SRFR1 Function in Plant Immunity

Subjects: Plant Sciences

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SUPPRESSOR OF rps4-RLD1 (SRFR1) is known as a negative regulator by forming an immune complex with resistance proteins and transcription factors of the plant immune system in Arabidopsis. Mutations in *SRFR1*, identified in a suppressor screen, activated EDS1-dependent ETI to *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000. Besides, mutations in *SRFR1* boosted defense responses to the generalist chewing insect *Spodoptera exigua* and the sugar beet cyst nematode *Heterodera schachtii*. In the current study, we report that mutations in *SRFR1* enhance susceptibility to the fungal necrotrophs Fusarium *oxysporum f. sp. lycopersici* (*FOL*) and *Botrytis cinerea* in Arabidopsis. The *slsrfr1* tomato mutants generated by a CRISPR/Cas9 system increased expression of SA-pathway defense genes and enhanced resistance to *Pto* DC3000. In contrast, *slsrfr1* mutants elevate susceptibility to *FOL*. Together, these data suggest that SRFR1 is functionally conserved in both Arabidopsis and tomato and functions antagonistically as a negative regulator to biotrophic pathogens and a positive regulator to necrotrophic pathogens.

Keywords: SRFR1 ; CRISPR/Cas9 ; tomato ; fungal necrotrophs ; plant resistance

1. Introduction

Arabidopsis *SUPPRESSOR OF rps4-RLD 1* (*SRFR1*) was identified from a suppressor screen using wild-type RLD, an accession that possesses missense mutations in *RPS4* [1]. In Arabidopsis, mutations in *SRFR1* enhanced resistance to *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 expressing *avrRps4* or *hopA1* when the corresponding *R* genes, *RPS4* and *RPS6*, are mutated, respectively [2][3]. *srfr1* mutants were equally susceptible as wild-type RLD to virulent *Pto* DC3000. The mutant *srfr1* alleles were recessive, suggesting that, genetically, *SRFR1* functions as a negative regulator of ETI [4].

SRFR1 functions as an adaptor protein by forming protein complexes containing the defense regulator ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and resistance proteins of the TNL class, such as RPS4, RPS6, and SNC1 [5][6]. SRFR1 contains a tetratricopeptide repeat (TPR) domain that has sequence similarity to that of *Saccharomyces cerevisiae* Ssn6, which functions as a transcriptional repressor [1]. Transcript levels of defense-related genes are induced in *srfr1* mutants [2]. In addition, SRFR1 physically interacts with the immune cochaperone, suppressor of G2 allele of skp1 b (SGT1b) [5], and members of TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factor family [6]. These suggest that SRFR1 functions as an adaptor protein that negatively regulates ETI-associated transcriptional immune responses in Arabidopsis.

Additionally, Arabidopsis *srfr1* (*atsrfr1*) mutants showed enhanced resistance to the generalist chewing insect *Spodoptera exigua* and the sugar beet cyst nematode *Heterodera schachtii* [7]. This, together with increased ETI to *P. syringae*, raised the possibility that SRFR1 has a broader role in determining a set-point of plant innate immunity to a wide variety of biotic stresses. Overall, characterization of SRFR1 in crops may provide potential clues to improve protection against invading plant pathogens. In this regard, advanced techniques such as genome editing offer ways to precisely edit *SRFR1* and further characterization in both model plant and crop species.

Since *SRFR1* is a well-conserved single-copy gene in several crop plants, a functional study of *Solanum lycopersicum SRFR1* (*SlSRFR1*) can shed light on its role in the crop immune system. Since *atsrfr1* mutants display broad-spectrum resistance to (hemi-) biotrophic pathogens and a pest, we hypothesized that disruption of *SlSRFR1* in tomato would alter defense responses to biotic stresses. In the present work, we generated *SlSRFR1* alleles using the CRISPR/Cas9 system with two single guide RNAs (sgRNAs) that target 5′-end sequences of *SlSRFR1*. Our data reveal that mutations in *SlSRFR1* increased the expression of *PR* genes involved in salicylic acid (SA)-dependent defense signaling and suppressed the growth of the virulent bacterium *Pto* DC3000. Interestingly, mutations in *SRFR1* in both Arabidopsis and tomato enhanced susceptibility to the necrotrophic fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*) and *Botrytis cinerea* (*B. cinerea*). This study provides molecular insights into *SlSRFR1* function and paves the way to modulate this well-conserved gene to fine-tune plant immune responses in plants.

