Calmodulin-binding transcription activator AtSR1/CAMTA3 fine-tunes plant immune response by transcriptional regulation of the salicylate receptor NPR1

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Abstract

Calcium signaling regulates salicylic acid (SA)-mediated immune response through calmodulin-meditated transcriptional activators, AtSRs/CAMTAs, but its mechanism is not fully understood. Here, we report an AtSR1/CAMTA3-mediated regulatory mechanism involving the expression of the SA receptor, NPR1. Transcriptional expression of *NPR1* increased in knockout mutant, *atsr1,* independently of SA biosynthesis. AtSR1 directly bound to a CGCG box in the *NPR1* promotor. The *atsr1* mutant exhibited resistance to the virulent strain of *Pseudomonas syringae* pv. *tomato* (*Pst*), however it was susceptible to an avirulent *Pst* strain carrying *avrRpt2*, due to the failure of the induction of hypersensitive responses. These resistant/susceptible phenotypes in the *atsr1* mutant were reversed in the *npr1* mutant background, suggesting that AtSR1 regulates NPR1 as a downstream target during plant immune response. The virulent *Pst* strain triggered a transient elevation in intracellular Ca^{2+} concentration, whereas the avirulent *Pst* strain triggered a prolonged change. The distinct Ca^{2+} signatures were decoded into the regulation of NPR1 expression through AtSR1's IQ motif binding to calcium-free-CaM2, while AtSR1's calmodulin-binding domain binding to calcium-bound-CaM2. These observations reveal a role for AtSR1 as a Ca^{2+}-mediated transcription regulator for controlling the NPR1-mediated plant immune response.

KEY WORDS: calcium signaling, basal resistance, hypersensitive response, plant immune

response, salicylic acid, AtSR1/CAMTA3, NPR1.

1 INTRODUCTION

Salicylic acid (SA) plays a key role during plant-pathogen interactions. A number of genes are involved in SA-mediated signaling pathway (Seybold et al., 2014; Yuan, Jauregui, Du, Tanaka, & Poovaiah, 2017). *Nonrace-specific disease resistance* (*NDR1*)*, enhanced disease susceptibility 1* (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*) are genes up-stream to the activation of SA biosynthesis in SA signaling pathway (Haitao et al., 2017; Yuan, Tanaka, Du, & Poovaiah, 2018). NDR1 is required as a downstream component for CC-NBS-LRR proteins that recognize pathogen effectors and activate the downstream of SA signaling pathway (Aarts et al., 1998). EDS1 partnered by PAD4 is required as a downstream component for TIR-NBS-LRR proteins, and is reported to positively regulate SA accumulation and *pathogenesis-related 1* (*PR1*) induction, a marker gene for SA-mediated immune response (Haitao et al., 2017; Zhang & Li, 2019). In *Arabidopsis thaliana*, SA is synthesized from chorismate through reactions catalyzed by isochorismate synthase (ICS) (Aarts et al., 1998; Du et al., 2009; Wildermuth, Dewdney, Wu, & Ausubel, 2001). During pathogen attack, SA is synthesized in the chloroplasts. The transportation of SA from the chloroplasts to cytoplasm is important for SA-mediated plant immune response. Enhanced disease susceptibility 5 (EDS5) was identified as an SA transporter which is required for SA accumulation in the cytosolic compartment (Nawrath, Heck, Parinthawong, & Métraux, 2002). The SA is perceived by nonexpresser of pathogenesis-related gene 1 (NPR1), an SA receptor, which acts as a co-transcriptional factor to establish a proper immune response to pathogen attack (Cao, Glazebrook, Clarke, Volko, & Dong, 1997; Fu et al., 2012; Spoel et al., 2009).

Plants deploy basal disease-resistance and hypersensitive response (HR) based on programmed cell death (PCD) (Fu et al., 2012; Kuai, MacLeod, & Després, 2015). Basal disease-resistance is mainly activated by virulent pathogens even in susceptible hosts (Jones & Dangl, 2006), in which pattern-triggered immunity (PTI) is induced by recognition of microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) (Zhou & Zhang, 2020). PTI is often targeted by pathogen effectors, which when released by host-specific pathogens, race to suppress plant immunity (Van de Weyer et al., 2019). Instead, plants have evolved various resistant proteins (R proteins) to perceive the effectors, and thereby inducing stronger and rapid immune response, known as effecter-triggered immunity (ETI) (Frantzeskakis et al., 2020; Fu et al., 2012; Jones & Dangl, 2006). ETI usually culminates to HR

(Jubic, Saile, Furzer, El Kasmi, & Dangl, 2019). NPR1 is required for basal resistance, since the *npr1* mutant displayed susceptibility during infection of a virulent pathogen (Aviv et al., 2002). In contrast, NPR1 suppressed HR, which is seen in *npr3 npr4* mutant plants, where the NPR1 protein is accumulated and plants displayed weakened HR (Fu et al., 2012).

It has been documented that calcium (Ca^{2+}) signaling plays a key role in regulating the production of SA (Lenzoni, Liu, & Knight, 2018; Tian, Wang, Gao, Li, & Luan, 2020). In the loss-of-function mutant *defense no death 1* (*dnd1*) plants, SA is known to accumulate (Moeder, Urquhart, Ung, & Yoshioka, 2011). *DND1*encodes cyclic nucleotide-gated ion channel 2 (AtCNGC2), as a Ca^{2+}-permeable channel (DeFalco, Moeder, & Yoshioka, 2016; Moeder et al., 2011; Tian et al., 2019). In addition, a double mutant of *Ca^{2+}-ATPase 4* (*ACA4*) and *ACA11* displayed high levels of SA (Boursiac et al., 2010). During plant-pathogen interactions, the increase of free intracellular Ca^{2+} concentration observed as an early and necessary event in plant immune response (Frei dit Frey et al., 2012; Lecourieux, Ranjeva, & Pugin, 2006; Whalley & Knight, 2013). This calcium-dependent signaling is decoded by several Ca^{2+} sensor proteins (Lenzoni et al., 2018; Marcec, Gilroy, Poovaiah, & Tanaka, 2019; Whalley et al., 2011);. CaM-binding protein 60a (CBP60a), is known to negatively regulate the expression of *ICS* gene by binding to its promotor (Truman et al., 2013), while CBP60g together with SARD1 positively regulate the expression of *ICS* in response to pathogen infection (Tongjun Sun et al., 2015). These observations suggest that Ca^{2+} is an essential messenger for SA production. Not only for SA production, recent studies have revealed that Ca^{2+} signaling is important also for controlling the SA signaling pathway (Du et al., 2009; Tongjun Sun et al., 2015; Truman et al., 2013). For example, Ca^{2+}-dependent protein kinases (CDPKs) phosphorylate the WRKY proteins to activate their function during pathogen attack (Gao et al., 2013), in which the WRKY transcription factors are found to regulate the expression of *NPR1* gene (Spoel et al., 2009), suggesting that Ca^{2+} signaling rigorously regulates the SA signaling.

