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SR1, a Calmodulin Binding Transcription Factor, Modulates Plant Defense and Ethylene-Induced Senescence by Directly Regulating NDR1 and EIN3

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ABSTRACT

Plant defense responses are tightly controlled by many positive and negative regulators to cope with attacks from various pathogens. Arabidopsis EDR2 (ENHANCED DISEASE RESISTANCE 2) is a negative regulator of powdery mildew resistance and edr2 mutants display enhanced resistance to powdery mildew (Golovinomyces cichoracearum). To identify components acting in the EDR2 pathway, we screened for edr2 suppressors and identified a gain-of-function mutation in SRI (SIGNAL RESPONSIVE 1), which encodes a calmodulin-binding transcription activator. The sr1-4D gain-of-function mutation suppresses all edr2-associated phenotypes, including powdery mildew resistance, mildew-induced cell death and ethylene-induced senescence. The sr1-4D single mutant is more susceptible to a Pseudomonas syringae pv. tomato (Pto) DC3000 virulent strain and to avirulent strains carrying avrRpt2 or avrRPS4 than wild type. We show that SR1 directly binds to the promoter region of NDR1, a key component in RPS2-mediated plant immunity. Also, the ndr1 mutation suppresses the sr1-1 null allele, which shows enhanced resistance to both Pto DC3000 avrRpt2 and G. cichoracearum. In addition, we show that SR1 regulates ethylene-induced senescence by directly binding to the EIN3 promoter region in vivo. Enhanced ethylene-induced senescence in sr1-1 is suppressed by ein3. Our data indicate that SR1 plays an important role in plant immunity and ethylene signaling by directly regulating NDR1 and EIN3.
INTRODUCTION

Plants encounter a wide variety of pathogens in the wild and to counter this threat, plants have evolved two layers of immune defenses, including pathogen/microbe associated molecular patterns (PAMP or MAMP) trigged immunity (PTI) and effector triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006). In ETI, pathogen effectors delivered into the plant cell are recognized by cognate cytoplasmic immune receptors traditionally called resistance (R) proteins, which subsequently triggers specific defense responses. In Arabidopsis, many R genes encode structurally related proteins containing NBS (nucleotide binding site) and LRR (Leucine-rich repeat) domains. Based on N-terminal sequences, the NBS-LRR proteins can be further divided into two subfamilies: proteins containing a coiled coil domain (CC-NBS-LRR) and proteins containing a domain homologous to Toll and Interleukin-1 receptors (TIR-NBS-LRR). In general, CC-NBS-LRR mediated resistance requires NDR1, a plasma membrane localized protein (Century et al., 1997; Coppinger et al., 2004), and TIR-NBS-LRR mediated resistance requires EDS1, a protein with similarity to lipases (Aarts et al., 1998). For instance, RPS2, RPM1 and RPS5 mediated resistance is dependent on NDR1, but RPP2, RPP4 and RPS4 mediated resistance is dependent on EDS1.

Based on their infection strategy, pathogens can be divided into two broad classes: the first class is biotrophic pathogens, such as the fungal pathogen powdery mildew; the second class is necrotrophic pathogens, such as Botrytis cinerea (Glazebrook, 2005). Biotrophic pathogens depend on living host cells for invasion and reproduction. Increasing evidence has shown that salicylic acid (SA) signaling usually is involved in the defense against biotrophic pathogens while jasmonic acid (JA) and ethylene (ET) signaling are involved in the defense against necrotrophic pathogens. Powdery mildew pathogens are obligate biotrophs that infect a broad range of crop species including barley, wheat, and grape, and cause large worldwide economic losses (Micali et al., 2008).
powdery mildew, three major types of mutants with altered responses to powdery mildew pathogens have been identified. The first class of Arabidopsis mutants shows defects in nonhost penetration resistance to the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*); these mutants include *pen1*, *pen2* and *pen3* (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). The second class of mutants show increased powdery mildew resistance, but without mildew-induced cell death; this class is represented by *pmr1* (*powdery mildew resistant 1*) to *pmr6* (Vogel and Somerville, 2000; Vogel et al., 2002; Nishimura et al., 2003; Vogel et al., 2004). The third class of mutants is represented by enhanced disease resistance (*edr*) mutants, including *edr1*, *edr2*, and *edr3*, which show increased disease resistance to powdery mildew that is accompanied by mildew-induced cell death (Frye et al., 2001; Tang et al., 2005, 2006). Like the *edr1* and *edr3* mutants, *edr2*-mediated resistance is dependent on an intact SA signaling pathway. The EDR2 protein contains a pleckstrin homology (PH) domain, a StAR transfer (START) domain and a plant specific domain of unknown function (Tang et al., 2005; Vorwerk et al., 2007). The PH domain binds to Phosphatidylinositol 4-phosphate (PI4P) *in vitro*. The EDR2 protein localizes to the endoplasmic reticulum, plasma membrane and endosomes (Vorwerk et al., 2007). However, the mechanism by which EDR2 regulates powdery mildew resistance is not clear.

