Arabidopsis FLOWERING LOCUS D influences systemic-acquired-resistance-induced expression and histone modifications of *WRKY* genes

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A plant that is in part infected by a pathogen is more resistant throughout its whole body to subsequent infections – a phenomenon known as systemic acquired resistance (SAR). Mobile signals are synthesized at the site of infection and distributed throughout the plant through vascular tissues. Mechanism of SAR development subsequent to reaching the mobile signal in the distal tissue is largely unknown. Recently we showed that *FLOWERING LOCUS D* (*FLD*) gene of *Arabidopsis thaliana* is required in the distal tissue to activate SAR. *FLD* codes for a homologue of human-lysine-specific histone demethylase. Here we show that FLD function is required for priming (SAR induced elevated expression during challenge inoculation) of *WRKY29* and *WRKY6* genes. FLD also differentially influences basal and SAR-induced expression of *WRKY38*, *WRKY65* and *WRKY53* genes. In addition, we also show that FLD partly localizes in nucleus and influences histone modifications at the promoters of *WRKY29* and *WRKY6* genes. The results altogether indicate to the possibility of FLD's involvement in epigenetic regulation of SAR.

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1. Introduction

Plants, when challenged with pathogens, induce resistance at the site of infection to control the spread of the pathogen. The cells of the infected tissues secrete antimicrobial phytoalexins and peptides to control growth of the pathogens (Spoel and Dong 2012). Certain secreted molecules by the infected cells are transported across the plant through vascular system and serve as mobile signal to activate systemic acquired resistance (SAR). Plants with induced SAR display higher level of resistance upon subsequent infections, compared to the naïve plants (Ross 1961; Sticher *et al.* 1997; Shah 2009). Transcriptional reprogramming plays a major role both in local resistance and SAR. Experiments suggest that mobile signals prime the SAR-induced plants to activate faster and elevated transcription of defence-related genes during subsequent infections (Fu and Dong 2013; Xin and He 2013).

The chemical nature of the mobile signals is still elusive and appears to be varied upon experimental materials and environmental conditions (Dempsey and Klessig 2012). Salicylic acid (SA) is transported through phloem subsequent to SAR induction in cucumber (Rasmussen *et al.* 1991). Mutants of the SA biosynthetic gene *ISOCHORISMATE SYNTHASE 1*(*ICS1*) and the SA signalling gene *NON-EXPRESSOR OF PR GENES 1* (*NPR1*) are defective in SAR (Cao *et al.* 1994; Wildermuth *et al.* 2001; Durrant and Dong 2004). Methyl salicylate (MeSA) esterase activity of SA BINDING PROTEIN 2 (SABP2) has been reported to be required for systemic signalling in tobacco upon *Tobacco Mosaic Virus* infection (Park *et al.* 2007). Homologues of SABP2 from *Arabidopsis* and potato are also reported to be required for SAR activation (Vlot *et al.* 2008; Manosalva *et al.* 2010). Analysis of vascular sap from the SAR-induced *Arabidopsis* plants identified other molecules such as azelaic acid, glycerol-3-phospahte, dehydroabietinal and pipecolic acid as potential

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SAR inducers (Nandi *et al.* 2004; Chaturvedi *et al.* 2008, 2012; Jung *et al.* 2009; Chanda *et al.* 2011; Navarova *et al.* 2012). Lipid metabolism is also associated with SAR activation. Mutants like *suppressor of fatty acid desaturase 1 (sfd1)*, *fatty acid desaturase 7* (*fad7*) and *monogalactosyldiacylglycerol synthase 1*(*mgd1*) affect plastidic lipid metabolism and are specifically defective in SAR activation (Nandi *et al.* 2004; Chaturvedi *et al.* 2008, 2012). The function of a putative lipid transfer protein coding gene *DEFECTIVE IN INDUCED RESISTANCE1(DIR1)* has been reported to be very crucial for activation of SAR in *Arabidopsis* (Maldonado *et al.* 2002) and has been proposed to be involved in making SAR signalling complex in vascular sap with other mobile signals (Jung *et al.* 2009; Chanda *et al.* 2011; Liu *et al.* 2011; Chaturvedi *et al.* 2012).