Calmodulin (CaM)-binding transcription activators (CAMTAs) have been shown to negatively regulate SA biosynthesis, in which CAMTAs suppress the expression of *EDS1* and *NDR1* genes by binding to the CGCG box (CAMTA-binding cis-element) in their promotors (Du et al., 2009; Nie et al., 2012). Recent studies revealed that CAMTAs also suppress the expression of CBP60g and SARD1 with direct and indirect interactions on their promoters, respectively (T. Sun et al., 2020), thereby suppressing the SA and pipecolic acid (Pip/NHP) accumulations.

Interestingly, NPR1 protein level is required to be high for SA- and Pip-mediated systemic acquired resistance (Y. Kim, Gilmour, Chao, Park, & Thomashow, 2020). These studies revealed that CAMTAs/AtSRs play a key role for SA-mediated systemic immune response. However, it is still not clear how plants establish proper immune response to a specific pathogen through the AtSR1 pathway. Here we report a mechanism illustrating how AtSR1 interacts with Ca^{2+}/CaM to regulate NPR1-mediated immune response.

Our *in silico* survey revealed that *NPR1* promoter contains a typical CGCG box (CAMTA-binding cis-element), leading us to hypothesize a direct transcriptional regulation of the *NPR1* gene by CAMTAs. Here, we demonstrate that Ca^{2+}/CaM-mediated signaling directly regulates the expression of *NPR1*, for which AtSR1/CAMTA3 plays a critical role through a dynamic change in the Ca^{2+}/CaM-AtSR1 complex and SA-mediated immune responses.

2 MATERIALS AND METHODS

2.1 Plant materials

The *Arabidopsis* lines used in this study are wild-type (WT) Columbia (Col-0) and loss-of function *atsr1* mutant (Salk_001152C). Loss-of function *npr1* line (Salk_204100C) and complimentary AtSR1 lines in *sr1 sr4* (CS71604), i.e., cW (CS71607), mIQ (A855V, CS71613), mCaMBD (K907E, CS71615) and mIQ+mCaMBD (K907E/A855V, CS71617) were ordered from ABRC. The homozygous knock-out mutants were verified by PCR and RT-PCR. The *atsr1 npr1* double mutants were generated by crossing *atsr1* and *npr1* single mutant lines.

Seeds were surface sterilized with 1/3 diluted bleach for 10min and germinated on half-strength MS medium (Caisson Laboratories Inc.) containing 0.05% MES and 1% sucrose, adjusting pH to 5.7 with KOH. One-week-old seedlings were transferred to pots containing soil mix. Plants were maintained in a growth chamber under a 12-h photoperiod at 20-22°C and plants were watered as needed.

2.2 Bacterial pathogen inoculation and disease resistance assays

Pseudomonas syringae pv. tomato DC3000 was cultured in King's B medium and inoculation was performed as previously described. Briefly, leaves of 4- to 5-week-old plants were infiltrated with *Pst* DC3000 at OD600 = 0.001 in 10 mM $MgCl_2$ and *Pst* DC3000 carrying avrRpt2 at OD600 = 0.01, respectively, using 1mL needleless syringe for time course induction and disease

resistance test. At 1 hour after inoculation (as day 0) and 3 days after inoculation, the leaf samples were harvested for disease resistance test, as 3 d.p.i. Data were shown as a average of six biological replicates; the results are presented as mean ± S.D.

2.3 RNA extraction and qRT-PCR.

Five-week-old *Arabidopsis* seedlings were used to test gene expressions. Control and infected leaves samples of different genotypes were collected and flash frozen in liquid nitrogen. The frozen tissues were ground to powder in 1.5 ml microfuge. Total RNA was prepared using TRIzol Reagent (Invitrogen) followed by DNase-I (Roche) treatment. 2 μg total RNA was used to synthesize cDNA with an oligo (dT) primer and random hexadeoxynucleotides primer. The cDNA was diluted 10 or 20 times and 1μL/reaction (10 μL) was used as a template. Real-time PCR was performed using a MyiQTM single-color real-time PCR detection system with SYBR Green Supermix (Bio-Rad). Target gene expression levels were normalized to that of *AtACTIN2* (AT3G18780) or *AtUBQ5* (AT3G62250). A minimum of two technical replicates and three biological replicates were used for each experiment.

2.4 EMSA for the AtSR1 protein on *NPR1* promotor fragment

The *E. coli* strain BL21(DE3)/pLysS carrying the pET32a-derived plasmid for expression of recombinant AtSR1 covering the CG-box binding domain (1-153 aa) were used. The *E. coli* strain was cultured in SOC medium for around 3-5 hours at 37°C to the concentration of OD600 = 0.5. Then, IPTG was added with a final concentration of 0.5 mM IPTG for 3 hours for induction. The cells were centrifuged at 4000 RPM for 10 min at 4 °C and the pellets were re-suspended at autoclaved PBS (pH 7.4) with a proteinase inhibitor. The re-suspended cells were lysed by sonicator to release the recombinant protein. 6His-tagged recombinant proteins were purified using Ni-NTA agarose affinity beads (Qiagen) as described by the manufacturer. The recombinant proteins were dialyzed in an EMSA buffer containing 50 mM Tris (pH=7.6), 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 25 mM HEPES (pH 7.6), 150 mM NaCl, and 10% ethylene glycol. The dialyzed proteins were quantified by Bradford assay and stored at -20°C. The recombinant AtSR1 was used for EMSA to detect its interaction with the promoter fragments of *NPR1*.

157 **2.5 Promoter activity assay**

158 *Arabidopsis* protoplasts cells (2 x 10^5/mL) from 3-week-old wild-type or the *atsr1* mutant grown
159 at 20ºC were transfected in four replicates with 20 µg of plasmid (WT or mutated AtSR1, GFP
160 empty vector as internal control or AtSR1 fused to GFP) and 10 µg of *NPR1P* and *mNPR1P*
161 (CCCG) plasmids with the 40% PEG-mediated transfection method. Overnight incubation at
162 room temperature in light, the transformed protoplasts were harvested, and luciferase assays
163 were performed using a luciferase assay kit (Promega). To account for variation in transfection
164 efficiencies, GFP signals were measured as internal control. The data presented are the average
165 of the Luc/GUS ratios of four replications.