The interaction between plants and powdery mildew pathogens is conserved among different plant species. For example, in barley, MLO, an integral membrane protein with seven transmembrane domains, acts as a negative regulator of powdery mildew resistance (Büschges et al., 1997; Kessler et al., 2010). In Arabidopsis, MLO2, the ortholog of barley MLO, plays a similar role (Consonni et al., 2006). Interestingly, ROR2, the barley ortholog of PEN1, is required for *mlo*-mediated penetration resistance in barley (Bhat et al., 2005). Also, calcium signaling is required for MLO signaling (Kim et al., 2002). Calcium is a second messenger in biotic and abiotic stress signaling; these stresses induce temporal changes in cytosolic free Ca$^{2+}$, which is called the calcium signature (Reddy, 2001; Hepler, 2005; Kim et al., 2009).
Calcium signatures are decoded by calcium sensors, a class of calcium binding proteins (Dodd et al., 2010). The predominant sensor is calmodulin, which has four EF-hands that binds to calcium and relays calcium signaling by binding to its target proteins. Several calmodulin binding proteins have been shown to play important roles in plant innate immunity. For instance, MLO binds to calmodulin in vitro, and loss of calmodulin binding activity affects MLO function (Kim et al., 2002). In addition, CBP60g, a member of the Arabidopsis CBP60 gene family, regulates MAMP signaling and SA accumulation through calcium dependent calmodulin binding (Wang et al., 2009). Furthermore, SR1, a calmodulin binding transcription factor, contributes to plant defense responses by binding to the CGCG box in the promoter of its target genes to regulate their expression (Yang and Poovaiah, 2002). One of its targets is EDS1, a positive regulator of SA signaling. SR1 calmodulin binding activity is essential for its function (Du et al., 2009).

To identify genes that are involved in plant defense responses, we screened for suppressors of edr2. Here, we show that a gain-of-function mutation in the calmodulin binding motif of SR1 suppressed edr2-mediated resistance to powdery mildew and enhanced ethylene-induced senescence in edr2. We also show that SR1 regulates plant defense responses and senescence by directly binding to the promoter regions of NDR1 and EIN3.

RESULTS

The sr1-4D Mutation Suppressed edr2-Mediated Powdery Mildew Resistance and Ethylene-Induced Senescence

Previously, edr2 has been shown to display enhanced disease resistance to powdery mildew pathogen Golovinomyces cichoracearum strain UCSC1 (Tang et al., 2005; Vorwerk et al., 2007). To identify components that are involved in EDR2 signaling, we screened for mutants that suppressed the edr2 enhanced resistance phenotype. In this screen, we identified a number of suppressors, including mutations in PAD4,
SID2, NPR1 and ALD1, indicating that the screen was highly efficient (Nie et al., 2011). Here, we describe one of these mutants, which we named sr1-4D based on our subsequent characterizations described below; other edr2 suppressor mutants will be described elsewhere.

To characterize the powdery mildew resistance of edr2 sr1-4D, four-week-old plants were inoculated with G. cichoracearum, and the disease symptoms were scored at 8 days post infection (dpi). The wild type plant was susceptible and intensive sporulation was observed on the leaves, but the edr2 plant showed dramatic necrotic lesion formation upon mildew infection, and little powder was produced. The edr2 sr1-4D plants displayed a wild type like phenotype that supported formation of a large number of conidia on the leaves, with no visible necrotic cell death observed at 8 dpi (Figure 1A), indicating that sr1-4D suppressed the edr2 powdery mildew resistance phenotype. To further characterize the edr2 sr1-4D disease phenotype, we examined plant host cell death and fungal pathogen growth by staining the infected leaves with trypan blue at 8 dpi. As shown in Figure 1B, infected leaves of edr2 displayed massive necrotic cell death, with many fewer spores produced by the fungus compared to those of wild type, but edr2 sr1-4D displayed extensive fungal growth with no obvious cell death, similar to the wild type plants. Previously, it was shown that the edr2-mediated powdery mildew cell death was accompanied by production of hydrogen peroxide (H2O2) in the cells that undergo cell death (Vorwerk et al., 2007).