2. Enhanced Susceptibility to the Necrotrophic Fungal Pathogens in Arabidopsis *srfr1* Mutants

Arabidopsis *srfr1* mutants were mainly involved in EDS1-dependent ETI responses against *Pto* DC3000 [1][3][8] and in the resistance to chewing insect *S. exigua* and the sugar beet cyst nematode *H. schachtii*, which is an obligate biotrophic pathogen [7]. *FOL* has been reported to cause vascular wilt disease in Arabidopsis and tomato. In *FOL* - plant interactions, initial fungal infection occurs primarily in the roots, resulting in disruption of vascular tissues, chlorosis, and necrosis, which leads to plant death [9],[10]. The Arabidopsis wild-type accessions Col-0 and RLD, and *srfr1-1* and *srfr1-2*, two recessive alleles of *SRFR1* in the RLD background, were plug-inoculated with *FOL*. The wild-type RLD was more resistant to *FOL* than Col-0 (Figure 1a). Lesion size in Col-0 was three times larger than in RLD (Figure 1b), suggesting RLD is naturally resistant to *FOL*. Interestingly, the *srfr1-1* and *srfr1-2* mutants displayed enhanced susceptibility to *FOL* compared to RLD, as reflected by more severe symptoms, such as increased chlorosis (Figure 1a) and increased lesion size (Figure 1b).

B. cinerea is a necrotrophic fungal pathogen that destroys plant cells at the early stage of infection resulting in widespread tissue injury [11][12]. The wild-type RLD and *srfr1* mutants were drop-inoculated with *B. cinerea*. As shown in Figure 1c, the lesion areas were more extensive in *srfr1* mutants than that in RLD. The lesion area in *srfr1* mutants was twice larger than that in the wild-type (Figure 1d). These results demonstrate that mutations in *SRFR1* enhanced susceptibility to *FOL* and *B. cinerea*, indicating SRFR1 functions as a positive regulator of plant disease resistance against necrotrophic fungal pathogens.