166

167 **2.6 ChIP-PCR**

168 5g of leaf tissue was harvested from 4-week-old *atsr1* mutant plants in which AtSR1-2HA was
169 transiently expressed. Leaf samples were resuspended in autoclaved PBS buffer (pH 7.5)
170 containing 1% formaldehyde and crosslinked by drawing vacuum for 10 min. Samples were then
171 quenched by using 2 M glycine under vacuum for 5 min. The chromatin samples were sheared
172 with a sonicator for 10 min on ice with an output setting at "power: 10", 10 sec "ON cycle", 50
173 sec "OFF cycle". The sheared chromatin samples were pre-cleared with Protein A agarose beads.
174 Pre-cleared chromatin samples were immunoprecipitated with Protein A agarose beads
175 conjugated with anti-HA-antibody overnight. The bound agarose beads were washed by the PBS
176 buffer, a low-salt buffer, a high-salt buffer, and a LiCl buffer. The DNA was eluted by in an
177 SDS-NaCl buffer at 65°C for 15 min. The immunoprecipitated -DNA was cleaned by PCR
178 Purification Kit (Qiagen) for PCR test.

179

180 **2.7 Calcium Measurements**

181 The calcium spikes in leaves were measured with aequorin (AEQ)-based calcium assay (Knight,
182 Trewavas, & Knight, 1996; Maintz et al., 2014; Tanaka, Choi, & Stacey, 2013). The *Arabidopsis*
183 Col-0 plants carrying AEQ were grown in soil. The leaf discs (5-mm diameter) obtained from 4-
184 week-old were immersed into 1mL of 5µM coelenterazine solution (NanoLight Technolgies) in
185 24-well microplates. The plate was left under vacuum for 10 min twice, and then further
186 incubated overnight in the dark at room temperature. The AEQ-based bioluminescence was
187 quantified in illuminometer for 5 min as baseline. An equal volume of double-strength pathogens

was added and quantified for 20 min, as L (luminescence intensity per second). The total remaining Ca^{2+} in each microplate well was discharged by treatment with equal volume of 2 M $CaCl_2$ in 20% ethanol to release remaining AEQ, as L_{max}. Ca^{2+} concentration in plant cells were calculated as described previously (Tanaka et al., 2013). The equation is: $[Ca^{2+}]_{cyt}$ (nM) = [X+ (X*55)-1]/(1-X)/0.02, where $X = (L/L_{max})^{1/3}$.

2.8 Recombinant protein purification and CaM-HRP binding assay

The *E. coli* strain BL21(DE3)/pLysS carrying the pET32a-derived plasmid for expression of WT and mutated recombinant AtSR1 proteins (740-922) containing IQ motif and CaMBD were used. The recombinant protein was expressed and purified as described above. Finally, the recombinant proteins were dialyzed in in a CaM buffer containing 50 mM Tris (pH = 7.6), 1 mM DTT, 1 mM EDTA, and 10% ethylene glycol. The dialyzed proteins were quantified by Bradford assay and stored at -20°C.

The AtCaM2 were expressed as described above and purified with CaM-Sepharose column (Amersham Biosciences) essentially as described (Yang & Poovaiah, 2002). The AtCaM2 conjugated with horseradish peroxidase (CaM-HRP) was used to study the CaM-binding property of WT and mutated recombinant proteins. 1 μg of the recombinant proteins were separated by SDS–PAGE (15%) and transferred into PVDF membrane. The membrane was blocked in a binding buffer (PBST with 0.2mM $CaCl_2$ or 5mM EGTA) containing 1.5% Bovine serum albumin (BSA) for 45 min at room temperature, then incubated with the milk containing binding buffer supplemented with AtCaM2-HRP (1:1000 dilution for Ca^{2+} present or 1:250 dilution for Ca^{2+} free) for 1 h at room temperature. The membrane was then washed three times in binding buffer for 10 min each. To detect the CaM binding signal, the BM chemiluminescence Western blotting kit (Roche Applied Science) was used according to instructions from manufacturer.

2.9 *Agrobacterium*-mediated transient expression assay in *Nicotiana Benthamiana* for promoter activity assay

Agrobacterium tumefaciens, LBA4404 was cultured on LB agar containing proper antibiotics and incubated at 28°C overnight. The LBA4404 was harvested using a centrifuge and re-suspended in 10 mL of infiltration media (10 mM $MgCl_2$, 0.5 μM acetosyringone), to a final

OD600 of 1.0, and incubated at room temperature without shaking for 2 h before infiltration. Infiltrations were carried out similar to the pathogen infiltration described above. Approximately 300 µL of the *Agrobacterium* mixture was infiltrated into 3-week-old leaves of *N. benthamiana*, and then covered to keep humidity high for 1 day in the dark. Consequently, transient expression was assayed starting 4 days after inoculation.

2.10 Statistical Analysis

Results were analyzed using Microsoft Excel. Error bars in all of the figures represent standard deviations. Number of replicates is described in the figure legends. For two group samples, statistical analyses were performed by one-way ANOVA analysis using Microsoft Excel ToolPak. Samples with statistically significant differences (asterisks, ***, indicate $p < 0.005$) of pairwise comparisons of each individual group. For multiple group samples, statistical analyses were performed by one-way ANOVA with Tukey's HSD (honest significant difference) test. The different letter (a, b, c) indicates samples with statistically significant differences ($p < 0.05$), while the same letter indicates no statistically significant difference.

3 RESULTS

3.1 AtSR1 regulates the transcriptional expression of *NPR1* through direct binding to its promoter.

Our previous finding in the microarray data revealed that the transcriptional expression of *NPR1* was induced in *atsr1* as compared to WT, suggesting that *NPR1* is a potential target regulated by AtSR1: (Yuan, Du, & Poovaiah, 2018). We tested the expression levels of *NPR1* in *atsr1* mutant plants and found that *NPR1* is significantly induced in the mutant plants (Figure 1a). However, it is known that the transcriptional expression of *NPR1* is also induced by SA (Cao et al., 1997), and enhanced SA accumulates in *atsr1* (Du et al., 2009; Y. S. Kim et al., 2017). Hence, to clarify whether upregulation of *NPR1* in *atsr1* is due to the enhanced accumulation of SA, we tested the expression of *NPR1* in the SA deficient mutant *ics1*. The data showed that the expression of *NPR1* was still induced in *ics1 atsr1* double mutant plants as compared to *ics1* single mutant plants (Figure 1b). These results suggest that the expression of *NPR1* is directly regulated by AtSR1.

