Global SUMOylome Adjustments in Basal Defenses of Arabidopsis thaliana Involve Complex Interplay Between SMALL-UBIQUITIN LIKE MODIFIERs and the Negative Immune Regulator SUPPRESSOR OF rps4-RLD1

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Steady-state SUMOylome of a plant is adjusted locally during developmental transitions and more globally during stress exposures. We recently reported that basal immunity in Arabidopsis thaliana against Pseudomonas syringae pv tomato strain DC3000 (PstDC3000) is associated with strong enhancements in the net SUMOylome. Transcriptional upregulations of SUMO conjugases, suppression of protease, and increased SUMO translations accounted for this enhanced SUMOylation. Antagonistic roles of SUMO1/2 and SUMO3 isoforms further fine-tuned the SUMOylome adjustments, thus impacting defense amplitudes and immune outcomes. Loss of function of SUPPRESSOR OF rps4-RLD1 (SRFR1), a previously reported negative regulator of basal defenses, also caused constitutive increments in global SUMO-conjugates through similar modes. These suggest that SRFR1 plays a pivotal role in maintenance of SUMOylation homeostasis and its dynamic changes during immune elicitations. Here, we demonstrate that SRFR1 degradation kinetically precedes and likely provides the salicylic acid (SA) elevations necessary for the SUMOylome increments in basal defenses. We show that SRFR1 not only is a SUMOylation substrate but also interacts in planta with both SUMO1 and SUMO3. In sum1 or sum3 mutants, SRFR1 stabilities are reduced albeit by different modes. Whereas a srfr1 sum1 combination is lethal, the srfr1 sum3 plants retain developmental defects and enhanced immunity of the srfr1 parent. Together with increasing evidence of SUMOs self-regulating biochemical efficiencies of SUMOylation-machinery, we present their impositions on SRFR1 expression that in turn counter-modulates the SUMOylome. Overall, our investigations reveal multifaceted dynamics of regulated SUMOylome changes via SRFR1 in defense-developmental balance.

Keywords: SUMOylation, SUMO isoforms, SRFR1, basal defenses, PstDC3000
INTRODUCTION

In higher eukaryotes, pivotal roles of post-translational modifications (PTMs) balance protein expressions, activities, interacting partners, localization, and proteostasis (Walsh et al., 2005; Millar et al., 2019). With structural similarities to ubiquitin, the SMALL UBIQUITIN-LIKE MODIFIERS (SUMOs) covalently attach to lysine (K) residues of substrates through a cascade of enzymatic reactions biochemically similar to ubiquitination cycles and termed as SUMOylation (Geiss-Friedlander and Melchior, 2007; Flotho and Melchior, 2013; Zhao, 2018). The partially conserved motif, ψ−K−X−D/E (ψ = hydrophobic amino acid, X = any amino acid, and D/E = aspartate/glutamate), most often harbors the SUMOylated lysine residue. SUMOs can also facilitate non-covalent protein–protein associations requiring the essential presence of hydrophobic core-containing SUMO-interaction motifs (SIMs), K−X−3−[V/I]−[I/L]−X−3−[D/E/Q/N]−[D/E]₂, in the cognate recipient (Hannich et al., 2005). Computational studies identify SUMOylation-annotated candidates as strategic central relay players of protein–protein interaction webs, and with noted predominance of DNA-modification enzymes and transcription factors (TFs), transcriptional processes are especially modulated (Duan and Walther, 2015).

Maintenance of SUMOylome homeostasis necessitates tight coordination of SUMO-conjugation/deconjugation cycles (Kurepa et al., 2003; Morrell and Sadanandom, 2019). SUMOylation requires the availability of processed SUMOs, with exposed diglycine (GG) residues at their C-terminus. SUMO proteases generate these conjugation-proficient SUMOs from precursors that contain extended C-terminus residues. The mature SUMOs through an ATP-dependent AMP–SUMO intermediate formation is linked via thiol–ester bond to the catalytic cysteine of SUMO E1 ACTIVATING ENZYME (SAE), a heterodimer of two subunits SAE1 and SAE2. SUMO E2 CONJUGATING ENZYME (SCF) then acquires the SUMO moiety from SAE via trans-esterification. Although SCE is capable of direct SUMOylation, its binding to SUMO E3 ligases, which also simultaneously interact with targeted substrates, augments the process and imparts specificity (Gareau and Lima, 2010). SUMOs are also self-SUMOylated at internal lysines forming poly-SUMO chains. These are catalyzed by SUMO E4 ligases that belong to the PIAL (protein inhibitor of activated stat-like) class of proteins (Tomanov et al., 2014). Some SUMO proteases remove SUMOs from SUMOylated substrates or disintegrate polySUMO chains, thus recycling free SUMOs (Chosed et al., 2006; Colby et al., 2006; Morrell and Sadanandom, 2019).

Insights from studies on the model plant Arabidopsis thaliana have provided vital clues into the complexity of SUMOylation and its genetic link to diverse cellular processes. Arabidopsis SUMO-machineries are mostly encoded by multiple genes showing tissue/stage-dependent, stress-inducible expressions and interaction/modification specificities with substrates. Although A. thaliana genome encodes eight SUMO isoforms, only four (SUM1, -2, -3, and -5) are expressed (van den Burg et al., 2010). SUMO1 and -2 proteins share considerable sequence identity (94%) and, in several instances, are functionally redundant or additive and surprisingly in some responses contrasting (Saracco et al., 2007; van den Burg et al., 2016; Castano-Miquel et al., 2011; Ingole et al., 2021b). SUMO3 and -5 proteins are more diverged with less than 50% identity to SUMO1. Simultaneous loss of SUM1 and SUM2 is embryonic lethal, indicating that plants require at least one functional copy of either of these isoforms. In plants, SUMO1/2 predominantly occur as free non-conjugated forms, which upon exposure to heat, peroxide, or ethanol stress are rapidly utilized for SUMOylating target proteins (Kurepa et al., 2003; van den Burg et al., 2010). The Arabidopsis sum3 mutant is viable with mild late-flowering phenotype (van den Burg et al., 2010). SUMO3, unlike SUMO1/2, cannot form poly-SUMO chains in vitro, is barely detected in plant extracts, and shows little or no change to heat shock treatments (Kurepa et al., 2003; Chosed et al., 2006; Colby et al., 2006; Budhiraja et al., 2009; van den Burg et al., 2010). SUMO1/2 but not SUMO3-modified targets are efficiently deconjugated by ULPs (Chosed et al., 2006; Colby et al., 2006). Lastly, SUMO1 and SUMO3 reciprocally influence each other’s conjugation efficiencies in vitro, implying functional cooperativity (Ingole et al., 2021b). HIGH PLOIDY 2/METHYL METHANE SULFONATE 21 (HPY2/MMS21) and SAP and MIZ 1 (SIZ1) remain the two well-characterized SUMO E3 ligases in Arabidopsis. Multiple Arabidopsis SUMO proteases have been identified till date and include EARLY IN SHORT DAYS 4 (ESD4), its closest homologs ELS1/2 (ESD4-LIKE SUMO PROTEASE1/2), OVERLY TOLERANT TO SALT 1/2 (OTS1/2), SUMO-PROTEASES RELATED TO FERTILITY 1/2 (SPF1/2), and DE-SUMOYLATING ISOPEPTIDASES (DeSIs), among others (Kurepa et al., 2003; Murtas et al., 2003; Chosed et al., 2006; Colby et al., 2006; Kong et al., 2017; Orosa et al., 2018). However, only a fraction of these remain functionally characterized (Morrell and Sadanandom, 2019; Srivastava et al., 2021).