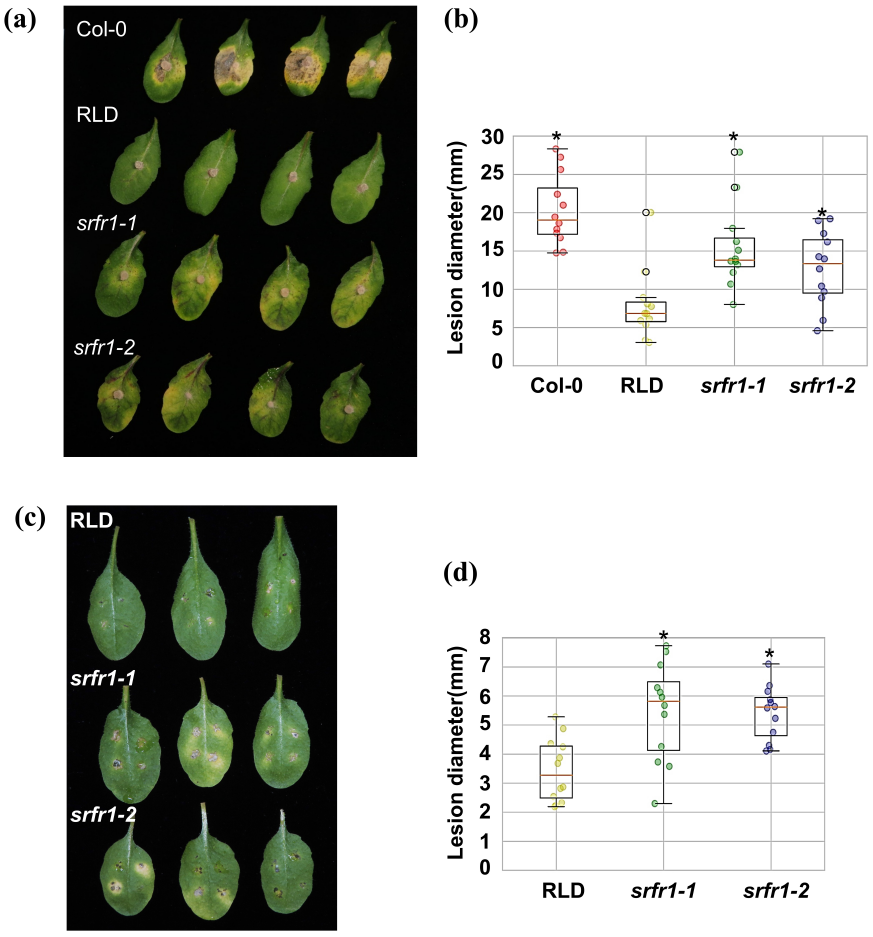

Figure 1. Response of Arabidopsis *srfr1* mutants to the necrotrophic fungal pathogens. (**a**) Infection with conidia and mycelial plugs on Arabidopsis RLD*, srfr1-1, and srfr1-2* mutants. Detached leaves from 4-week-old plants were inoculated with 4 mm-diameter plugs of *F. oxysproum*. Photographs were taken at 14 DPI; (**b**) Box plots of lesion size at 14 DPI in Col-0, RLD*, srfr1-1*, and *srfr1-2.* The y-axis displays the measured diameter of disease lesion (mm, n=12) in each plant. The box ranges were determined from the twenty-fifth to the seventy-fifth percentiles (*P<0.05*).; (**c**) Fungal spores of *B. cinerea* grown on PDA were harvested and inoculated onto detached leaves of Arabidopsis RLD*, srfr1-1, and srfr1-2* mutants at a concentration of 1 X 10^8 spores/ml. Photographs were taken at 5 DPI; (**d**) Box plots of lesion size at 5 DPI in RLD*, srfr1-1*, and *srfr1-2.* The y-axis displays the measured diameter of disease lesions (mm, n=12) in each plant. The box ranges were determined from the twenty-fifth to the seventy-fifth percentiles (*P<0.01*).

3. Altered Morphology and Expression of Defense Marker Genes in CRISPR/Cas9-Edited *slsrfr1* Plants

In Arabidopsis, RLD *srfr1-1* and *srfr1-2* mutants exhibit normal and wild-type-like morphology, although there is a slight decrease in growth. However, as shown in *srfr1-4*, a mutation in *SRFR1* in Col-0 leads to extreme stunting and abnormal growth because of the constitutive activation of the Col-0-specific resistance gene *SNC1* [13]. *G1-slsrfr1* plants showed weak growth reduction, not severe stunting, reminiscent of RLD *srfr1* phenotype (Figure 2a). As shown in Figure 2b, like Arabidopsis *srfr1* mutants [2], *SlPR1* and *SlPR2* expression were significantly increased in two independent *G1-slsrfr1* mutants, *slsrfr1-1* and *slsrfr1-2*, compared to wild-type M82. Consistent with this, SlPR1 protein was strongly accumulated in *G1-slsrfr1-1* lines (Figure 2c). These suggest that CRISPR/Cas9-mediated mutations in *SlSRFR1* upregulate the expression level of SA-dependent defense markers both transcriptionally and translationally. In addition, consistent with *atsrfr1, TomloxD*, a JA signaling marker gene, was induced in *slsrfr1* mutants (Figure 2b) [7], suggesting both SA- and JA-dependent defense is upregulated in untreated CRISPR/Cas9-edited *slsrfr1* lines.