AtSR1 was reported to regulate gene expression through binding to the CGCG box in

promoter regions (Du et al., 2009). *In silico* analysis revealed that *NPR1* promoter contains a typical CGCG box at -1166 to -1161 relative to the putative translation initiation codon. To determine whether AtSR1 binds to the promotor of *NPR1* gene, the electrophoretic mobility shift assay (EMSA) was performed using a recombinant protein of AtSR1 DNA-binding domain (1-153 amino acid) fused with 6X His-tag and a radiolabeled *NPR1* promoter DNA fragment (from -1176 to -1148) that includes the CGCG box. As shown in Figure 1c, the AtSR1 protein fragment was able to interact with the *NPR1* promoter fragment (compare lane 1 and 2 in Figure 1c). In contrast, the AtSR1 protein fragment failed to interact with a mutated *NPR1* promoter fragment, where CGCG element was mutated to CCCG (lane 3 in Figure 1c). When a non-labeled *NPR1* promoter fragment was used as a competitor, the intensity of the shifted radioactive band was reduced in a dose-dependent manner (compare lane 1 with lane 4 and 5 in Figure 1c). These results demonstrated that AtSR1 protein directly interacts with the CGCG box in the *NPR1* promoter *in vitro*.

Arabidopsis protoplast-mediated transient expression assay was performed to evaluate the interaction between the *NPR1* promoter and the AtSR1 protein *in vivo*. The *NPR1* promoter (from -1500 bp to -1bp) was fused with luciferase (Luc) report gene and the *AtUBQ5* promoter was fused with GFP report gene as an internal control. These two genes were co-expressed in WT or *atsr1* protoplasts. As shown in Figure 1d, in comparison with the Luc activities in *atsr1* protoplasts, the relative Luc activities was reduced to around 40% in WT protoplasts, indicating that AtSR1 suppressed the expression of *NPR1*. Furthermore, the suppression of *NPR1* expression was compromised as we used mutated *NPR1* promotor (CCCG instead of CGCG). At the same time, the interaction between *NPR1* promoter and AtSR1 protein *in vivo* was further determined using the *Agrobacterium*−mediated transient expression assay in *Nicotiana Benthamiana*. Similarly, over-expression of AtSR1 protein suppressed *NPR1* promoter activity as compared to expression of GFP only, whereas activity of the mutated *NPR1* promotor was not suppressed by AtSR1 (Figure S1). In conclusion, AtSR1 protein suppressed the transcriptional expression of *NPR1* through direct binding to the CGCG box in the *NPR1* promoter *in vivo*.

In addition, we performed chromatin immunoprecipitation coupled with PCR (ChIP-PCR) to test the interaction between AtSR1 protein and *NPR1* promoter *in planta*. The *atsr1* mutant complemented with 35S::AtSR1-HA were generated in which the *AtSR1* coding region was fused to 2X HA-tag. Non transgenic *atsr1* mutant plants were used as a negative control and

EDS1 promoter was used as positive control as reported previously (Du et al., 2009). The ChIP-PCR results revealed that AtSR1 protein interacts with the *NPR1* promotor in a similar manner as that with the *EDS1* promoter (Figure 1e).

3.2 NPR1 is required for the auto-immune phenotype in *atsr1*

To test whether AtSR1 regulated NPR1-mediated immune response in plants, we generated *atsr1 npr1* double mutants (Figure S2) and confirmed that the expressions of both *AtSR1* and *NPR1* genes were not detected in the double mutant (Figure S2c). When these plants were grown in a relatively cool temperature (at 20°C), *atsr1* showed the typical auto-immune phenotype with upregulation of the SA marker genes, *PR1, PR2* and *PR5* (Figure 2). Interestingly, the *atsr1 npr1* double mutant rescued autoimmune phenotype that seen in the *atsr1* single mutant (Figure 2a) and alleviated constitutive upregulation of the SA marker genes (Figure 2b, 2c and 2d). Similar results were obtained when we used another allelic mutant line, *npr1-3* mutant in which a mutation (C1198T) causes a premature stop code that generates a truncated NPR1 protein (Figure S3). These results confirmed that NPR1-mediated signaling pathway is downstream of AtSR1 in plant immune system.

3.3 AtSR1 plays a role in the regulation of NPR1-mediated immune response

NPR1 functions as a co-transcription factor playing a positive role in basal resistance (Fu et al., 2012), whereas NPR1 plays a negative role in ETI-mediated HR since the *npr1* mutant displayed a stronger HR as compared to WT (Rate & Greenberg, 2001).

To test the effect of AtSR1 on NPR1-mediated basal resistance, we infiltrated rosette leaves of *atsr1*, *npr1*, and their double mutant plants with virulent strain of *Pst* DC3000 (OD600 = 0.001) to avoid triggering HR. Three days after inoculation, WT plants exhibited enhanced chlorotic spots on leaves, while *atsr1* mutant plants exhibited scarce chlorotic spots in their leaves (Figure 3a). Based on the measurement of bacteria grown in the infected leaves, the *atsr1* plants were more resistant (~10 times less infected) to the virulent *Pst* strain as compared to WT plants (Figure 3a). These results demonstrate that AtSR1 play an important role in the basal resistance. Interestingly, the *atsr1 npr1* double mutants reverted the phenotype seen in the *atsr1* single mutant, i.e., the double mutant showed enhanced chlorotic spots on leaves and more susceptible to the virulent *Pst* strain in comparison to the WT plants (Figure 3a and 3b),

demonstrating that *NPR1* is required for enhanced basal resistance in the *atsr1* mutant. Moreover, the *atsr1 npr1* double mutants exhibited reduced chlorotic areas and slightly more resistance to the virulent *Pst* strain in comparison to the *npr1* single mutant (Figure 3a and 3b). This result implies that AtSR1 negatively regulates NPR1-mediated signaling pathway in basal resistance.

To investigate the role of AtSR1 for NPR1-related ETI response, we infiltrated rosette leaves with avirulent strain *Pst* DC3000 carrying *avrRpt2* (OD600 = 0.01) to trigger ETI-mediated HR. Three days after inoculation, strong HR was observed in WT, while *atsr1* exhibited some chlorotic spots, but no HR in its leaves (Figure 3c). This non-HR phenotype were reverted to the phenotype causing HR in *atsr1 npr1*, i.e., *npr1* background. Bacterial measurement showed that the *atsr1* mutant plants were ~10 times more infected by the avirulent *Pst* strain than WT (Figure 3d), which was reduced in *npr1* background. The trypan blue staining assay revealed that the avirulent *Pst* strain induced strong HR in WT and *npr1*, and *atsr1 npr1* mutants, but not in *atsr1* mutant (Figure 3e). Intensity of HR was the strongest in the *npr1* mutant in consistency with the previous report (Rate and Greenberg, 2001). The HR intensity in the *atsr1 npr1* mutant was slightly reduced in comparison to the *npr1* single mutant. These results suggested that AtSR1 is positively regulates ETI response, where AtSR1 negatively regulates a NPR1 function.