In broadly understood layers of plant defenses, conserved molecular signatures or pathogen-associated molecular patterns (PAMPs) present on microbes are sensed by extracellular transmembrane pattern-recognition receptors (PRRs) to transduce downstream induction of defense-associated genes (Jones and Dangl, 2006; Bentham et al., 2020). This route of immune signaling comprise the PAMP-triggered immunity (PTI). Intracellular perception of a pathogen attack is performed by strategically deployed resistance (R) proteins that directly or indirectly sense manipulations by the invader-secreted effectors. Defense responses elicited downstream of these perceptions are termed as effector-triggered immunity (ETI) and include heightened production of defensive hormone salicylic acid (SA), and prolonged and aggravated expression of PTI-responsive markers such as PATHOGENESIS-RELATED PROTEINS (PRs) and SA-biosynthesis SALICYLIC ACID-DEFICIENT 2/ISOCHORISMATE SYNTHASE 1 (SID2/ICS1) gene, among others. Basal and ETI mediated by the Toll–interleukin1 (TNL)-type of R proteins require ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) to potentiate SA-based defenses (Aarts et al., 1998; Falk et al., 1999).

Disturbances in SUMO pathway affect immune responses often with developmental costs to the host (Lee et al., 2007; van den Burg et al., 2010; Bailey et al., 2016; Orosa et al., 2018;...
Verma et al., 2018). Studies overall suggest that SUMO1/2 suppresses whereas SUMO3 potentiates immunity primarily through modulation of SA-signaling networks (van den Burg et al., 2010; Saleh et al., 2015; Ingole et al., 2021b). However, adjustments of a host SUMOylome in response to pathogen attack or in SUMOylation-perturbed mutants present a more complicated involvement not only of SUMO isoforms but also of SUMO-machineries. For example, globally reduced SUMOylome in siz1-2 or enhanced SUMO1/2-conjugates in esd4-2 or ots1 ots2 both lead to elevated SA levels with constitutive activation of defenses (Villajuanà-Bonequi et al., 2014; Bailey et al., 2016). ESD4 and SIZ1 are SUMOylated and non-covalently bind SUMOs, implying that their activities are self-regulated by SUMOylation (Miller et al., 2010; Mazur et al., 2017). Our studies recently identified both positive and negative immune regulators as differentially SUMOylated candidates upon a pathogen attack (Ingole et al., 2021a). Further, we also revealed that SUMO-conjugation efficiencies are affected by the crosstalk between SUMO isoforms independent of their covalent-modification activities. Taken together, how a plant maintains SUMOylome

Salicylic Acid Measurements
Free and total SA (SA + glucose-conjugated, SAG) measurements were performed according to Defraia et al. (2008) using the Acinetobacter sp. ADPWHLux biosensor system. Briefly, 100 mg of frozen tissue was homogenized in 250 µl of acetate buffer (0.1 M, pH 5.6) and clarified by centrifugation at 12,000 rpm for 15 min to remove cell debris. A 100 µl of the supernatant was kept for measuring free SA, while a similar volume was treated with 6 U of β-glucosidase (Sigma-Aldrich, St. Louis, MO, United States) for 90 min at 37°C for total SA determination. A 20 µl aliquot of plant extract was mixed with 50 µl of Acinetobacter suspension (grown to OD600 = 0.4) along with 60 µl of fresh Luria–Bertani (LB) broth. Standard curve was generated with sid2-1 tissue extracts spiked with known amounts of SA (Sigma-Aldrich, United States). After incubation for 1 h at 37°C, luminescence was measured using a Luminometer (POLARStar Omega, BMG Labtech, Ortenberg, Germany). At least three biological replicates were used for each measurement, and data were reported as mean ± SD.

Total RNA Extraction and Quantitative Real-Time PCR
Total RNA extraction from plant tissues was performed using RNAiso Plus reagent (Takara, Taabashi, Japan), DNase-treated (TURBO™ DNA-free kit, Thermo Fisher Scientific, Wilmington, DE, United States), and then reverse transcribed (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA, United States) according to the manufacturer’s instructions. All qPCR primers used here are listed in Supplementary Table 1. Real-time PCRs were performed with HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) as suggested by the manufacturer and on a QuantStudio 6 Flex machine (Applied Biosystems, Foster City, CA, United States). Expressions were normalized to the endogenous control MOn1 (At2g28390) levels and calculated according to the (PCR efficiency)−ΔΔ Ct formula (Kim et al., 2010). Each experiment was repeated at least twice with three biological and technical replicates.

Protein Extraction, Immunoprecipitation, and Immunoblotting
Sample processing for anti-SUMO1/2 immunoblots is described in Ingole et al. (2021a). In brief, tissues snap frozen and stored in −80°C were homogenized in protein extraction buffer (PEB) [50 mM of Tris–HCl pH 8.0, 8 M of urea, 50 mM of NaCl, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate (SDS) and 1 mM of EDTA, and 20 mM of N-ethylmaleimide (NEM)] containing indicated plants discussed in the respective results sections were crossed. From the segregating F2 or F3 populations, the desired genotype was identified and propagated for experiments. To generate plants co-expressing EDS1-YFP and His-H89R-SUM1, the parental EDS1-YFP (Garcia et al., 2010) and His-H89R-SUM1 (Miller et al., 2010) plants were crossed. F1 plants were verified for the presence of both transgenes before analysis. Primers for genotyping are listed in Supplementary Table 1.

MATERIALS AND METHODS

Plant Materials and Growth Conditions
Mutants of A. thaliana used here, namely, sum1-1, sum3-1, sum1-1 sum3-1, srfr1-4, sid2-1, srfr1-4 esd1-2, srfr1-4 snc1-11, and esd4-2, have been described earlier (van den Burg et al., 2010; Bhattacharjee et al., 2011; Villajuanà-Bonequi et al., 2014; Ingole et al., 2021b). Plants were propagated under short day (SD; 8-h light; 16-h dark) conditions at 22°C (or 24°C for PstDC3000 infection-based assays) with 70% relative humidity in controlled growth chambers with a light intensity of 100 µmol photons m−2 s−1. To generate double mutants srfr1-4 sid2-1, srfr1-4 sum3-1, or HA-SRFR1 expressing plants in SUMOylation-disturbed (sum1-1, sum3-1, esd4-2, or esd4-2 sum3-1) mutants,
freshly added 1 × plant protease inhibitors (Sigma-Aldrich, United States) and 2% w/v polyvinylpyrrolidone (PVPP). For anti-HA, anti-EDS1, anti-GFP, or anti-PR1/2 immunoblots, tissues were ground with 6 M of urea. Clarified homogenates of extracts were mixed with loading dye, boiled, resolved in SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane via wet transfer. After blocking with 5% w/v non-fat skimmed milk powder in 1 × Tris-buffered saline (TBST; containing 0.1% w/v Tween® 20), membrane was incubated overnight with indicated primary antibodies [anti-SUMO1 (Abcam, Cambridge MA, United States), anti-PR1/2 (Agrisera, Vännäs, Sweden), anti-EDS1 (custom generated against full-length Arabidopsis EDS1 from BioBharati LifeScience, Kolkata, India), anti-HA (Sigma, United States), or anti-GFP (BioBharati LifeScience, India)] in 1 × TBST. Membranes were washed thrice the next day with TBST, incubated at room temperature (RT) with secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotech, Dallas, TX, United States), and developed with α-FLAG (Sigma-Aldrich, United States) and 2% w/v polyvinylpolypyrrolidone (PVPP). For MG132 treatments, the proteasome inhibitor at a dose of 100 µM in 10 mM of MgCl₂, 10 mM of 2-(N-morpholino)ethanesulfonic acid (MES) was infiltrated into plant tissues were searched for peptide sequences (MASCOT software, Matrix Science, Boston, MA, United States) mass spectrometer instrument. Raw MS data files were searched for peptide sequences (MASCOT software, Matrix Science, Boston, MA, United States) against the Arabidopsis protein database1.

Construction of Clones for Escherichia coli SUMOylation Assay
SUM1 cDNA sequences in pCDFDuet TM1 vector were mutagenized via overlapping oligos at position 91 to incorporate arginine (R) replacing the threonine (T) residue. This resulted in SUMO1 T91R, which imparts smaller SUMOylation footprint of RGG on the modified lysine. For generating FLAG-SRFR1, cDNA sequence was directionally cloned as a NotI restriction fragment at the identical site of pFLAG-TEV vector (BioBharati LifeScience, India). For generating T7-EDS1, the cDNA sequence was cloned as a SalI–Xhol fragment into the pET28a(+) vector (Novagen, Madison, WI, United States). Details of primers used here are listed in Supplementary Table 1.