To examine whether H2O2 production was suppressed in edr2 sr1-4D, we monitored the H2O2 production in wild type, edr2 and edr2 sr1-4D by staining infected leaves with 3,3’-diamino benzidine hydrochloride (DAB) at 2 dpi. As shown in Figure 1C and 1D, edr2 accumulated more H2O2 than wild type, but edr2 sr1-4D accumulated H2O2 to a much lower level than edr2, indicating that the accumulation of H2O2 in edr2 was suppressed by the sr1-4D mutation. To further assess the effects of sr1-4D on the resistant phenotype of edr2, we monitored fungal growth by counting the conidiophores (asexual reproductive structures) per colony in wild type, edr2 and edr2 sr1-4D leaves at 7 dpi. The edr2 mutant supported significantly fewer
conidiophores than wild type, but \textit{edr2 sr1-4D} supported a much higher number of conidiophores than \textit{edr2} or wild type (Figure 1E).

The disease resistance mediated by \textit{edr2} is correlated with activation of the salicylic acid (SA) signaling pathway. The defense related gene \textit{PRI} is induced more quickly and to higher levels in \textit{edr2} than in wild type (Tang et al., 2005; Vorwerk et al., 2007). To investigate whether \textit{sr1-4D} affects \textit{PRI} expression in \textit{edr2}, we used quantitative RT-PCR to examine \textit{PRI} transcript levels in wild type, \textit{edr2} and \textit{edr2 sr1-4D} at different time points after powdery mildew infection. As shown in Figure 1F, the \textit{PRI} transcript level was very low in all plants in the absence of pathogen. However, at 4 dpi the \textit{PRI} transcript level was much higher in \textit{edr2} than wild type, but was much lower in \textit{edr2 sr1-4D} than in \textit{edr2} and wild type, indicating that \textit{sr1-4D} fully suppressed the accumulation of \textit{PRI} transcripts upon powdery mildew infection in \textit{edr2}.

In addition to powdery mildew resistance, \textit{edr2} also shows an enhanced ethylene-induced senescence phenotype (Tang et al., 2005). To investigate whether \textit{sr1-4D} suppressed the \textit{edr2} senescence phenotype, four-week-old wild type, \textit{edr2} and \textit{edr2 sr1-4D} were treated with 100 $\mu$g l$^{-1}$ ethylene for 3 days. In wild type, ethylene induced senescence in old leaves, but the \textit{edr2} mutant displayed more severe senescence phenotypes, and the senescence occurred in much younger leaves (Figure 1G). In contrast, the \textit{edr2 sr1-4D} mutant displayed delayed senescence compared to \textit{edr2} and wild type, indicating that \textit{sr1-4D} also suppressed the \textit{edr2}-mediated ethylene-induced senescence. To quantify this phenotype, we measured the senescence-associated decline in chlorophyll content and found that \textit{edr2} lost more chlorophyll than wild type, but \textit{edr2 sr1-4D} had significantly more chlorophyll than wild type and \textit{edr2} after ethylene treatment (Figure 1H). Taken together, these data indicated that \textit{sr1-4D} fully suppressed all \textit{edr2}-associated phenotypes, and \textit{sr1-4D} conferred enhanced disease susceptibility, further delayed ethylene-induced leaf senescence even in the \textit{edr2} background in comparison with wild type (Figure 1).
Identification of the sr1-4D Mutation

Genetic analysis showed that sr1-4D acts as a dominant mutation, as the original edr2 sr1-4D mutant segregated both edr2 and suppressed plants. Also, the edr2/edr2 SR1/sr1-4D plants displayed the same phenotypes as edr2/edr2 sr1-4D/sr1-4D plants. To map the sr1-4D mutation, we crossed a homozygous edr2 sr1-4D plant with Landsberg erecta (Ler) to generate a mapping population. Initially, we mapped the sr1-4D mutation to a region on Chromosome 2 between markers T26C24 and F3N11. Using a large number of F3 plants, we narrowed down the sr1-4D mutation to about 100kb (Figure 2A). We then sequenced the candidate genes in this region. A single nucleotide (C to T) change was identified in At2g22300 at 2564nt in the coding sequence; this change was predicted to produce an amino acid change (A855V) (Figure 2A).