The transcriptional expression of *NPR1* was highly upregulated in *atsr1* mutant plants, as compared to WT, but not in the *npr1* background (Figure 3f), which further induced the enhanced BR and reduced HR due to the disruption of the *NPR1* expression in *atsr1*. Higher transcriptional expression of *EDS1* and *PR1* in *atsr1* mutant plants was observed, no matter what *Pst* strains were used (Figure 3f). Only *EDS1* was induced by the avirulent *Pst* strain in the *atsr1 npr1* mutant, but not *NPR1*, which further confirmed that the failure to trigger HR in *atsr1* resulted from the mis-regulation of *NPR1,* but not only due to the SA accumulation.

3.4 IQ motif and CaM-binding domain are involved in AtSR1-regulated NPR1-mediate immune response

AtSR1 contains a CaM-binding domain (CaMBD) and an IQ-motif, both of which plays an important role for its function as a transcription factor (Doherty, Van Buskirk, Myers, & Thomashow, 2009; Du et al., 2009), although the function of the IQ motif is not clear. To test the

role of the IQ motif and CaMBD in plant immune response to pathogens infection, we used four complementation lines in the *atsr1 atsr4* (= *camta3 camta2*) double mutant background (ABRC: https://abrc.osu.edu/). Transgenes in the complementation lines were as follows: *cW* (WT AtSR1), *mIQ* (AtSR1^{A855V}), *mCaMBD* (AtSR1^{K907E}), and *mIQ+mCaMBD* (AtSR1$^{K807E/A855V}$). In consistency with the previous report (Kim et al., 2017), the complimentary line with *mIQ* and the double mutant *mIQ+mCaMBD* restored autoimmune phenotype of *atsr1 atsr4* to WT (Figure 4a) and suppressed the upregulation of the SA mark genes, *PR1*, *PR2* and *PR5* (Figure 4b). However, Complementation with the single mutant *mCaM* still displayed the auto-immunity phenotype and failed to suppressed the SA mark genes (Figure 4a and 4b). Furthermore, like as *atsr1 atsr4* mutant plants (non-complementation line), *mCAMBD* was more resistance to the infection by the virulence *Pst* strain, but neither *mIQ* nor *mIQ+mCaMBD* (Figure 4c and 4d).

The inoculation with the avirulent *Pst* strain (carrying *avrRpt2*) triggered HR in *mIQ* and *mIQ+mCaMBD*, but not in *mCaMBD* (Figure 4e). Bacterial measurement assay revealed that *mCaM* was more susceptible to the infection by the avirulence *Pst* strain due to failure to induce HR in infection site (Figure 4f). The trypan blue staining revealed that the avirulent *Pst* strain induced strong HR in *cW, mIQ and mIQ+mCaMBD* mutants as seen in non-transgenic WT, whereas it did not induce HR in the *mCaMBD* mutant as seen in the non-complementation line of *atsr1 atsr4* (Figure 4g). The transcriptional analysis revealed that both virulent and avirulence *Pst* strains induced the expression of *EDS1*, *NPR1,* and *PR1* higher in *mCaMBD*, like as that in the non-complementation line of *atsr1 atsr4* (Figure 4h). These observations suggested that CaMBD is required for AtSR1-mediated suppression of plant immune response through NPR1. In contrast, the IQ domain may be required for limiting the function of AtSR1 as an immune suppressor, given that immune phenotypes in *mCAMBD* were reverted in the *mIQ+mCAMBD* line.

3.5 ETI induced strong, prolonged Ca^{2+} signaling in plants

Distinct Ca^{2+} transients are formed during basal resistance- and ETI-mediated responses (Edel, Marchadier, Brownlee, Kudla, & Hetherington, 2017; Kudla et al., 2018; Lecourieux et al., 2006). Here, we performed aequorin-based Ca^{2+} measurement to test if the virulent *Pst* strain and the avirulent *Pst* strain trigger different Ca^{2+} signature. When the virulent *Pst* strain was applied, Ca^{2+} transient was induced at 3 min after inoculation, reached peak at 5 min and returned to basal

level at 7 min. (Figure 5a). When the avirulent *Pst* strain was applied, distinct Ca^{2+} transient was observed with induction started at 2min after inoculation, a peak at 8 min, and fluctuation at high levels until the end of the test (Figure 5b). Total integrated Ca^{2+} transient in 20 min after inoculation of the avirulent strain was greatly abundant in comparison to that of the virulent strain (Figure 5c). These Ca^{2+} transients were completely attenuated when a Ca^{2+} channel blocker, lanthanum chloride ($LaCl_3$) was applied 1h before the pathogen inoculation (Figure 5a-c). Taken together, our data indicated that different patterns of the Ca^{2+} signatures were generated for basal resistance response (by the virulent strain) and ETI-mediated HR response (by the avirulent strain).

3.6 Ca^{2+}/CaM plays a key role in AtSR1-dependent regulation of NPR1 expression.

To test how calmodulin (CaM) proteins interact with the IQ motif or CaMBD on AtSR1, we performed protein-protein interaction assay using a recombinant AtSR1 protein fragment ($AtSR1^{740-922}$), which contains the IQ motif and CaMBD (Figure 5d), and a known AtSR1-binding CaM, AtCAM2 (Du et al., 2009; Nie et al., 2012). Our result showed that AtCaM2 interacted with WT AtSR1 protein and its mutant at the IQ motif (mIQ) in the presence of Ca^{2+}, but not with the mutant protein mCaMBD (Figure 5e), suggesting that AtCaM2 is binding to CaMBD in a Ca^{2+} dependent manner. In contrast, AtCaM2 interacted with WT protein and mCaMBD in the absence of Ca^{2+} (in the presence of the Ca^{2+} chelator EGTA), but not with the mIQ protein (Figure 5e), suggesting that the IQ motif is responsible for the interaction with AtCaM2 in this case (Figure 5e).

To test the role of the IQ motif and CaMBD for AtSR1-mediated *NPR1* expression, we co-expressed the intact or mutated AtSR1 (mIQ and mCaMBD) with the *NPR1* promoter::*Luc* gene in the protoplasts of the *atsr1* mutant plants. The empty vector only expressing GFP was used as control and the relative *NPR1* promoter activity was measured. As shown in Figure 5f, WT-AtSR1 strongly suppressed the *NPR1* promoter activity. In addition, mIQ still suppressed the *NPR1* promoter activity. Interestingly, mCaMBD failed to suppress the promoter activity, whereas the double mutant (mIQ+mCaMBD) suppressed the *NPR1* promoter activity (Figure 5f), suggesting that the IQ motif inhibits the function of CaMBD. These observations suggest that Ca^{2+}/CaM plays a key role in the AtSR1-mediated *NPR1* expression.