The SUMOylation assay was performed according to Okada et al. (2009). Briefly, BL21 (DE3) cells containing pCDFDuet TM1-SUMO1/2/3 GG form together with SCE1, or pCDFDuet TM1-SUMO1/2/3 AA form co-cloned with SCE1, and pACYCDuet TM1-SAE2 and SAE1 were co-transformed with either pFLAG-SRFR1 or pT7-EDS1 plasmid. Heterologous protein expression was induced with 0.5 mM of IPTG overnight at 28°C. The cell pellet harvested the next day was resuspended in phosphate-buffered saline (0.137 M of NaCl, 0.0027 M of KCl, 0.01 M of Na₂HPO₄, and 0.0018 M of KH₂PO₄, pH 7.4), lysed by boiling in 1 × loading dye, and then used for immunoblots with anti-FLAG (Sigma, United States) or anti-T7 antibodies (Merck Millipore, Kenilworth, NJ, United States).

Sample Preparation and Liquid Chromatography–Tandem MS Analysis for Determination of SUMOylation Sites
Processing for in-gel trypsin digestion and MS analysis was according to Ingle et al. (2021a). Briefly, proteins from in vitro SUMOylation reaction were separated on 6% SDS-PAGE, stained with Coomassie brilliant blue (CBB R-250) solution, and destained; and gel slices were excised as 1-mm³ pieces with sterile surgical blade. After processing as earlier, trypsin digestion (Promega, Madison, WI, United States) was performed; samples were desalted with C18 tips (Thermo Fisher Scientific, United States) according to manufacturer’s instructions and dried in a speed-vac centrifuge. Tandem MS (MS/MS) analysis was done using TripleTOF® 5600+ (AB SCIEX, Redwood City, CA, United States) mass spectrometer instrument. Raw MS data files were searched for peptide sequences (MASCOT software, Matrix Science, Boston, MA, United States) against the A. thaliana protein database1.

Construction of Bimolecular Fluorescence Complementation, GFP-Tagged SUM1 GG/AA Vectors, and in planta Assays
Bimolecular fluorescence complementation (BiFC) vectors for SRFR1, EDS1, SUMO1 GG/AA, and SUMO3 GG/AA have been described earlier (Bhattacharjee et al., 2011; Ingle et al., 2021b). Clones were introduced into Agrobacterium tumefaciens GV3101 strain via electroporation. For in planta interaction assays, Agrobacterium strains harboring the indicated BiFC vectors were cultured overnight in LB broth, centrifuged, and resuspended in 10 mM of MgCl₂, 10 mM of MES containing 150 mM of acetosyringone. After induction for 3–4 h, indicated combinations were mixed at equal bacterial density and infiltrated in fully expanded leaves of 4-week-old

1www.NCBI.nlm.NIH.gov/RefSeq/
**Construction of HA-SRFR1**

The construction of HA-SRFR1 expressing native promoter-driven HA-epitope tagged genomic clone of SRFR1 has been described earlier (Kim et al., 2010). With the use of this vector as a backbone, the indicated lysine residues were converted to arginine using overlapping primers (listed in **Supplementary Table 1**). pDONR201 clone of SUMO1 GG or AA, SUMO3-GG described earlier (Ingole et al., 2021b), was subcloned via Gateway reaction into pSITE-2CA binary vector that carries an N-terminal GFP tag (Chakrabarty et al., 2007). The above clones were confirmed by sequencing and then electroporated into Agrobacterium GV3101 strain. Transformants obtained were cultured; and srfr1-4 or sum3-1, as appropriate, was transformed via floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on kanamycin (for HA-SRFR1 or GFP-SUMO3 transformations) containing plant growth media and then propagated to T2 and T3 generations and genotyped. Simultaneously, expression of GFP-SUMO3 was also determined via anti-GFP immunoblots. GFP-SUM3 Line#1 was crossed to HA-SRFR1 expressing plants and F2/F3 population genotyped for sum3-1, srfr1-4, mutations, and HA-SRFR1 or GFP-SUM3 transgene homozygosity. The plants were then used for further analysis, as indicated.

For co-expression assays with HA-SRFR1, Agrobacterium strains with GFP alone (pSITE-2CA) or GFP-SUM1 GG or AA forms (in pSITE-2CA backbone) was co-infiltrated as above. Total protein from infiltrated tissues was isolated at 48 hpi and used for immunoblot with anti-HA antibodies. Decrease in SRFR1 protein levels was noted as early at 1 hpi and persisted to near wild-type levels was noted by 24 hpi (**Figure 1A**).

**In planta Qualitative and Quantitative Bacterial Growth Assay**

Bacterial growth assays were performed according to standardized protocol in Kim et al. (2010). Briefly, fully expanded leaves of 3- to 4-week-old SD-grown plants were infiltrated with a needleless syringe at a bacterial density of 10^6 cfu/ml (half-leaf infiltrated for qualitative disease symptom assay) or 5 × 10^4 cfu/ml (full leaf infiltrated for quantitative bacterial growth assays) with virulent PstDC3000 or avirulent PstDC3000 (avrRps4) strains. For disease symptom determinations, infiltrated leaves were detached 4 days post-infiltration (dpi) and imaged. For quantitative measurements on bacterial growth, leaf disks of defined area were harvested at 0 and 3 dpi, macerated in 10 mM of MgCl₂, and plated with serial dilution on appropriate antibiotic containing media plates. Anti-SUMO1/2 immunoblot on Col-0, sum1-1, sum2-1, and sum3-1 post-PstDC3000 challenge (10^6 cfu/ml bacterial inoculum) was performed at 24 hpi.
FIGURE 1 | SRFR1 protein instability promotes salicylic acid (SA) and SUMOylome elevations in basal defenses. At progressive time points (hpi, hours post-infection) upon PstDC3000 challenge (A) immunoblot of HA-SRFR1, (B) expression levels of SRFR1 transcripts, (C) SUMO1/2-conjugates and free SUMO1/2 levels, (D) free SA and total SA + SAG levels, and (E) expression of SID2/ICS1. Blots were probed with anti-HA or anti-SUMO1/2 antibodies, as indicated. Ponceau S-stained membrane or anti-actin immunoblot shows comparable protein loading across samples. Numbers above the HA-SRFR1 protein bands in panel (A) are densitometric quantification values relative to actin expression levels in the same extracts. Transcript abundance is relative to internal control MON1 gene expressions and presented as fold-change relative to uninfected (0 hpi) samples (n = 3). Statistical significance is by pairwise comparison with 0-hpi sample with Student’s t-test (**p < 0.01, ****p < 0.0001, and ns = not significant).