Despite having ample information on mobile signals, mechanism of development of infection memory subsequent to receiving the mobile signal in distal tissues is largely unknown. Recent studies, mostly with model plant *Arabidopsis*, indicate that epigenetic modifications contribute towards infection memory formation. Promoters of plant-specific WRKY class of transcription factors accumulate elevated level of modified histones that are normally associated with epigenetic control of gene expression (Jaskiewicz *et al.* 2011). It has also been reported that upon severe infections, plants can transmit such epigenetic modifications and provide SAR protection to the immediate next generations (Luna *et al.* 2012; Slaughter *et al.* 2012). WRKY transcription factors are important modulators of plant immune response (Eulgem and Somssich 2007; Pandey and Somssich 2009). While several WRKY proteins such as WRKY3, 4, 6, 29, 33, 52 and 70 function as positive regulator of immune response, WRKY7, 11, 17, 18, 23, 25, 27, 38, 40, 41, 48, 53, 58, 60 and 62 show negative regulatory roles (Robatzek and Somssich 2002; Pandey and Somssich 2009).

Recently, we reported that *REDUCED SYSTEMIC IMMUNITY 1* (*RSI1*, alias *FLOWERING LOCUS D*) is required for SAR development in *Arabidopsis* (Singh *et al.* 2013). Vascular sap from SAR-induced wild-type plants fails to activate SAR in the *rsi1* mutant, suggesting that FLD/RSI1 function is required in the distal tissue subsequent to receiving the mobile signal. In addition, the *rsi1* mutants are defective in SAR-induced SA accumulation in the distal tissue and priming of SA-inducible *PATHOGENESIS RELATED 1*(*PR1*) gene (Singh *et al.* 2013). FLD function has been associated with histone methylation and acetylation to control flowering time epigenetically (He *et al.* 2003; Liu *et al.* 2007; Yu *et al.* 2011). Here we show that RSI1/FLD influences basal and as well as SAR-induced priming of *WRKY* genes and histone modifications at their promoters.

2. Materials and methods

2.1 *Plant growth conditions and pathogen inoculation*

Arabidopsis plants were grown in controlled environment chamber at 21–22°C temperature, 65% relative humidity and 12–12 h light (80 μE m^{-1} s^{-1}) dark cycle as described previously (Swain *et al.* 2011). Bacterial pathogens were grown in liquid King's medium overnight and resuspended in 10 mM $MgCl_2$ at desired dilutions. The bacterial suspensions were pressure infiltrated in the abaxial leaf surfaces.

2.2 *mRNA expression analysis by RT-qPCR*

By using total RNA, cDNA synthesis was carried out with MMLV reverse-transcriptase (MBI Fermentas, USA). Relative abundance of mRNA for each gene was determined by reverse transcriptase quantitative PCR (RT-qPCR) (7500 Fast, Applied Biosystem, USA) and plotted as fold difference with *ACTIN 2* (At3g18780; Giri *et al.* 2014). Each experiment was repeated at least two times with similar results. Primers used in this study are mentioned in the supplementary table 1.

2.3 *ChIP assay*

Five-week-old, soil-grown *Arabidopsis* plants were inoculated with Avr-Pst, or as a negative control with 10 mM $MgCl_2$. Three days later, the distal uninoculated leaves were harvested and ChIP assay conducted as previously described (Saleh *et al.* 2008). Antibodies used for ChIP included anti-H3K4me2 (Cat # 16-157, Millipore, USA) and anti-H3K14Ac (Cat# 07-030, Millipore, USA). The relative quantity of precipitate for each target gene was determined by RT-qPCR and plotted as fold difference with *ACTIN 2* (At3g18780) promoter. Primers used in this study are mentioned in the supplementary table 1.

Each ChIP experiment was repeated twice with similar results.