4 DISCUSSION

AtSR1 has been studied in connection with SA-mediated plant immunity since its mutant, *atsr1*, displays an autoimmune phenotype with semi-dwarfism due to accumulation of SA as well as spontaneous lesions in leaves (Bruggeman, Raynaud, Benhamed, & Delarue, 2015; Rodriguez, El Ghoul, Mundy, & Petersen, 2016). In this study, the *atsr1* mutant displayed less chlorosis in infected leaves exhibited resistance to the virulent *Pst* strain in comparison to WT due to constitutively high basal resistance therein (Figure 3a and 3b). Inoculation of the avirulent *Pst* strain carrying *avrRpt2* induced a strong ETI-mediated PCD in WT, but a noticeable PCD was not manifested in the *atsr1* mutant plants (Figure 3c-3e). Thus, the *atsr1* mutant was susceptible the the avirulent *Pst* strain as compared to WT. Our current observations, together with previous studies, suggest that AtSR1 negatively regulates SA-mediate basal resistance, while it is involved in positive induction of ETI-mediated HR.

Based on the observation in the *atsr1 ics1* double mutant, a deficiency of SA biosynthesis appeared to restore the dwarf phenotype of *atsr1*, indicating that AtSR1 is a molecular component regulating the SA biosynthesis. However, the resistant phenotype of *atsr1* to virulent *Pst* strain is not affected even in the *ics1* background (Du et al., 2009), implying that there is another mechanism, by which *atsr1* shows resistance to the virulent *Pst* strain. Our previous study revealed that *NPR1* transcription was increased in *atsr1* mutant plants as compared to WT (Yuan, Du, et al., 2018). In this study, we further confirmed that AtSR1 directly regulates the transcriptional expression of *NPR1* through binding to the CGCG box in its promoter (Figure 1).

As mentioned above, ETI-mediated HR is not induced in the *atsr1* mutant plants. Similar results were reported in *npr3 npr4* double mutant plants (Ding et al., 2018). It is known that the stability of the SA receptor, NPR1, plays a key role in the establishment of HR (Bruggeman et al., 2015; Dong, 2004; Stael et al., 2015), in which NPR1 protein is degraded upon SA-triggered interaction with NPR3, an SA-dependent ubiquitin E3 ligase (Kuai et al., 2015). We observed that the autoimmune phenotype of *atsr1* was restored to that of WT in the *npr1* mutant background (Figure 2). In addition, when treated with the avirulent *Pst* strain, the *atsr1 npr1* double mutant plants displayed a strong ETI-mediated PCD at the infected site and consequently showed resistance to the pathogen in contrast to the *atsr1* single mutant plants (Figure 3c). Opposite results were observed during treatment with the virulent *Pst* strain (Figure

3a). These observations revealed that AtSR1 negatively regulates the expression of *NPR1* for fine-tuning plant immune responses, where NPR1 plays a positive regulator of basal resistance but a negative regulator of ETI-mediated HR.

It has been reported that PAMPs and pathogen-derived effecters induce Ca^{2+} transients (DeFalco et al., 2017; Downie, 2014; Keinath et al., 2015; Lammertz et al., 2019), however the duration and amplitude of Ca^{2+} transients are different. It has been well documented that Ca^{2+} transients are early events and are required for the activation of pattern-induced immunity for basal resistance in plants (Marcec et al., 2019). In contrast, the prolonged calcium transients were observed during the process of programmed cell death (Ngo, Vogler, Lituiev, Nestorova, & Grossniklaus, 2014; Zhivotovsky & Orrenius, 2011). NLRs function as receptors of pathogen elicitors to activate ETI (Castel et al., 2019; Maekawa, Kufer, & Schulze-Lefert, 2011). Recently, the structural analysis of CC-NLRs, ZAR1, revealed that oligomerization of ZAR1 forms a pentangular resistosome, a plant inflammasome (Wang et al., 2019). In mammalian cells, Ca^{2+} transients play a key role in inflammasome activation, such as NLRP3 inflammasome. However, it was not clear that Ca^{2+} transients are essential for the activation of resistosome in plants (Wang et al., 2019). We observed a prolonged and strong Ca^{2+} influx during ETI-induced PCD in plants (Figure 5), which provides further insights into the role of Ca^{2+} transients in ETI.

Ca^{2+} binding proteins, such as CaMs, CMLs, CPKs and CBL, are identified as cellular Ca^{2+} sensors which decrypt the distinct Ca^{2+} signatures into downstream signaling pathways to establish immune responses (Fischer et al., 2017; Poovaiah & Du, 2018; Weinl & Kudla, 2009; Yuan, Yang, & Poovaiah, 2018). CaMs have four EF-hand motifs that fall into two pairs: low Ca^{2+}-binding affinity sites (Kd ~10-12 μM) in the N-terminal domain and high-affinity sites (Kd ~ 1-2 μM) in the C-terminal domain (Hoffman, Chandrasekar, Wang, Putkey, & Waxham, 2014), suggesting that the dynamics of Ca^{2+} binding to each binding sites is also dramatically distinct. The N-terminal of CaM displays association and dissociation rates for Ca^{2+} of $>1x10^8$ $M^{-1}s^{-1}$ and >500 s^{-1}, whereas the C-terminal displays association and dissociation rates of ~ 0.05 x 10^8 $M^{-1}s^{-1}$ and ~10 s^{-1} (Hoffman et al., 2014; Liu, Whalley, & Knight, 2015; Whalley & Knight, 2013). This means that during transient Ca^{2+} flux, Ca^{2+} would bind to the N-terminal first before binding to the C-terminal, while Ca^{2+} would release from the N-terminal before releasing from the C-terminal, as transient Ca^{2+} flux ends. These traits enable CaM and other Ca^{2+} sensors, to decode different Ca^{2+} signatures.