To directly correlate SA increase due to SID2/ICS1 upregulation as the causal factor of SUMOylome enhancements in srfr1-4, we generated srfr1-4 sid2-1 plants by genetic crossing. Unlike srfr1-4 eds1-2 that are developmentally similar to Col-0, srfr1-4 sid2-1 plants retained stunted stature-like srfr1-4 (Figure 2A). Thus, growth deficiencies in srfr1-4 are SID2/ICS1-independent. Total (SA + SAG) and free SA levels in srfr1-4 sid2-1 resembled those of Col-0 (Supplementary Figure 1A).
To determine global SUMOylome profile in srfr1-4 sid2-1, anti-SUMO1/2 immunoblots were performed. We also included srfr1-4 snc1-11 plants in this analysis. The snc1-11 is a knockout mutation in SNC1, the R gene responsible for growth abnormalities in srfr1-4 (Kim et al., 2010). The srfr1-4 snc1-11 plants developmentally resemble Col-0 but retain intermediate upregulation of defense-associated markers and enhanced resistance than srfr1-4 (Kim et al., 2010). We noted that sid2-1 or snc1-11 mutation completely abolished SUMO1/2-conjugate or free SUMO1/2 enhancements of srfr1-4 to Col-0 levels (Kim et al., 2010). Noted that sid2-1 or snc1-11 mutation completely abolished SUMO1/2-conjugate or free SUMO1/2 enhancements of srfr1-4 to Col-0 levels. We also included srfr1-4 snc1-11 plants in this analysis. The snc1-11 is a knockout mutation in SNC1, the R gene responsible for growth abnormalities in srfr1-4 (Kim et al., 2010). We noted that sid2-1 or snc1-11 mutation completely abolished SUMO1/2-conjugate or free SUMO1/2 enhancements of srfr1-4 to Col-0 levels (Figure 2B and Supplementary Figure 1B). Further, elevated free SA and total SA were also restored to Col-0 levels in the srfr1-4 eds1-2 or srfr1-4 snc-11 plants (Supplementary Figure 1A). Additionally, increased expressions of SUMOylation-promoting genes shown earlier (SIZ1, SCE1, or SUM3) and PRI transcripts were considerably downregulated to Col-0 levels in srfr1-4 sid2-1 plants (Ingole et al., 2021a; Figures 2C,D and Supplementary Figure 1C). Expression of SUM1 remained unaltered in all plant genotypes. These results implied that SA upregulation via heightened expression of SID2/ICS1 was mainly responsible for SUMOylome enhancements in srfr1-4 in SNC1- and EDS1-dependent manner.

**SRFR1 Is a SUMOylation Candidate**

SRFR1 harbors several intrinsically disordered regions (IDRs) especially between its multiple TPRs and the C-terminal domain (Gassmann and Bhattacharjee, 2012). Protein with IDR features often has various interacting partners adjusted according to the cellular signaling needs (Haynes et al., 2006; Liu et al., 2009). Most often, IDRs are also enriched for motifs that...
attract PTM changes (Gao and Xu, 2012; Narasumani and Harrison, 2018). In SRFR1, two sites that match canonical SUMOylation motifs (LK\textsuperscript{325}EE and LK\textsuperscript{427}QE) are predicted by the SUMOsp2.0 tool (Ren et al., 2009; Figure 3A).

![Figure 3A](image-url)

Plus, at least two each of high-scoring SIMs and non-consensus SUMOylation sites are also present in SRFR1. To test whether SRFR1 is SUMOylated by the Arabidopsis SUMO isoforms, we utilized the Escherichia coli SUMOylation reconstitution system.

**TABLE 3**

| Position | Peptide | Score | Cutoff | Type |
|----------|---------|-------|--------|------|
| 26 - 30  | SRNWSKA IRVLD SLLAKES | 31.775 | 29.92 | SUMO Interaction |
| 325      | SIFDKVLKEETYPE | 26.755 | 2.13 | SUMOylation Concensus |
| 427      | KDLSCIKLQEKDNKS | 15.671 | 2.13 | SUMOylation Concensus |
| 642      | KLKQDL | 3.588 | 3.33 | SUMOylation Nonconcensus |
| 686 - 690 | HRMAGLA VIELA OKVSKAW | 39.97 | 29.92 | SUMO Interaction |
| 780 - 784 | ISEPCDP VVWN KLSEEFN | 38.04 | 29.92 | SUMO Interaction |
| 838 - 842 | VRSKKDK VIDLS KDEKIEK | 42.463 | 29.92 | SUMO Interaction |
| 846      | IDLSKDEKIEKIMRA | 6.436 | 3.32 | SUMOylation Nonconcensus |

**TABLE 4**

| Position | Peptide | Score | Cutoff | Type |
|----------|---------|-------|--------|------|
| 375 - 379 | RVENQKK HIQYI QOERFLK | 33.563 | 29.92 | SUMO Interaction |
| 478      | ANYHRLKNEQDTGY | 14.669 | 2.13 | Sumoylation Concensus |

**FIGURE 3**

SRFR1 is a SUMOylation and SUMO-binding candidate. (A) Prediction of putative SUMOylation and SIM motifs in SRFR1 and EDS1. In silico prediction was performed using SUMOsp2.0 tool. (B) SRFR1 is SUMOylated by SUMO1 T91R, SUMO2, or SUMO3 GG forms in Escherichia coli SUMOylation reconstitution system. Immunoblot was probed with anti-FLAG antibodies to detect FLAG-SRFR1. Migration position of molecular weight standards (in kDa), SUMOylated or non-SUMOylated SRFR1 (SRFR1\textsuperscript{SUMO} or SRFR1, respectively), is indicated. (C) Bimolecular fluorescence complementation (BiFC) panels of co-expressed n\textsuperscript{Venus}-SRFR1 with CFP-clones of SUMO1 GG/AA, SUMO3 GG/AA, or negative control GUS (β-glucuronidase). Images were acquired using a confocal microscope with GFP or bright-field filters. Merged images of YFP with bright field are shown. Scale bar = 50 µm.
system, with minor modifications (Okada et al., 2009). The N-terminal 91st residue N-terminal to GG in SUMO1 was modified from threonine to arginine (T91R) to facilitate smaller SUMO footprints on targets and improved identification via MS. SUMO3 contains a relatively proximal arginine at the 88th position to the diglycine G\textsubscript{2}G\textsubscript{3} ends. MY3B0, a previously known target tested by Okada et al. (2009), was SUMOylated by SUMO1 T91R variant (Supplementary Figure 2). SRFR1 cDNA sequences were co-expressed with a FLAG-epitope tag (FLAG-SRFR1) in the presence of SUMO1 T91R, SUMO2, or SUMO3 SUMOylation-competent (containing diglycine, GG) or SUMOylation-deficient (diglycine replaced by dialanine, AA) isoforms. Induced E. coli extracts probed with anti-FLAG antibodies showed a slower migrating band than FLAG-SRFR1 in the presence of only GG, but not AA forms of the co-expressed SUMOs (Figure 3B). This band was more intense when SUMO3 was co-expressed than with SUMO1/2. These results suggested that SRFR1 is a potential SUMOylation candidate of multiple SUMO isoforms.

We subjected the slower migrating SRFR1 protein bands to trypsin digests and liquid chromatography (LC)–MS/MS analysis. SUMO1, but not SUMO2 or SUMO3, footprints on selective lysine residues of SRFR1 were detected in the identified peptides (Supplementary Figure 3). Remarkably, K\textsubscript{325} in the predicted LK\textsubscript{325}EE SUMOylation motif was identified with SUMO1-modifications. In addition, SRFR1 K\textsuperscript{229} was also covalently modified by SUMO1, though conserved features of a SUMOylation motif were absent for this lysine (CK\textsuperscript{229}PC). These results demonstrated SRFR1 as a SUMOylation candidate, at least in the E. coli system. To test the functional relevance of in silico predicted SRFR1 SUMOylation sites, we generated native promoter-driven HA-SRFR1 expressing transgenic plants containing K\textsubscript{325}R, K\textsubscript{427}R, or K\textsubscript{325}R + K\textsubscript{427}R mutations (named as HA-SRFR1\textsuperscript{K325R}, HA-SRFR1\textsuperscript{K427R}, or HA-SRFR1\textsuperscript{K325R + K427R}, accordingly), in the srfr1-4 background. Functional complementation of the expressed SRFR1 variant is expected to abolish growth defects of srfr1-4. Transgenic plants expressing HA-SRFR1\textsuperscript{K325R}, HA-SRFR1\textsuperscript{K427R}, or HA-SRFR1\textsuperscript{K325R + K427R} versions of SRFR1 restored wild-type growth in srfr1-4 (Supplementary Figure 4). These results implied that R substitutions of at least these K residues do not affect SRFR1 function. With one additional lysine residue (K\textsuperscript{229}) identified in SRFR1 as a potential SUMO-acceptor, similar mutational and complementation studies are needed to decipher its functional relevance. Detection in planta of SUMO1-modifications on K\textsubscript{721} of TPR1, which is not a predicted SUMOylation site, encourages possibility of a non-conventional motif in SUMO-influences on SRFR1 (Niu et al., 2019).

