1-mM solution in 5 mM morpholinoethanesulfonic acid buffer, pH 6.5 (Jung et al. 2009). These solutions were infiltrated with a needleless syringe into three lower leaves to induce SAR. SAR assays were carried out as mentioned above.

mRNA expression analysis by real-time PCR.

Total RNA was isolated from leaf samples with a mix of guanidine-phenol-chloroform, as previously described (Chomczynski and Sacchi 1987). cDNA synthesis was carried out with Moloney murine leukemia virus reverse-transcriptase (MBI Fermentas, Amherst NY, U.S.A.). Relative quantity of mRNA for each gene was plotted as fold difference with *ACTIN2* (At3g18780). Real-time PCR was carried out by a 7500 Fast Real-Time PCR machine (Applied Biosystems, Foster City, CA, U.S.A.) using Power SYBR Green master mix (Applied Biosystems). All analyses were with the system software v2.0.5. Specificity of each primer set was validated by sequencing the amplified product. A nontemplate control was included in all real-time PCR reactions. The melting curve generated by the real-time software was followed to ensure the presence of a single product in each lane.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Arabidopsis Information Resource website: www.arabidopsis.org
Salk Institute of Genome Analysis website: signal.salk.edu