CaM-binding proteins contain different domains that interact with CaM (Abdel-Hameed, Prasad, Jiang, & Reddy, 2020; Gilroy et al., 2014). CaMBD were found in most CaM-binding proteins, such as CBP60, CCaMKs and AtSRs/CAMTAs. CaMBDs only interact with Ca^{2+}-bound CaM, but not apo-CaM (Ca^{2+}-free CaM) (Miller et al., 2013; Reddy, Ali, Celesnik, & Day, 2011). Our data of in vitro binding assay demonstrated that Ca^{2+}-bound AtCaM2 directly binds to CaMBD of AtSR1 as we expected (Figure 5). The AtSR1-mediated suppression of *NPR1* expression was attenuated by a mutation at the CaMBD (Figure 4). There results suggested that CaMBD is activated by Ca^{2+}-bound CaM, which is required for AtSR1-mediated suppression of plant immune-related genes including *NPR1*.

The IQ motifs also interact with CaM either in the presence of Ca^{2+} (Fischer, Kugler, Hoth, & Dietrich, 2013; Jacob et al., 2017) or in the absence of Ca^{2+} (Thomas A DeFalco et al., 2016; Tian et al., 2019). Here, we observed that the IQ motifs in AtSR1 interact with AtCaM2 in the absence of Ca^{2+}, but not in the presence of Ca^{2+} (Figure 5). Moreover, the AtSR1 mutated at the IQ motif still suppressed the *NPR1* promoter activity, although intensity of the suppression was slightly stronger than WT AtSR1. Previous studies revealed that the gain-of-function mutant, *atsr1-4D*, which has A855V mutation in IQ motif, displayed opposite phenotype in comparison to the loss-of-function *atsr1* mutant, in terms of susceptibility to pathogen infection (Y. S. Kim et al., 2017; Nie et al., 2012). Our data also demonstrated that the complementation line with mutated transgene $AtSR1^{A855V}$ (*mIQ* in Figure 4) was more influenced on the immune phenotype than WT AtSR1 did. Interestingly, complementation with mutated transgene, $AtSR1^{A855V/K907E}$ (mIQ+mCaMBD in Figure 4), restored *atsr1* to WT phenotype, although complementation with the single mutant $AtSR1^{K907E}$ (mIQ) could not restore the *atsr1* phenotype. These data suggested that the IQ domain play a role for limiting the function of AtSR1 as an immune suppressor.

In conclusion, we document that AtSR1 directly suppresses the transcriptional expression of *NPR1*. NPR1 is known to enhance basal resistance but also negatively regulates ETI-mediated HR. Based on our data, together with the evidence reported previously, we propose a speculative model in Figure 6. The transient Ca^{2+} signature caused by virulent *Pst* strain creates a condition for the dynamic formation of both Ca^{2+}-bound CaM and apo-CaM, which regulate the function of AtSR1 by binding to the CaMBD and the IQ motif, respectively. That condition could transiently limit the AtSR1 function followed by de-regulation of *NPR1*, which eventually

confers basal disease resistance to plants. This scheme could also apply to the mechanism of AtSR1-mediated de-suppression of *ICS1* and *EDS1* for SA-mediated basal resistance. The auto-immune phenotype in the *atsr1* mutant can be explained by constitutive de-suppression of *NPR1* (and also *ICS1* and *EDS1*) that provides more resistance to virulent *Pst* strain due to higher basal resistance. On the other hand, the prolonged, strong Ca^{2+} signature caused by the avirulent *Pst* strain likely activates AtSR1 function through interaction between Ca^{2+}-bound CaM and the CaMBD on AtSR1. Activated AtSR1 suppresses the expression of NPR1, the negative regulator of ETI, and subsequently enhances HR. In *atsr1* plants, *NPR1* was constitutively de-suppressed (i.e., upregulated) due to the absence of functional AtSR1, which makes the *atsr1* mutant susceptible to avirulent *Pst* strain due to the failure to trigger ETI-mediated HR. Our present study provides insights into the mechanism of the Ca^{2+}/CaM-mediated functional switch in AtSR1 for plant immune response.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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741 **FIGURE LEGENDS**

742

743 **FIGURE 1.** AtSR1 regulates the transcriptional expression of *NPR1*. (a) and (b) Basal
744 expression of *NPR1* gene was compared between WT and *atsr1* mutant or *ics1* and *ics1 atsr1*
745 mutants. Rosette leaves of 4- or 5-week-old plants were harvested for real-time PCR analysis.
746 Expression of the *NPR1* gene was normalized to that of the *Actin2* gene. Values are means ± SD
747 of three biological replicates. Asterisks (***) indicate statistical significance based on a one-way
748 analysis of variance (one-way ANOVA) test ($p < 0.005$) of pairwise comparisons for each
749 individual group. (c) Lane 1, ^{32}P-labelled *NPR1 Promoter fragment (NPR1P,* -1164 to -1158) +
750 AtSR1; Lane 2, ^{32}P-labelled *NPR1P* only; Lane 3, ^{32}P-labelled mutated *NPR1P* (*mNPR1P*,
751 C<u>G</u>CG to C<u>C</u>CG) + AtSR1; Lane 4 and 5, ^{32}P-labelled *NPR1P* + AtSR1 mixed with unlabeled
752 *NPR1P* (10 and 200 times the amount of radiolabeled fragment, respectively). (d) The relative
753 transcriptional activity of intact *NPR1* promoter (*NPR1P::Luc*) or its mutated version
754 (*mNPR1P::Luc*) was measured in the protoplasts isolated from WT or the *atsr1* mutant. *AtUBQ5*
755 promotor fused to GFP was used as an internal control. The ratio of Luc/GFP was measured as
756 the *NPR1* promoter activity. Data are expressed as mean ± SD (n = 4). Different letters indicated
757 statistically significant differences among treatments with one-way ANOVA ($p < 0.05$)
758 followed by Tukey test. (e) Chromatin immunoprecipitation (ChIP) associated with AtSR1-HA
759 protein in *atsr1* background was measured in the sample shown in the left lane in each photo,
760 while non-transgenic vector control was shown in the right lane. The precipitated DNA was
761 amplified using primers flanking the "CGCG" box in the *NPR1* promoter (left panel) and the
762 *EDS1* promoter (as control, right panel).

763

764 **FIGURE 2.** The suppression of *atsr1/camta3* autoimmunity in *npr1*. (a) For phenotypic
765 comparison, 5-week-old plants of WT, *atsr1, npr1* and the *atsr1npr1* mutants grown at 20°C.
766 (b)-(d) qRT-PCR revealed the transcriptional expression of *PR1* (B), *PR2* (C), and *PR5* (D) in
767 the WT and mutant plants. The gene expressions were normalized to that of the *UBQ5* gene.
768 Data are expressed as mean ± SD (n = 3). Different letters indicated statistically significant
769 differences among treatments by one-way ANOVA ($p < 0.05$) with Tukey test.