2.4 *GFP-FLD localization*

For GFP-FLD fusion, the *FLD* coding sequence was amplified using end primers (supplementary table 1) and proofreading capable DNA polymerase Pfu (NEB, USA) and cloned between the *XcmI* sites of the vector pCXDG (Tsuchiya *et al.* 2010). Fleshy onion scales were co-cultivated with the *Agrobacterium* for 3 days and thoroughly washed with sterile water. The epidermal layer was peeled off using forceps and observed under fluorescence microscope (Nikon Eclipse TiE, Japan) and analysed with the system software NIS-elements-AR 4.00.00.

3. Results

3.1 *FLD/RSI1 influences expression of WRKY genes*

We examined the influence of RSI1/FLD in the systemic expression of several *WRKY* genes. Both WT and *rsi1* plants were SAR induced by infiltrating three lower leaves with *Pseudomonas syringae* pv *tomato* DC3000 carrying *AvrRpt2* gene (Avr-Pst) at 10^7 CFU/mL suspended in 10 mM $MgCl_2$. The control plants received only 10 mM $MgCl_2$. Accumulation of *WRKY* transcripts, especially the ones that are implicated in SAR, was determined at 3 days post inoculation (dpi) by RT-qPCR. The systemic expression of all the *WRKY* genes tested were significantly induced except for *WRKY6* in the WT plants (Figure 1 A to F). SAR-induced expression of *WRKY29* was abolished in the *rsi1* mutant (figure 1A). *WRKY6* expression was lower both in mock and SAR induced *rsi1* plants than WT plants (figure 1B). On the contrary, expression of *WRKY38* and *WRKY65* was higher in the *rsi1* than WT plants (figure 1C and D). The SAR-induced tissues of *rsi1* but not the mock tissues showed elevated expression of *WRKY53* gene (figure 1E). However, we did not observe any effect of *rsi1* mutation on the expression of *WRKY18* gene (figure 1F).

3.2 *SAR-induced priming of WRKY29 and WRKY6 is compromised in rsi1*

Expression of defence-related genes in SAR-induced plants are primed for higher expression during challenge inoculation compared to naïve plants. To investigate the possible role of RSI1 in priming of these *WRKY* genes, three lower leaves of WT and *rsi1* plants were inoculated with Avr-Pst