To evaluate non-covalent binding of SUMOs to SRFR1, we utilized the BiFC assays. N. benthamiana leaves transiently expressing SRFR1 showed restoration of YFP signal with SUMO1 or SUMO3 co-expressed as GG (SUMOylation-competent) forms (Figure 3C). When SUMOs were expressed as AA (SUMOylation-deficient) forms, only SUMO3 but not SUMO1 bound SRFR1. As controls, neither SRFR1 nor SUMOs interacted with the negative control beta-glucuronidase (GUS) protein or the corresponding empty vector (Figure 3C and Supplementary Figure 5). These results suggest that SRFR1 is a likely SUMOylation target as well as SUMO-binding protein.

**EDS1 K\textsuperscript{478}, a Predicted SUMOylated Residue, Is Essential for Interaction With SRFR1**

To test for possible functional relevance of SRFR1 SIMs, we utilized its previously known interaction with EDS1 (Bhattacharjee et al., 2011). As a negative immune regulator, SRFR1 likely sequesters EDS1 from activation of defenses. A high-scoring LK\textsuperscript{478}E NE matching the consensus SUMOylation motif is identified in the EDS1 protein sequence (Figure 3A). EDS1 K\textsuperscript{478} is located in the EP domain and is bracketed by residues R\textsuperscript{475} and D\textsuperscript{481} that form salt bridges with the corresponding loop residues in its partner SAG101 (Wagner et al., 2013). When tested in the E. coli–SUMOylation system, EDS1 was not SUMOylated by any of the SUMO isoforms (Supplementary Figure 6). Interestingly, in BiFC assays, co-expression of EDS1 and SUMO1 GG but not SUMO1 AA or SUMO3 GG/AA allowed reconstitution of YFP fluorescence, hinting that SUMO1 is a direct modifier of EDS1 (Figure 4A). To test the relevance of EDS1 K\textsuperscript{478} in plant defenses, we generated binary vectors that express CaMV 35S promoter-driven Myc-epitope tagged wild-type (Myc-EDS1\textsuperscript{WT}) or K\textsuperscript{478}R (lysine replace by arginine; Myc-EDS1\textsuperscript{K478R}) versions of EDS1 cDNA. The binary vectors were then used to generate transgenic plants in the EDS1-null (eds1-2) background (Cui et al., 2017). Transgenic plants obtained were then tested in qualitative disease assays with the virulent PstDC3000 or avirulent PstDC3000 (avrRps4) strains. While Myc-EDS1\textsuperscript{WT} expression in eds1-2 reinstated the basal defenses causing considerably lower chlorotic symptoms, Myc-EDS1\textsuperscript{K478R} remained similar to hyper-susceptible and collapsed eds1-2 leaves indicative of enhanced bacterial accumulations (Figure 4B). To the avirulent PstDC3000 (avrRps4) challenges, Myc-EDS1\textsuperscript{K478R} remained as hyper-symptomatic as eds1-2, while Myc-EDS1\textsuperscript{WT}/eds1-2 displayed Col-0-like resistance. Comparable Myc-EDS1\textsuperscript{WT} and Myc-EDS1\textsuperscript{K478R} protein expressions were noted between the transgenic plants (Figure 4C). Overall, these results indicated that EDS1K\textsuperscript{478R} is functionally deficient in supporting defenses. To test whether interactions with SRFR1 are affected for EDS1K\textsuperscript{478R}, we co-expressed Myc-EDS1 (wild type or K\textsuperscript{478}R) with HA-SRFR1 in N. benthamiana leaves. Immunoenrichment of HA-SRFR1 detected Myc-EDS1\textsuperscript{WT} but not Myc-EDS1\textsuperscript{K478R}, indicating that EDS1 K\textsuperscript{478} is important for SRFR1 interaction (Figure 4D). Interestingly, PAD4, a known partner of EDS1 (Fey et al., 2001), when transiently co-expressed (as HA-PAD4) in N. benthamiana leaves showed comparable interaction with either Myc-EDS1\textsuperscript{WT} or Myc-EDS1\textsuperscript{K478R}, implying that EDS1 K\textsuperscript{478} is not essential for their association (Figure 4E).

To detect for SUMOylated EDS1 in planta, we obtained F1 plants that co-expressed EDS1-YFP and His-H89R-SUM1. The crossed parents expressed EDS1-YFP or His-H89R-SUM1 that functionally complement the loss of respective endogenous EDS1 or SUM1 (Garcia et al., 2010; Miller et al., 2010). Expression of His\textsubscript{6}-SUM1-H89R facilitates improved affinity-based enrichment.
of SUMO1-SUMOylated proteins. **EDS1-YFP** (control) or **EDS1-YFP/His-H89R-SUM1** plants were sprayed with SA to mimic defense responses and then enriched for His-SUMO1-conjugated proteins with Ni²⁺-affinity chromatography, as earlier (Ingole et al., 2021a). Eluates showed enrichment of SUMO1-conjugates and when probed with anti-GFP antibodies identified a protein band at expected migration positions for SUMOylated EDS1-YFP (EDS1SUMO1) in both EDS1-YFP and EDS1-YFP/His-H89R-SUM1 samples (Supplementary Figure 7). Input extracts from both samples displayed similar levels of EDS1-YFP expression. Because of their low amounts, we were unable to perform MS analysis on the hypothesized EDS1SUMO1 protein bands. In a parallel approach, we transiently co-expressed Myc-EDS1WT or Myc-EDS1K478R with GFP-SUMO1 or His-StrepII-SUM3g (Ingole et al., 2021b) in *N. benthamiana* leaves and probed whether SUMO1/3 co-expression caused reduced migration of EDS1 indicative of its SUMOylation. Immunoblot, however, showed similar migration of Myc-EDS1WT across all SUMO combinations (Supplementary Figure 8). Thus, despite our attempts, reasonable doubt whether in planta EDS1 is
SUMOylated (on K478) persists, and further investigations to elucidate this are therefore warranted.

**sum1-1 or sum3-1 Affect Endogenous Levels and Dynamics of SRFR1 During Basal Defenses**

With indications of SRFR1 as a potential SUMOylation and SIM-harboring candidate, we tested whether enhanced or compromised defenses, respectively, in *sum1-1* or *sum3-1* (Ingole et al., 2021b), are the result of changes in SRFR1 levels. Toward this, we generated HA-SRFR1/sum1-1 plants by crossing *sum1-1* to the HA-SRFR1 line. Plants with homozygous *sum1-1* mutation displayed reduced HA-SRFR1 protein in comparison with the HA-SRFR1 parent (Figure 5A). To determine whether the lower protein levels were due to transcriptional or post-transcriptional effects, we measured SRFR1 transcript abundance. In *sum1-1* plants, SRFR1 transcripts displayed only a slight reduction (~0.8-fold), suggesting that reduced SRFR1 protein is mostly due to post-transcriptional effects (Figure 5B). Addition of the 26S proteasome inhibitor MG132 slightly improved HA-SRFR1 levels in HA-SRFR1/sum1-1 plants. Furthermore, GFP-SUMO1 GG or AA variant co-expressed with HA-SRFR1 in *N. benthamiana* leaves caused an increase in HA-SRFR1 protein abundance in comparison with the GFP alone control (Figure 5C). Taken together, our results imply that SUM1 role as a negative immune regulator may be via maintaining the steady-state levels of SRFR1.