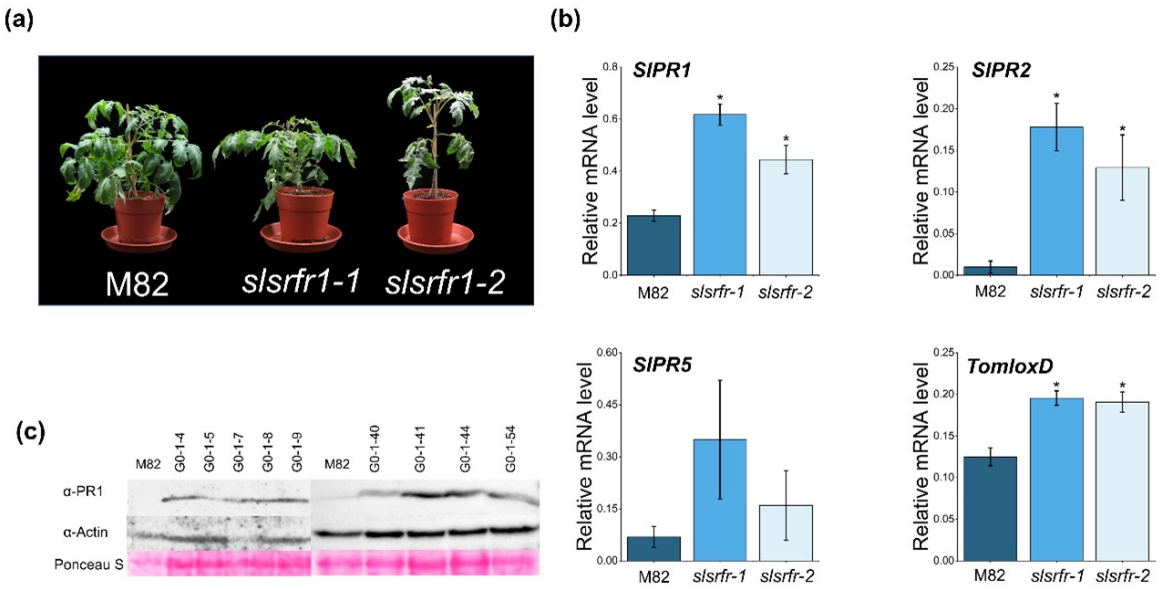

Figure 2. Altered morphology and expression of defense marker genes in CRISPR/Cas9-edited *slsrfr1* plants. (**a**) Growth phenotype of 6-week-old M82 and *slsrfr1* lines grown in 16 h light/8 h dark long-day photoperiod; (**b**) Relative mRNA expression of defense-related genes. The genes used for gene expression analysis refer to *SlPR1 (Solyc09g007010.1), SlPR2 (Solyc01g008620.2), SlPR5(Solyc08g080640), and TomloxD (Solyc03g122340.2).* Gene expression levels of each gene were normalized with *SlACT (Solyc04g011500.3.1)* as an internal control. Error bars represent standard deviation. A statistically significant difference was determined by the Student's t-test *(P<0.01*). This experiment was repeated twice with similar results; (**c**) Proteins were extracted from 6-week-old *slsrfr1-1* G1 plants and were detected by western blot using α-PR1 and α-Actin (an internal control).

4. Enhanced Resistance to *Pto* DC3000 in CRISPR/Cas9-Edited *slsrfr1* Plants

The absence of increased resistance to virulent *Pto* DC3000 is observed in Arabidopsis *srfr1* mutants, even though defense-related genes are constitutively upregulated [2][13]. Both CRISPR/Cas9-Edited *slsrfr1-1* and *slsrfr1-2* leaves showed enhanced resistance to *Pto* DC3000 in contrast to wild-type M82. Disease symptoms in M82, such as chlorosis and water-soaked lesions, were dramatically reduced in *G1- slsrfr1* lines (Figure 3a). Consistent with the visible symptoms*, slsrfr1-1* and *slsrfr1-2* showed approximately 100-fold lower *Pto* DC3000 growth than wild-type M82 (Figure 3b). These results suggest that mutations in *SlSRFR1* increased SA-pathway defense genes, leading to enhanced resistance against the (hemi-)biotrophic pathogen *Pto* DC3000.

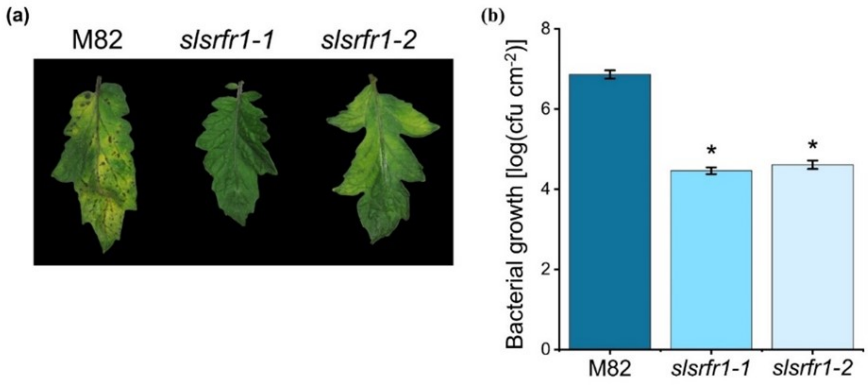

Figure 3. Response of *slsrfr1* lines to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. (**a**) Disease symptoms of parental M82 (left), *slsrfr1-1* (middle), and *slsrfr1-2* (right) dip-inoculated with *Pto* DC3000. Disease symptoms (leaf chlorosis) were recorded at 5 DPI. Only the fourth trifoliate leaflet of the plants was inoculated; (b) *In planta* bacterial growth was measured in indicated plant lines on day 5 after inoculation with *Pto* DC3000 at a density of 2 × 10^8 cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from 4 replicas, and error bars denote standard deviation. Asterisks indicate that the growth of DC3000 was significantly different between M82 and *slsrfr1* mutants as determined by a two-tailed Student's *t*-test (* $P < 0.01$). This experiment was repeated twice with similar results.