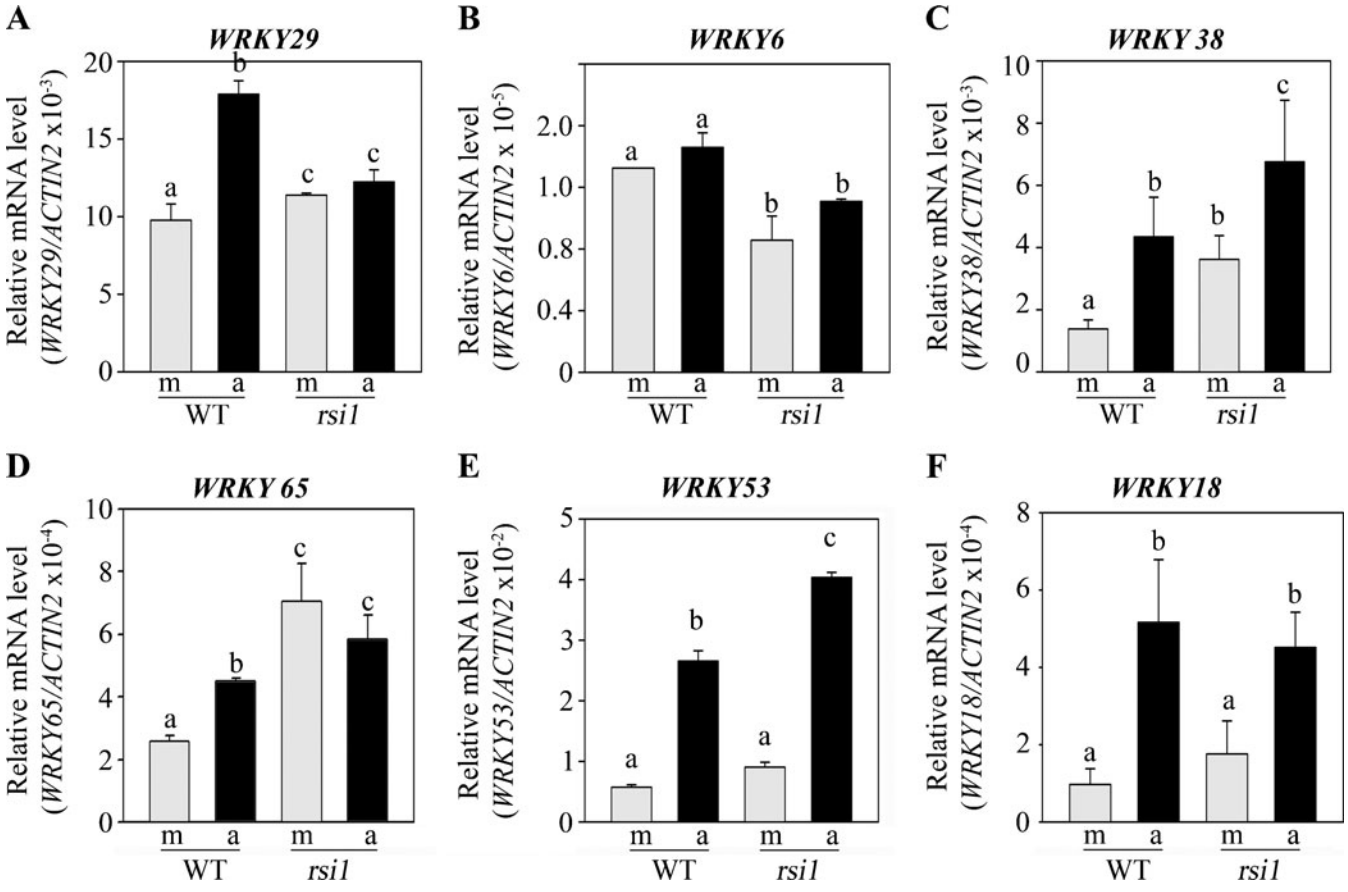

Figure 1. Expression of *WRKY* genes in the distal leaves of mock and SAR-induced WT and *rsi1* plants. Five-week-old soil grown plants were either SAR-induced by inoculating with Avr-Pst (a) at 10^7 CFU/mL or mock-induced with 10 mM $MgCl_2$ (m). After 3 days, distal systemic tissues were harvested and expression of *WRKY* genes (**A** to **F**, name of the *WRKY* genes are mentioned above each panel) were determined by RT-qPCR. Each bar represents the mean fold difference with *ACTIN2*±SD of 3 biological samples with two 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 ANOVA (Holm-Sidak method). The experiment was repeated 3 times with similar results.

or mock-inoculated with 10 mM $MgCl_2$. After 3 days all plants were inoculated with virulent pathogen *P. syringae* pv *maculicola* (Psm). After 4 h of second inoculation, expressions of *WRKY* genes were monitored by RT-qPCR. The WT plants but not the *rsi1* plants showed SAR-induced priming of both *WRKY29* and *WRKY6* genes (figure 2A and B)

3.3 *RSI1/FLD influences histone modification at the promoter of WRKY29 and WRKY6 genes*