770

771 **FIGURE 3.** The *npr1* mutant suppresses the phenotype of the *atsr1* mutant in hypersensitive

response and SA-mediated immunity. (a) and (b). The SA-mediated basal resistance responses in WT, *atsr1*, *npr1*, and *atsr1 npr1* mutants were evaluated at 3 days post inoculation (d.p.i.). Virulent pathogen *Pst* DC3000 (OD600 = 0.001) was infiltrated into rosette leaves to avoid HR. The colony forming units (c.f.u.) at 3 d.p.i. are shown. The gene expressions were normalized to that of the *UBQ5* gene. Results are expressed as mean ± SD (n =3). (c) and (d). The hypersensitive responses (HR) in WT, *atsr1*, *npr1*, and *atsr1 npr1* mutants were evaluated at 3 d.p.i. Avirulent pathogen *Pst* DC3000 (*carrying* avrRpt2, OD600 = 0.01) was infiltrated into rosette leaves for inducing HR. Data are expressed as mean ± SD (n = 6-8). (e)**.** Trypan Blue Staining Assay were performed to evaluate *Pst*-induced HR. (f). qRT-PCR revealed the transcriptional expression of *EDS1, NPR1 and PR1* in the WT and mutant lines at 12 h post inoculation with the avirulent *Pst* strain (carrying *avrRpt2*) or the virulent strain *Pst* strain. The gene expressions were normalized to that of the *UBQ5* gene. Results are expressed as mean ± SD (n = 3). Different letters indicated statistically significant differences among treatments analyzed by one-way ANOVA ($p < 0.05$) with Tukey test.

FIGURE 4. Involvement of IQ motif and CaM-binding domain in AtSR1-regulated NPR1-related immune response. (a)**.** Five-week-old plants of WT, *atsr1 atsr4,* and complementation lines in *atsr1 atsr4* background grown at 20°C. (b)**.** qRT-PCR revealed the transcriptional expression of *PR1, PR2, and PR5* in the WT and the complementation lines. The gene expressions were normalized to that of the *UBQ5* gene. Results are expressed as mean ± SD (n = 4). (c). The pictures show rosette leaves of WT and the complementation lines at 3 days post inoculation (d.p.i.) with the infiltration of the avirulent strain of *Pst* DC3000 (OD600 = 0.001). (d). The colony forming units (c.f.u.) at 3 d.p.i. are shown. (e). The pictures show rosette leaves of WT and mutant at 3 days post inoculation (d.p.i.) with the infiltration of the avirulent strain *Pst* DC3000 carrying *avrRpt2* (OD600 = 0.01) to induce HR. (f). The colony forming units (c.f.u.) at 3 d.p.i. are shown. (g)**.** Results from Trypan Blue Staining are shown. Genotype is marked beneath the panel. (h). qRT-PCR revealed the transcriptional expression of *EDS1, NPR1, and PR1* in the WT and mutant at 12 h post inoculation with the avirulent *Pst* strain, *Pst* DC3000, and the virulent *Pst* strain. The gene expressions were normalized to that of the *UBQ5* gene. Results are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences among treatments analyzed by one-way ANOVA ($p < 0.05$) with

Tukey test.

FIGURE 5. Ca^{2+}- and CaM-dependent activities of AtSR1 on the *NPR1* transcription. Pathogen-induced cytosolic Ca^{2+} elevation in leaves of aequorin-expressing *Arabidopsis* plants. (a), (b) The virulent Pst strain (OD = 0.001) (a) and the avirulent Pst strain carrying avrRpt2 (OD = 0.01) (b) were added with or without 10mM $LaCl_3$. The signals shown are mean values ± SD (n = 4). (c)The histogram shows total $[Ca^{2+}]_{cyt}$ 20 min after pathogen addition. (d) Amino acid sequences surrounding IQ motif (First IQ and second motifs are enclosed by orange color and green borders, respectively) and CaMBD (enclosed by a red border) are shown in which the mutated amino acids targeted are represented in red text. (e) HRP-labelled AtCaM2-binding assay. The recombinant proteins of the wild-type AtSR1 (WT) and three mutants were immobilized on PVDF membrane followed by incubation with the HRP-labelled AtCaM2 protein. Bound CaM-HRP was visualized using a chemiluminescent reagent. Coomassie staining was used as a loading control (Coomassie blue staining). (f) The reporter assay of the *NPR1* promoter using *NPR1P*::Luc was performed by co-expression of intact or mutated AtSR1 fused to GFP in the *atsr1* protoplasts. Empty GFP was used as negative control. The ratio of Luc/GFP activities was measured. Data are expressed as mean ± SD (n = 4). Different letters indicate statistically significant differences among treatments analyzed by one-way ANOVA ($p < 0.05$) with Tukey test.

FIGURE 6. Proposed model explaining how Ca^{2+}/CaM-mediated AtSR1/CAMTA3 regulates NPR1-mediated plant immune response. In the absence of pathogens, there is no $[Ca^{2+}]_{cyt}$ dynamics. Hence, the status of CaM is not in a saturated phase at four Ca^{2+} binding sites, designated as as apo-CaM. Apo-CaM interacts with the IQ motifs of AtSR1. This CaM-AtSR1 complex may have an effect on the stability of AtSR1, which suppresses the transcriptional expression of *NPR1* as an autoregulator. Thus, suppression of the NPR1-mediated plant immunity maintains normal plant growth and development. Under the virulent pathogen infection, $[Ca^{2+}]_{cyt}$ is transiently increased which confers a status of mixture of Apo-CaM and Ca^{2+}-saturated CaM. Ca^{2+}-saturated CaM interacts with the CaMBD of AtSR1, while Apo-CaM interacts with the IQ motifs. This CaM-AtSR1 complex loses its binding activity to the cis-element "CGCG", and de-regulates the transcriptional expression of *NPR1*. This eventually

activates the NPR1-mediated basal resistance. Under avirulent pathogen infection, $[Ca^{2+}]_{cyt}$ is constitutively increased, which leads to stable binding of Ca^{2+} ions to CaM. The Ca^{2+}-saturated CaM interacts only with the CaMBD of AtSR1. This CaM-AtSR1 complex strongly suppresses the transcriptional expression of *NPR1*, and thereby prevents NPR1 from negatively regulating the ETI-triggered PCD. In the *atsr1* mutant, due to loss of negative regulation of the *NPR1* transcription by AtSR1, the plants constitutively activate basal immune response, resulting in the autoimmune phenotype. Under pathogen infection in the *atsr1* mutant due to the lack of fine-tuning for transcriptional regulation of *NPR1*, the immune response is disturbed in the mutants, i.e., enhanced basal resistance and failure of ETI-induced PCD to the virulent and avirulent bacterial pathogens, respectively.