5. Enhanced Susceptibility to *Fusarium oxysporum* f. sp. *lycopersici* in CRISPR/Cas9-Edited *slsrfr1* Plants

As shown in Figure 1, the Arabidopsis RLD *srfr1* mutants display enhanced susceptibility against *FOL* and *B. cinerea*. To test the functional conservation of SRFR1 in tomato, we analyzed the resistance response of *G1-slsrfr1* lines after inoculation with the necrotrophic fungal pathogen *FOL*. Three days after plug-inoculation with *FOL*, all *slsrfr1* mutants, *slsrfr1-1, slsrfr1-2, slsrfr1-3,* and *slsrfr1-4*, displayed enhanced susceptibility compared to wild-type M82, as indicated by severe necrosis and enlarged lesion area (Figure 4a and 4c). Trypan blue staining of the infected leaves showed that extensive development of fungal hyphae was observed in *slsrfr1-1* and *slsrfr1-2* with expanding lesion areas (Figure 8b). These results demonstrate that SlSRFR1 positively regulates the immune response against the necrotrophic pathogen *FOL,* and that SRFR1 function is conserved between Arabidopsis and tomato.

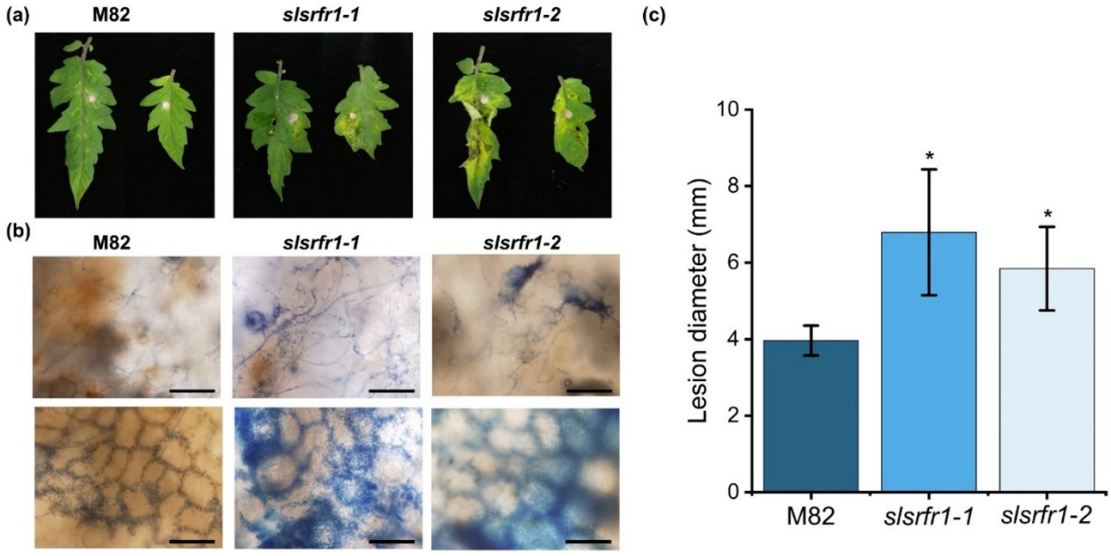

Figure 4. Plant response to *Fusarium oxysporum* f. sp. *lycopersici* in *slsrfr1* lines. Plant response was analyzed at 3 days after plug inoculation of *FOL* in *slsrfr1* lines. (**a**) Detached leaves from **6**-week-old M82, *slsrfr1-1,* and *slsrfr1-2* were inoculated with 4 mm-diameter plugs of *F. oxysproum*. Photographs were taken at **3** DPI; (**b**) Trypan blue staining in *FOL*-inoculated *slsrfr1* lines. The bar represents 200 µm in the upper layer and 100 µm in the lower layer; (**c**) Lesion size at 3 DPI in *slsrfr1* lines. Plant response of *slsrfr1-1* and *slsrfr1-2* against *FOL* was repeated three times and once, respectively, with similar results. A statistically significant difference was determined by the Student's t-test *(P<0.01*).