Biochemical function of FLD is not known. Based on altered nucleosomal composition at *FLOWERING LOCUS C* gene and structural similarity with human-lysine-specific histone demethylase (LSD1) gene, FLD function has been predicted to reduce histone 3 acetylation and dimethylation (He *et al.* 2003; Liu *et al.* 2007; Yu *et al.* 2011). To know whether these predicted FLD functions are associated with observed differences in the expression of *WRKY* genes in *rsi1* mutant, we determined the accumulation of dimethyl H3K4 (H3K4me2) and acetylated histone 3 at lysine 14 (H3K14Ac) by chromatin immuno-precipitation (ChIP) using specific antibodies followed by RT-qPCR. WT plants showed enhanced level of H3K4me2 occupancy at *WRKY29* and *WRKY6* promoters in the distal tissues upon SAR induction (figure 3A and B). Distal tissues of both mock- and SAR-induced *rsi1* plants showed reduced H3K4me2 occupancy at *WRKY29* and *WRKY6* promoters when compared to corresponding tissues of WT plants (figure 3A and B). As a positive control of our experiment we monitored H3K4me2 accumulation at *FLC* locus. As expected, the *rsi1* plants showed higher level of H3K4me2 accumulation than WT plants at the *FLC* locus (figure 3C). H3K14Ac occupancy at *WRKY6* promoter of systemic tissue of *rsi1* was reduced when compared to that of wild-type plants, while it was comparable for *WRKY29* gene (figure 3D and E). Thus, the overall impact of RSI1/FLD on histone deacetylation was lower compared to that of histone demethylation on *WRKY* genes. As expected, H3K14Ac occupancy at FLC locus in *rsi1* was higher than WT plants, which served as positive control for this experiment (figure 3F).

3.4 *GFP-FLD is partly localized in the nucleus*

Even though FLD was known to influence histone modification and expression of the target genes, subcellular localization of FLD was not known. To visualize the subcellular localization, FLD was fused with the C terminal of green fluorescent protein (GFP) under *Cauliflower Mosaic Virus* 35S promoter and transiently expressed in onion epidermis cells. As a control we expressed free GFP under the same promoter. As expected, the free GFP was localized throughout the cytoplasm (figure 4). In contrast, the GFP-FLD was mostly localized in the nucleus and partly in cytoplasm (figure 4).

4. Discussion

Previously we had shown that FLD/RSI1 is required for development of SAR in *Arabidopsis* (Singh *et al.* 2013). Unlike WT plants, the *rsi1* mutants were defective in accumulating SA in the systemic tissue subsequent to localized inoculation with Avr-Pst. However, surprisingly, the

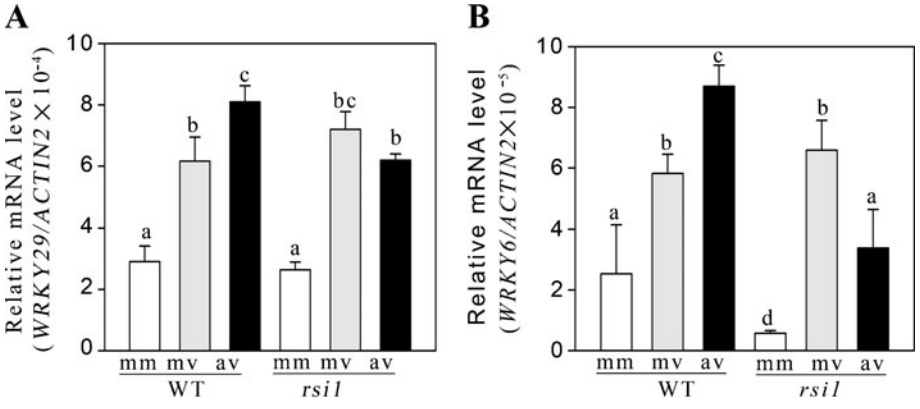

Figure 2. Priming induced expression of *WRKY29* and *WRKY6* in WT and *rsi1* plants. lower The three leaves of five-week-old plants were primary inoculated with either Avr-Pst at 10^7 CFU/mL or mock-inoculated with 10 mM $MgCl_2$. Three days later the upper leaves were secondary inoculated with virulent pathogen Psm or mock-inoculated with 10 mM $MgCl_2$. Samples were harvested 4 h post secondary inoculation and expression of *WRKY29* (**A**) *WRKY6* (**B**) was determined by RT-qPCR. Each bar represents the mean fold difference with *ACTIN2*±SD of 3 biological samples with two 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 ANOVA (Holm-Sidak method). mm – primary mock, secondary mock; mv – primary mock, secondary Psm; av – primary Avr-Pst, secondary Psm. Experiment was repeated 3 times with similar results.