Increased SA levels in *sum1-1* result in upregulated SUM3 expression (van den Burg et al., 2010; Ingole et al., 2021b). To test whether reduced SRFR1 in *sum1-1* is a consequence of elevated SUM3 expression, we generated HA-SRFR1/sum3-1 plants. From the segregating population, plants expressing HA-SRFR1 in *sum3-1* background showed significantly lower (~2-fold) SRFR1 transcript and protein levels than Col-0 (Figures 5A,B). Addition of MG132 did not improve HA-SRFR1 levels in *sum3-1*, implying that SUM3 regulates SRFR1 transcript abundance (Figure 5A). Since we did not have the HA-SRFR1/sum1-1 *sum3-1* plants to investigate their cumulative effect on SRFR1 protein, we checked SRFR1 transcript abundance in the *sum1-1 *sum3-1* plants that we reported recently (Ingole et al., 2021b). SRFR1 transcripts in *sum1-1 *sum3-1* were intermediate between *sum1-1* and *sum3-1* levels, suggesting interplay among the SUMO isoforms on its steady-state expressions (Figure 5B). SUM3 overexpression enhances basal defenses in *Arabidopsis* (van den Burg et al., 2010). With SUM3 role in SRFR1 transcripts indicated from our results, we generated HA-SRFR1 plants that overexpressed GFP-tagged SUMO3 (GFP-SUM3). These plants (GFP-SUM3/HA-SRFR1 Lines#1 and #2) were homozygous for the *sum3-1* mutation. Anti-GFP immunoblot detected overexpressed GFP-SUM3 in extracts from the transgenic lines (Supplementary Figure 9). We continued with Line#1 for further assays. Although downregulated SRFR1 transcripts noted in *sum3-1* restored to Col-0 levels in the GFP-SUM3 transgenic line, HA-SRFR1 protein were lower than the parental HA-SRFR1 (Figures 5B–D). Unlike what was noted for HA-SRFR1/sum3-1, MG132 treatment stabilized HA-SRFR1 protein in the GFP-SUM3 overexpressing plants, suggesting that as in *sum1-1*, the proteasome pathway was responsible for lower SRFR1 accumulations. Together with known SUM3 overexpression enhancing basal defenses, our results connect these responses again to consequences of reduced SRFR1 levels (van den Burg et al., 2010).

To further substantiate this, we challenged HA-SRFR1/sum1-1 or HA-SRFR1/sum3-1 plants with virulent *PstDC3000* and evaluated SRFR1 protein changes (Figure 5E). Interestingly, lack of SUM1 delayed SRFR1 restoration, and even at 24 hpi, HA-SRFR1 levels were barely detectable. Contrastingly, HA-SRFR1 recovery was rapid in *sum3-1* reaching native levels by 6 hpi. Accumulation of SUMO1/2-conjugates with *PstDC3000* challenge was dramatically lower in *sum3-1* and in agreement with our earlier observations (Ingole et al., 2021b; Figure 5F). Together, these data demonstrated intricate interplay between SUMO isoforms in basal defenses with SUM1 essential for SRFR1 restitution at post-transcriptional level and SUM3 modulating transcriptional efficiency as a part of feedback loop mechanism.

**Upregulated SUM3 Maintains SRFR1 Protein Levels in Autoimmune esd4-2 Plants**

The SUMO-protease ESD4 mutant (*esd4-2*) has increased SUMO1/2-conjugates, elevated SA, and enhanced basal defenses (Villajuana-Bonequi et al., 2014). We showed earlier that ESD4 transcriptions are downregulated during *PstDC3000* infections (Ingole et al., 2021a). Therefore, with overall similarities to basal defense patterns, we investigated the fate of SRFR1 in *esd4-2*. SRFR1 transcripts were significantly higher (~2-fold) likely due to higher SUM3 expressions in *esd4-2* (Figure 6A; Villajuana-Bonequi et al., 2014). To assess protein levels, we generated HA-SRFR1/esd4-2 plants by crossing HA-SRFR1/sum3-1 to *esd4-2*. In F2 plants with loss of *ESD4* but wild-type SUM3, SRFR1 protein levels were unchanged (Figure 6B). This contrast between SRFR1 transcriptional upregulations versus its unchanged protein levels in *esd4-2* plants once again indicated complex regulations on SRFR1 expressions by SUMOylome perturbations.

To directly determine whether SUM3 contributed to this process, from the above cross, we identified HA-SRFR1/esd4-2 *sum3-1* plants in the F2 and F3 populations. These plants were genetically similar to *esd4-2 *sum3-1* double mutant. Growth deficiencies including early bolting and enhanced global SUMO1/2-conjugates in *esd4-2* were not affected by SUM3 loss (Figures 6C,D). Levels of free SUMO1/2, however, showed lower levels than in Col-0, suggesting that ESD4 SUMO-protease functions are essential for maintaining these pools of SUMOs. *sum3-1* reduced total but not free SA levels and also lowered the protein or transcript abundance of several positive defense-associated players (EDS1, PR1, or PR2) that were elevated in the *esd4-2* background (Figures 6E,F and Supplementary Figure 10A). Introducing *sum3-1* in *esd4-2* reduced SRFR1 transcripts to Col-0 levels, whereas protein abundance was lower than that in HA-SRFR1 or HA-SRFR1/esd4-2 plants (Figures 6A,B). We accredit this to transcriptional promotion of SUM3 on SRFR1 observed earlier. Overall, these data reiterated that relative levels of SUMO1/3 module SRFR1 expression.
FIGURE 5 | Steady-state levels of SRFR1 are lower in sum1-1 or sum3-1 plants. (A) HA-SRFR1 protein detection in Col-0 (negative control), wild-type (HA-SRFR1), HA-SRFR1/sum1-1, and HA-SRFR1/sum3-1 plants. (B) Relative abundance of SRFR1 transcripts in Col-0, sum1-1, sum3-1, sum1-1 sum3-1, or GFP-SUM3 overexpressing plants. MON1 expressions were used as the internal control, and values are mean ± SD (n = 3). Statistical significance is according to post hoc Tukey’s test (p < 0.05) and represented by different alphabets. HA-SRFR1 protein levels with overexpressed (C) GFP alone, or GFP-SUMO1 GG or AA forms. (D) GFP-SUMO3. (E) Changes in HA-SRFR1 protein at 0, 1, 3, 6, and 24 hpi PstDC3000 (Pst EV) infection (hpi) in wild-type (HA-SRFR1), HA-SRFR1/sum1-1, or HA-SRFR1/sum3-1 plants. (F) Accumulation of SUMO1/2-conjugates in Col-0, sum1-1, sum2-1, or sum3-1 at 24 hpi. Immunoblots were probed with anti-HA or anti-SUMO1 antibodies as mentioned. Ponceau S staining show protein loadings. Migration position of molecular weight standards (in kDa) is indicated. MG132 treatments, where indicated, was performed at 12 h prior to respective analysis. Numbers above the HA-SRFR1 bands are densitometric quantification values relative to actin expression levels in the same extracts.
Several TFs are SUMOylation targets (Miller et al., 2010). SUMOylome disturbances cause expression differences of several SUMOylation-associated genes (Ingole et al., 2021b). To evaluate this in esd4-2 combination mutants, we performed quantitative real-time PCRs (qRT-PCRs). Expressions especially for SIZ1 and HPY2, the two E3 ligases, were higher and SUM3-independent in...
esd4-2 (Supplementary Figure 10C). SUM1 transcripts remain unaffected in all plants tested (Supplementary Figure 10B). Taken together, these data reflected consequences of deficient ESD4 activity rather than increased SUMOylation efficiencies by upregulated SIZ1/HYP2 or due to SUM3 involvement in their expressions as the primary cause of elevated SUMO1/2-conjugates in esd4-2 plants.

Enhanced Basal Defenses in srfr1-4 Are Partially SUM3-Dependent

Our results here and earlier suggested mutual expression influences between SRFR1 and SUMOylation-associated genes (Ingole et al., 2021a). To evaluate this interplay in the context of enhanced immunity in srfr1-4, we attempted to generate srfr1-4 sum1-1 or srfr1-4 sum3-1 double mutants. Considering SUM1 role as a negative immune regulator and SUM3 as a positive immune regulator, we tested whether pathogenesis outcomes of srfr1-4 are altered. From the F2/F3 segregating population of sum1-1 crossed with HA-SRFR1 plants used earlier, we screened for srfr1-4 sum1-1 double mutants. Even after extensive screening, srfr1-4 sum1-1 plants were not obtained, suggesting their embryonic lethality. It remains a possibility that developmental consequences that occur in srfr1-4 are compounded by the loss of SUM1. Observed growth and immune enhancements that we reported recently in sum1-1 support our speculation (Ingole et al., 2021b).