6. Current Insights

Plant resistance responses against a biotrophic pathogen are generally mediated by induction of the SA-signaling pathway and are partly due to the hypersensitive response. On the other hand, resistance to necrotrophic pathogens is dependent on the JA and/or the ethylene signaling pathway, suggesting the opposing mechanism of plant immune responses to two different pathogens with distinct attack strategies [14]. To explore the role of SRFR1 in responses to necrotrophic fungal pathogens, we performed pathogenesis assays in wild-type RLD, *atsrfr1-1,* and *atsrfr1-2* by inoculating with *FOL* and *B. cinerea*. The *atsrfr1* mutants showed enhanced susceptibility with increased necrosis, enhanced development of fungal hyphae, and expanded lesion areas compared to wild-type in response to invading necrotrophic fungal pathogens. The transcripts of TNL type *R* genes and *PR* genes were upregulated in the *atsrfr1* mutants [1][2], suggesting boosted SA-pathway defense genes in *atsrfr1* might suppress the plant defense against a necrotrophic fungal pathogen. In addition, it was shown previously that *atsrfr1-1* mutants display downregulation of the JA/ethylene response pathway genes *ORA59* and *PDF1.2* in response to the chewing insect *S. exigua*, whereas, at resting state, these genes were upregulated in *atsrfr1-1*. At the same time, *atsrfr1-1* showed upregulation of the JA pathway gene *VSP2* upon induction by *S. exigua*. This correlated with increased resistance of *atsrfr1-1* to insect herbivory. The increased mRNA levels of JA/ethylene genes in uninduced *slsrfr1* mutants and the increased susceptibility of Arabidopsis and tomato *srfr1* mutants to necrotrophic pathogens reported here are consistent with these earlier studies and highlight the conservation of *SRFR1* functions on multiple levels.

Besides, in Arabidopsis, there are several well-studied mutants that show enhanced SA defense signaling and suppressed JA defense signaling [15]. For instance, a mutation in *BOTRYTIS-INDUCED KINASE1* (*BIK1*) exhibited elevated SA accumulation and SA-pathway defense genes and attenuated expression of *PDF1.2a*, leading to increased resistance to *Pto* DC3000 and enhanced susceptibility to *B. cinerea* [16]. Loss-of-function in *BOTRYTIS SUSCEPTIBLE 1 (BOS1)* resulted in growth inhibition of *Pto* DC3000 but increased susceptibility to the necrotrophic pathogens *B. cinerea* and *Alternaria brassicicola* [17]. Furthermore, a *wrky33* mutant displayed boosted susceptibility to *B. cinerea* compared to wild-type with increased levels of SA-pathway genes and SA accumulation [18]. In tomato, pre-treatment of SA enhanced the development of a necrotrophic pathogen, *Alternaria solani* [19]. Moreover, Hernández-Aparicio and colleagues showed that a susceptible tomato cultivar displayed elevated SA levels in untreated plants and increased expression of *PR1* and of the ethylene signaling gene *1-aminocyclopropane-1-carboxylate synthase 2* (*ACS2*) in response to *FOL* compared with a resistant tomato cultivar [20]. Consistent with these, our findings indicate that the expression level of *SlPR1, SlPR2,* and *SlPR5* in the SA response pathway was upregulated, and resistance response to *Pto* DC3000 was enhanced in the CRISPR/Cas9-induced *slsrfr1* mutants. Conversely, susceptibility of *slsrfr1* and *atsrfr1* mutants was promoted in response to necrotrophic fungal pathogens. The antagonistic role of SRFR1 to pathogens with different attack strategies allows us to set up a new model for SRFR1; it functions as a negative regulator to (hemi-) biotrophic pathogens and a positive regulator to necrotrophic pathogens in plant disease resistance, and its function is possibly conserved in most crop plants.

Indeed, the data indicate that the function of SRFR1 in tomato and Arabidopsis is evolutionarily conserved. Interestingly, Arabidopsis SRFR1 was reported to associate physically with TCP transcription factors and interacts with the TOPLESS family genetically, which likely allows SRFR1 to function as a negative regulator in plant immunity [6][21]. These findings combined with our functional analysis of tomato SRFR1 suggest that SlSRFR1 potentially interacts with some transcription factors acting as positive regulators of tomato resistance against (hemi-) biotrophic pathogens to sequester them away from defense gene promoters. To further validate the role of SlSRFR1 in SA-based defense, determining the concentrations of endogenous SA in *slsrfr1* mutants is of particular interest. On the other hand, based on the positive function of SRFR1 in the JA signaling pathway, we suggest that SRFR1 may coordinate with JA-responsive proteins. It would be fascinating to identify whether SRFR1 interacts with JA biosynthesis enzymes and transcriptional regulators inducing JA-responsive genes. To further investigate the role of SRFR1 in JA-based defense, measurements of the expression of JA-responsive genes and endogenous JA concentration are required.

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