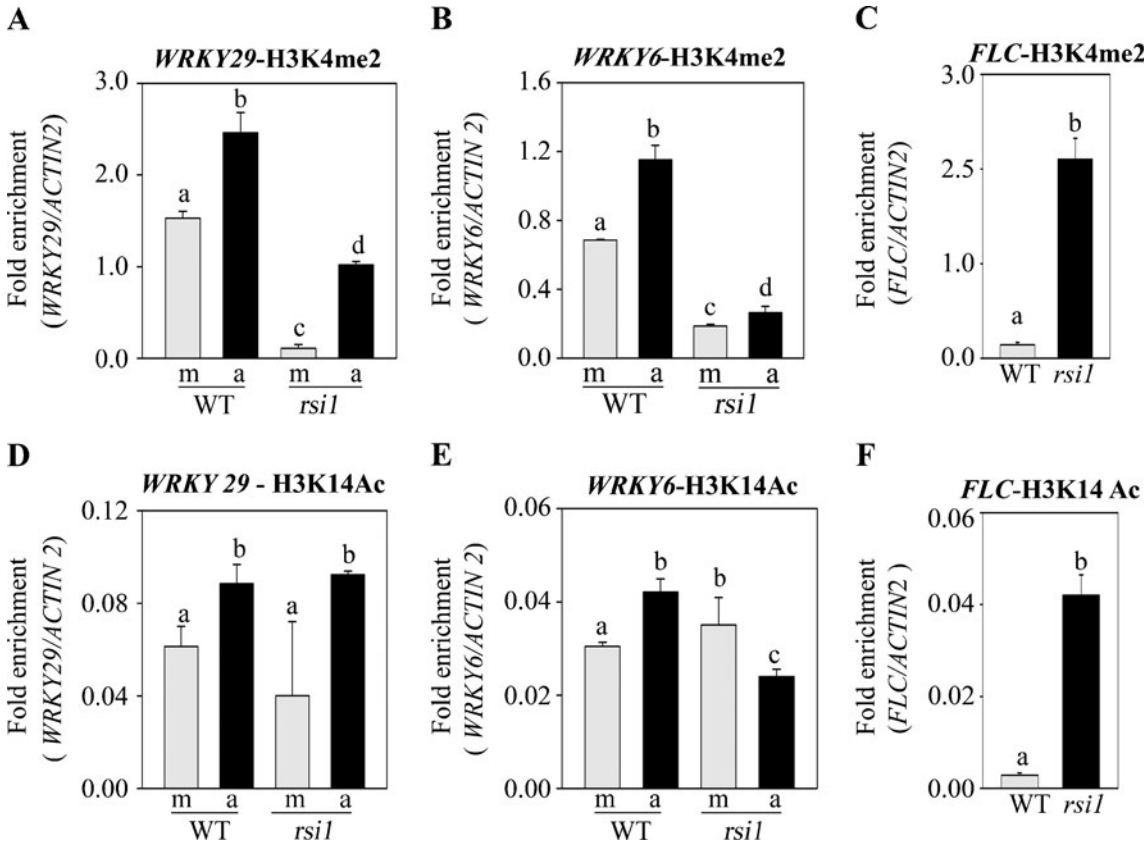

Figure 3. H3K4me2 and H3K14Ac occupancies at *WRKY*29 and *WRKY6* promoters. **A**, **B** and **C**, H3K4me2 occupancy at *WRKY29*, *WRKY6* and FLC promoters respectively; **D**, **E** and **F**, H3K14Ac occupancy at *WRKY29*, *WRKY6* and FLC promoters respectively Distal leaves of WT and *rsi1* plants that were treated on their lower leaves with Avr-Pst (10^7 CFU/mL) (a) or 10 mM $MgCl_2$ (m) were harvested 3 dpi. Chromatin isolated from these leaves was precipitated with anti-H3K4me2 or anti-H3K14Ac antibody. Relative abundance *WRKY* promoters in the precipitates were determined by RT-qPCR. Each bar represents the mean of fold difference with *ACTIN2*±SD of 3 biological replications with 2 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 ANOVA (Holm-Sidak method). The experiment was repeated twice with similar results.