We identified srfr1-4 sum3-1 in the segregating populations of sum3-1 crossed with HA-SRFR1. As observed for srfr1-4 sid2-1 plants, sum3-1 mutation does not restore growth defects of srfr1-4 (Figure 7A). Enhanced SUMO1/2-conjugates were also unaffected by introducing sum3-1 in srfr1-4 (Figures 2B–D). Heightened expression of SCE1 but not SIZ1 in srfr1-4 was abolished in srfr1-4 sum3-1 plants, meaning that mis-regulations of some SUMOylation-associated genes in srfr1-4 are SUM3-dependent. To compare defense responses, these plants were challenged with virulent PstDC3000 or avirulent PstDC3000 (avrRps4) strains. Surprisingly, unlike deficient immunity in sum3-1 plants (Ingole et al., 2021b), bacterial accumulation for both strains in srfr1-4 sum3-1 was lower than in Col-0 but higher than in srfr1-4 (Figure 7B). Thus, srfr1-4 mutation was epistatic to sum3-1. Free SA elevations were abolished, whereas total SA levels though are reduced than srfr1-4 and remained significantly (~20-fold) higher in srfr1-4 sum3-1 to Col-0 (Figure 7C). Relative expression levels of PR1, PR2, and SID2/ICS1 transcripts were also higher than those of Col-0 in srfr1-4 sum3-1 but lower than in srfr1-4 plants (Figure 7D). Similarly, protein levels of PR1, PR2, or EDS1 were also elevated than Col-0 but lower than srfr1-4 in the double mutant (Figure 7E). These results indicated that upregulated defenses in srfr1-4 only partially involved SUM3 contributions. Together, our data suggested that SRFR1 dynamics primarily modulated defense-associated SUMOylome changes and immune amplitudes in partial SUM3-dependent and SA-dependent manner. Overall, with our investigations, here we reveal an intricate molecular crosstalk between SRFR1 role in SUMOylome homeostasis/adjustments during defense and counter-repercussions on SRFR1 expressions by the SUMOylation changes overall to modulate immune amplitudes.

DISCUSSION

Pleiotropic phenotypes in the context of host SUMOylome alterations have remained restricted to studies in mutants of SUMOylation pathway genes (Lee et al., 2007; van den Burg et al., 2010; Villajuana-Bonequi et al., 2014; Bailey et al., 2016). We first demonstrated the role of a negative immune regulator SRFR1 in maintenance of host SUMOylome homeostasis (Ingole et al., 2021a). That loss of SRFR1 caused upregulated SUMOylation-promoting and downregulated SUMO protease expressions, a consequence mirrored in basal defenses, genetically placed SRFR1 as a transcriptional modulator of the host SUMOylome (Ingole et al., 2021a). Also, with our demonstration that SUM1 transcripts are preferentially loaded onto polysomes in srfr1-4 plants, we further expanded SRFR1 involvement also at the post-transcriptional level. Here, using srfr1-4 sid2-1 or srfr1-4 snc1-11 plants, we reaffirmed that similar to srfr1-4 eds1-2, elevated SA through SID2/ICS1, and SNC1 upregulated expressions in srfr1-4 are the principal cause of increased SUMO1/2-conjugates (Ingole et al., 2021a). Taken in the context of SUMO1/2-conjugation increase observed upon SA treatment, SRFR1 suppression of SID2/ICS1 expressions likely maintains global SUMOylome and mis-priming of immunity (Kim et al., 2010; Bhattacharjee et al., 2011; Bailey et al., 2016; Ingole et al., 2021a).

SA targets SUMO proteases OTS1/2 for proteasome-mediated degradation, thus enhancing SUMO1/2-conjugate levels (Bailey et al., 2016). We show here that SRFR1 undergoes transient instability when basal defenses are induced upon PstDC3000 exposure. The reduction in SRFR1 correlates with subsequent increase in SA levels and SUMO1/2-conjugate enhancements. Considering SA degrades OTS1/2, SRFR1 changes via downstream SA increase may drive the condemned fates of OTS1/2, presenting another example of its host SUMOylome adjustment role at the post-transcriptional level (Bailey et al., 2016). Recuperation of SRFR1 levels at latter time points of basal defense elicitation perhaps is indicative of fine-tuning of immune amplitudes, preventing overshoots or pleiotropic consequences like in srfr1-4 or in the SUMOylation-perturbed mutants such as sid2-1 or eds4-2 (Lee et al., 2007; Kim et al., 2010; Villajuana-Bonequi et al., 2014).

SUMOylome changes in turn regulate SRFR1 expressions. Firstly, in sum1-1 plants, unchanged SRFR1 transcripts but reduced protein levels are stabilized by the proteasome inhibitor MG132. Secondly, increased SRFR1 protein accumulation is detected by transient overexpression of SUM1 in N. benthamiana leaves. Thirdly, SRFR1 restoration is deficient in sum1-1 plants when challenged with PstDC3000. Last but not the least, gradual restoration of SRFR1 protein levels parallels progressive increase in SUMO1/2-conjugates during PstDC3000 challenges. Overall, these results suggest that SUM1 affects SRFR1 at a post-transcriptional level, possibly regulating protein turnover. Unlike sum1-1, reduced SRFR1 protein in sum3-1 matches its lower transcript levels. Taking into consideration our recent data
FIGURE 7 | Enhanced defenses in srfr1-4 are partially SUM3-dependent. (A) Growth phenotypes of 3- to 4-week-old plants of indicated genotypes. (B) Bacterial growth measurements with virulent PstDC3000 or avirulent PstDC3000 (avrRps4) strains; (C) free and total SA levels; (D) transcript abundance of PR1, PR2, or SID2/ICS1; and (E) protein levels of EDS1, PR1, or PR2 in Col-0, srfr1-4, sum3-1, or srfr1-4 sum3-1 plants. Growth of bacterial strains at day 0 or day 3 post-challenge is shown. Statistical significance in transcript expression levels is via post hoc Tukey’s test (p < 0.05) and marked by different letters (n = 3).
that SUM3 potentiates SUMO1/2-conjugation efficiencies and in sum3-1 plants endogenous as well as defense-induced increase in SUMO1/2-conjugates are deficient, lower SRFR1 levels in this mutant likely also incorporate additive effects from this intersection (Ingole et al., 2021b). Thus, sum3-1 partially mimics sum1-1 consequences in destabilizing SRFR1. This notion is supported by our observation that MGI32 treatment improves SRFR1 protein stability to a modest extent in sum3-1. Immune responses, however, remain deficient in sum3-1 likely because of functional inadequacies in the SA-signaling sector through NPR1, a known exclusive SUMO3-substrate (Saleh et al., 2015).

SUM3 expression is induced transiently at early time points of SA application (van den Burg et al., 2010). Increased SA levels and concomitant upregulation of SRFR1 transcripts during basal defenses noted in our data imply transcriptional contributions of SUM3 as we suggested above. However, SRFR1 instability also noted at same time points leads us to speculate that SUMO3, antagonistic to SUMO1, may negatively affect SRFR1 protein accumulation upon SUMOylation. Our data that SRFR1 is unstable in SUMO overexpressing transgenic lines are in accordance with this hypothesis. Presence of multiple predicted SUMOylation motifs and in vitro SUMOylation by SUMO3 more prominent than for SUMO1, indeed, present SRFR1 as a candidate whose functions/stabilities may in turn be affected by host SUMOylome/SUMO isoform ratio changes. Stimulus-driven SUMO isoform switches that affect the stability of a substrate have been widely documented in animal systems (Meulmeester et al., 2008; Zhu et al., 2008, 2009). The mammalian GTPase activating protein RanGAP1, although is equally modified by SUMO1/2/3 in vitro, conjugation in vivo to SUMO1, but not SUMO2, imparts more stability from isopeptidases (Zhu et al., 2009). Similarly, HDAC1 is targeted for degradation upon SUMOylation by SUMO1, but not SUMO2 (Citro et al., 2013). Reduced SRFR1 levels in sum1-1 plants can therefore be also attributed to increased SUM3 expressions (Ingole et al., 2021a). However, that the same is not noted for esd4-2 plants with elevated SUM3 levels indicates that ESD4 functions are necessary for this process. Indeed, involvement of a SUMO-protease has been reported recently for stimulus-dependent SUMO paralog switching and its impact on the substrate stability (Fasci et al., 2015).

Bioinformatics predictions of SIMs in SRFR1 taken together with non-covalent binding of multiple SUMO isoforms also present another mode by which a change in SUMO homeostasis may impact SRFR1 activities. Our observation that SRFR1 interaction with EDS1K478R, a speculated SUMOylation-deficient version, is abolished indicates biological implication of SRFR1 SIMs in interaction with positive defense regulators. The placement of a predicted SIMs (I49-LIDIC43) in the first TPR motif of SRFR1 (residues 39–72, TPRpred, toolkit.tuebingen.mpg.de), a well-known platform for protein–protein interaction, introduces encouraging direction to pursue further. Precise mutagenesis of this and other predicted SIMs followed by interaction analysis with EDS1 or other known interactors (such as RPS4/6, or SNC1) is needed to be tested in vivo (Kim et al., 2010; Bhattacharjee et al., 2011). With our assays, here, we are, however, unable to convincingly demonstrate EDS1 as an in vivo SUMOylation candidate. Since only a minor pool of EDS1 interacts with SRFR1, in planta identification of SUMOylated EDS1 may prove challenging (Bhattacharjee et al., 2011). SNC1 or TCP8/14/15 also contains multiple predicted SUMOylation motifs. Although SUMOylated SNC1 or TCPs has been detected in vivo or in vitro, respectively, their functional relevance remains untested (Gou et al., 2017; Mazur et al., 2017). Consolidated SUMO1/3 SUMOylome changes have strong potential to interfere with SRFR1 properties at multiple levels and modulate immune signaling. A mechanistic implication into this is presented in the schematic (Figure 8).

In summary, our results here provide a unique molecular basis into SRFR1 role in the SUMO-immune balances. With regulatory influences on expression of several SUMOylation-associated genes countered by SRFR1’s own stability determined by SUMO isoform crosstalk, we highlight its mediation of immune response amplitudes in plants (Ingole et al., 2021a). In addition to positive and negative immune regulators that are directly affected, SUMOylation efficiencies are also modulated by SUMO changes. This self-regulation is evident from the observation that SIZ1, SCE1, and ESD4 are themselves substrates for SUMOylation, and depending on the SUMO isoform they associate with, their localization or specificities are affected (Miller et al., 2010; Mazur et al., 2017). For example, SUMO1 bound to SCE1 SIM localizes the ternary complex (SUMO-SCE1-SIZ1) to nuclear bodies, whereas SUMO3 binding partitions it as nucleocytoplasmic. Overall, defense amplitudes therefore rely on strict harmony between SUMOylome modulators.

**FIGURE 8** | Simplified schematic representation of crosstalk between SRFR1 and SUMOylome maintenance in defense-developmental balance. Under homeostasis, SRFR1 prevents SUMOylome perturbations via its suppression of SID2/ICS1 expression that drive SA responses. This mode of negative regulation incorporates transcriptional suppression of SUMO-conjugases (such as SIZ1 and SCE1, among others), SUM3, and protein stabilities of SUMO proteases OTS1/2. Basal SUMOylation adjustments in turn reciprocates on SRFR1 function at multiple levels. As SUMOylation and/or SUMO-binding candidates, intermolecular SUMO-SIM-type associations of SRFR1 with SNC1, TCPs, and EDS1 prevent mis-primed immune activations. Similar nature of association occurring between these immune players and their downstream targets (TPRs and HDA19) regulates expression of defense-responsive genes such as DND1/2 and PRF1/2, among others. SUMO1/2 or SUMO3 isoforms with their respective roles as negative or positive immune modulators balance the association stoichiometry through SRFR1 expression/stability. During a pathogen threat, transient reduction in SRFR1 initiates SUMOylome perturbations and activates defense players to execute defense signaling. A feedback signaling loop also initiated restoration of SRFR1 levels to avoid development penalties of constitutive immunity.
such as SRFR1, (de)SUMOylation efficiencies, localization of SUMOylation-machines, and selection of substrates (Ingole et al., 2021a). Comprehensive elucidation of these events requires not only qualitative (i.e., which SUMO isoform-modified) but also quantitative (ratio of SUMOylated versus non-SUMOylated) evaluation of a host SUMOylome adaptation during immunity. While SUMO1/2-modified protein list is ever-increasing, SUMO3-targets remain grossly underrepresented. Development of an efficient SUMO3-enrichment system, on the similar theme to Miller et al. (2010), is therefore a necessity. Equally important is the characterization of protein–protein interactome changes that are defined by the SUMO-SIM nature. Lastly, functional interaction among SUMO-machines, especially the SUMO-ligases/proteases, recently identified DeSIs (de-SUMOylating isopeptidases) (Orosa et al., 2018), STUbLs (SUMO-targeted ubiquitin ligases; Elrouby et al., 2013), and PIAlS (E4-type SUMO ligases) (Tomanov et al., 2014), and their cognate substrates are warranted to decipher the net impact on immune signaling. Toward this endeavor, we present SRFR1 role in SUMOylome regulations and the srfr1-4 mutant as a promising system to pursue these investigations.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRIDE Archive, accession no: PXD026117.

**AUTHOR CONTRIBUTIONS**

SB and WG conceived the research. MK, KI, SR, and SB designed the research. SR generated T91R SUMO1 clone. MK and KI generated other clones and plants used here and performed the experiments. MK, KI, and SB analyzed the data. MK, WG, and SB generated other clones and plants used here and performed the research. SR generated T91R SUMO1 clone. MK and KI conceived the research. MK, KI, SR, and SB designed the research.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.680760/full#supplementary-material

**SUPPLEMENTARY FIGURE 1 |** arc1-11 abolishes enhanced SUMO1/2-conjugates and restores elevated SA to Col-0 levels in srfr1-4.

**SUPPLEMENTARY FIGURE 2 |** MYB30 is SUMOylated in the E. coli SUMOylation reconstitution system by the T91R variant of SUMO1.

**SUPPLEMENTARY FIGURE 3 |** A consensus-type and a non-conventional lysine residue in SRFR1 is SUMOylated by SUMO1 in the E. coli SUMOylation reconstitution system.

**SUPPLEMENTARY FIGURE 4 |** Growth defects of srfr1-4 is abolished by the expression of HA-SRFR1K427R, HA-SRFR1K427R+, or HA-SRFR1K427R+K427R variant.

**SUPPLEMENTARY FIGURE 5 |** EDS1 does not interact with SUMO1 AA or SUMO3 GG/AA.

**SUPPLEMENTARY FIGURE 6 |** EDS1 SUMOylation is not detected in the E. coli SUMOylation reconstitution system.

**SUPPLEMENTARY FIGURE 7 |** Enrichment of SUMO1-conjugates from SA-treated plants detect possible SUMOylated EDS1.

**SUPPLEMENTARY FIGURE 8 |** Over-expression of SUMO1 or SUMO3 in plants does not detect SUMOylated EDS1.

**SUPPLEMENTARY FIGURE 9 |** GFP-SUM3 overexpressing plants show detectable levels of GFP-SUM3 proteins.

**SUPPLEMENTARY FIGURE 10 |** PR1, PR2, but not SIZ1 or HPY2 upregulation in esd4-2 plants is SUMS-dependent.

**SUPPLEMENTARY TABLE 1 |** List of oligonucleotide primers.
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