expression of *PR1* gene prior to secondary inoculation was higher in *rsi1* mutant than WT plants (Singh *et al.* 2013). Thus the role of RSI1/FLD in the systemic tissues prior to secondary inoculation was ambiguous. Development of SAR is controlled at several levels such as synthesis of mobile signal(s), transport of the mobile signal(s) to the distal tissues and responding to the signal at the receivers end. All the mutants reported earlier except *rsi1* are defective in generation of mobile signal, because the vascular sap collected from the primary inoculated leaves of mutants such as *sfd1*, *mgd1*, *fad7,* etc., fail to induce SAR in the WT plants (Maldonado *et al.* 2002; Nandi *et al.* 2004; Chaturvedi *et al.* 2008). In contrast, the *rsi1* mutant is capable of generating the mobile signal (Singh *et al.* 2013). Our results on the expression pattern of *WRKY* genes reinstate that RSI1/FLD functions downstream to receiving mobile SAR signal in the systemic tissue. SAR induced enhanced expression of *WRKY38* and *WRKY53,* suggest that in *rsi1* plants, mobile SAR signal was generated and also transported to the distal tissues.

WRKY genes play important roles in SAR induction. Several *WRKY* genes show increased expression in the systemic tissues after SAR induction with biological or chemical agents, which is further enhanced upon secondary pathogen inoculation (Conrath 2011; Jaskiewicz *et al.* 2011). Our results show that SAR-induced priming of *WRKY6* and *WRKY29* genes depend on RSI1/FLD function. *WRKY6* and *WRKY29* are both important regulators of plant immune response (Asai *et al.* 2002; Bonardi *et al.* 2011; Jaskiewicz *et al.* 2011; Luna *et al.* 2012). *WRKY6* positively regulates expression of *PR1* and *NPR1* genes (Bonardi *et al.* 2011). Similarly, *WRKY29* over-expression constitutively activates defence in *Arabidopsis* (Asai *et al.* 2002). Thus, reduced expression of these *WRKY* genes especially during challenge inoculation

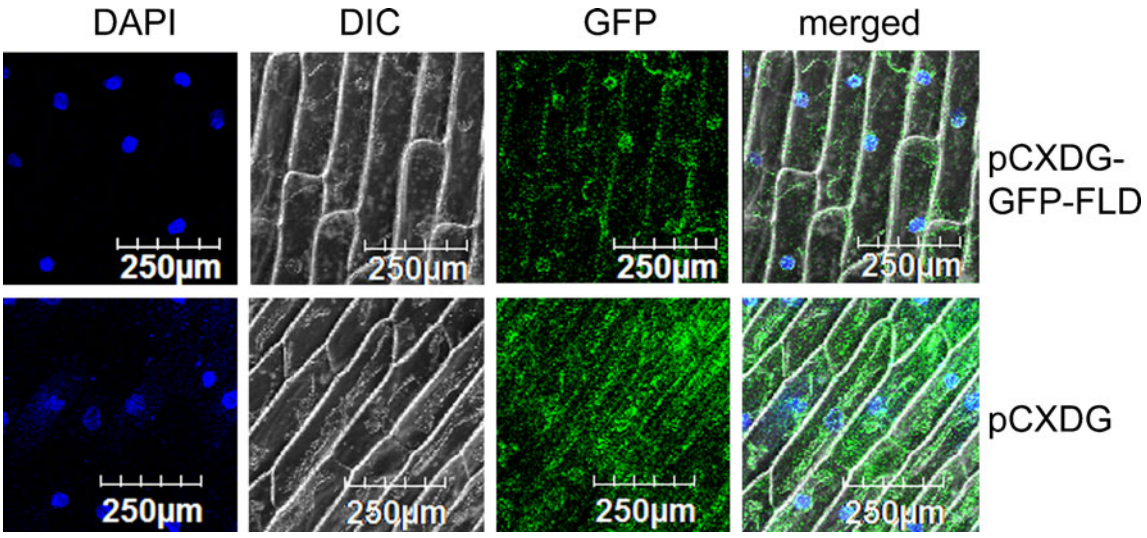

Figure 4. Subcellular localization of FLD-GFP in onion epidermal cells. Onion scales were transformed with pCXDG-GFP-FLD or the empty vector pCXDG by *A. tumefaciens*. The epidermal cells were observed under fluorescence microscope. DAPI – 4′,6-diamidino-2-phenylindole; DIC – differential interference contrast, GFP – green fluorescent protein. All the images were taken at the same magnification.

may explain the compromised SAR phenotype of *rsi1*. Our results show that FLD/RSI1 negatively regulates basal and SAR-induced expression of *WRKY38* and *WRKY65* and only SAR-induced expression of *WRKY53*. WRKY genes are known to regulate defence responses both positively and negatively and sometimes differentially against biotrophic and necrotrophic pathogens (Kim *et al.* 2006; Xu *et al.* 2006; Chen *et al.* 2012; Bhattacharjee *et al.* 2013). WKRY38 functions as a negative regulator of basal defence against bacterial pathogens (Kim *et al.* 2008). Expression of WRKY38 is induced upon pathogen or SA application in NPR1 dependent manner. While the WRKY38 over-expression lines are susceptible to PstDC3000, the mutant *wrky38* plants are resistant to it (Kim *et al.* 2008). The increased expression of *WRKY38* mRNA in *rsi1* mutant is in agreement with its negative regulations of basal defence. Further investigations are required to know whether WRKY38 or others such as WRKY65 and WRKY53 are targets of *RSI1/FLD* for activation of SAR.

Both H3K4me2 and H3K14Ac modifications are associated with actively transcribing genes (Liu *et al.* 2007; Yu *et al.* 2011).The observed reduced accumulation of these epigenetic marks in *rsi1*, especially for H3K4me2, is in agreement with the mRNA expression level of these two *WRKY* genes. FLD is known to influence histone methylation and acetylation (He *et al.* 2003; Liu *et al.* 2007; Yu *et al.* 2011). Studies on global accumulation of modified histones suggested that H3K4me2 is the major substrate of the FLD-dependent demethylase activity (Liu *et al.* 2007). Our results indicate that *FLD* influences chromatin modification at the promoters of *WRKY6* and *WRKY29* genes. A similar chromatin modification at *WRKY6* and *WRKY29* promoters after SAR induction was reported earlier (Jaskiewicz *et al.* 2011). However, unlike the documented function of the FLD-dependent activity in promoting histone demethylation at the *FLC* locus and thus limiting levels of methylated histones, FLD was required for promoting accumulation of H3K4me2 at the promoters of *WRKY6* and *WRKY29*. Thus, these *WRKY* gene promoters are unlikely to be the direct targets of the FLD-dependent demethylase activity. It is plausible that similar to its impact on negatively regulating activity of the *FLC* promoter during transition to flowering, increased FLD activity during SAR represses expression of an unknown demethylase that under normal circumstances, limits accumulation of H3K4me2 marks at the *WRKY6* and *WRKY29* promoters, thereby preventing inappropriate expression of these genes. In the *fld* mutant, increased expression of this demethylase would thus result in the inability of the SAR signals to promote priming of these *WRKY* genes. Alternatively, in response to the SAR signal, *FLD* could stimulate expression/activity of histone methylases that promote H3K4me2 marks at *WRKY6* and *WRKY29* promoters.

5. Conclusion

Upon receiving the SAR signal in the distal tissues, FLD indirectly modulates nucleosome composition of some of the

WRKY transcription factors, which in turn facilitate enhanced expression of these genes and provide higher level of resistance during secondary inoculations.

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