Because sr1-4D is a dominant mutation, it cannot be tested by traditional complementation. Instead, to confirm that At2g22300 is the gene responsible for the sr1-4D mutant phenotype, we tested whether introduction of the sr1-4D mutant genomic sequences could suppress edr2. To that end, we generated a genomic clone of At2g22300 by amplification of the genomic sequence from a homozygous edr2 sr1-4D mutant plant. This genomic clone contained the full-length At2g22300 gene, consisting of the coding sequence flanked by a 1.4kb upstream promoter region and a 0.8kb downstream sequence. We then transformed this genomic clone into the edr2 mutant and the transgenic lines exhibited susceptibility to powdery mildew (Figure 2B), indicating that this particular mutation in the At2g22300 gene suppressed the edr2 phenotype. Therefore, suppression of edr2 phenotype in edr2 sr1-4D was caused by a mutation in the At2g22300 gene.

The At2g22300 gene was previously designated SRI (SIGNAL RESPONSIVE 1) (also known as CAMATA 3); we therefore designated the edr2 suppressor sr1-4D. SR1 is a transcription factor that contains two IQ motifs, which are known to be calmodulin
binding domains (Yang and Poovaiah, 2002). The \( sr1-4D \) mutation (A855V) is in the first IQ motif (Figure 2C), in an amino acid that is highly conserved in the SR1 homologs in multiple plant species (Figure 2D).

**Responses of \( sr1-4D \) and \( sr1-1 \) to Bacterial and Fungal Pathogens**

To investigate whether \( SR1 \) expression is induced by pathogens, we examined the \( SR1 \) transcript levels in plants inoculated with the bacterial pathogen \( Pto \) DC3000 or the fungal pathogen \( G. cichoracearum \). The levels of \( SR1 \) transcript were higher at 5 days post inoculation by \( G. cichoracearum \) (Supplemental Fig. S1A) and 9 hours post inoculation by \( Pto \) DC3000 (Supplemental Fig. S1B).

Previously it was shown that the loss-of-function mutant \( sr1-1 \) displayed enhanced resistance to \( Pto \) DC3000 and \( Botrytis cinerea \) (Galon et al., 2008; Du et al., 2009). To further investigate the role of SR1 in plant innate immunity, we tested the responses of both \( sr1-1 \) and \( sr1-4D \) mutants to virulent and avirulent strains of \( Pto \) DC3000 and to the fungal pathogens \( G. cichoracearum \) and \( B. cinerea \). The \( sr1-1 \) mutant was more resistant to virulent \( Pto \) DC3000 and to the avirulent strains \( Pto \) DC3000 (avrRpt2) and \( Pto \) DC3000 (avrRPS4), which carry effectors that are recognized by the CC-NBS-LRR protein RPS2 or TIR-NBS-LRR protein RPS4 respectively. In contrast, the \( sr1-4D \) mutant displayed enhanced susceptibility to these bacterial strains (Figure 3A, 3B and 3C).

Similarly, for the fungal pathogen \( G. cichoracearum \), \( sr1-1 \) displayed \( edr2 \)-like powdery mildew resistance and mildew-induced necrotic cell death, but \( sr1-4D \) was highly susceptible and supported significantly more conidiophore formation than wild type (Figure 3D, 3E, 3F). \( sr1-1 \) was also more resistant than wild type to the necrotrophic pathogen \( B. cinerea \) (Galon et al., 2008) (Figure 3G, 3H); by contrast, \( sr1-4D \) was more susceptible to this pathogen. Taken together, these data indicate that SR1 plays an important role in plant innate immunity by negatively regulating defense responses. Also, the loss-of-function mutant \( sr1-1 \) displayed opposite phenotypes to
the sr1-4D mutant, suggesting that sr1-4D is a gain-of-function mutation.

Both edr1 and edr2 show enhanced disease resistance to powdery mildew, mildew-induced cell death, and ethylene-induced senescence. To examine whether the sr1-4D mutation can suppress edr1 phenotypes, we infected the edr1 sr1-4D double mutant with G. cichoracearum and assessed the disease phenotype by staining the infected leaves at 8 dpi. The edr1 sr1-4D double mutant was susceptible to powdery mildew, supporting extensive fungal growth and showing no necrotic cell death at 8 dpi, indicating that the sr1-4D mutation also fully suppressed the edr1 mutant phenotype (Supplemental Fig. S2A, S2B).

Previously, it was shown that the sr1-1 mutant accumulates high levels of SA and has a temperature dependent growth phenotype (Du et al., 2009). To examine the growth phenotypes of sr1-4D, we grew wild type, sr1-4D and sr1-1 plants at lower (19-21°C) or higher (25-27°C) temperature. At 25-27°C, the growth of wild type, sr1-4D and sr1-1 plants was similar, and no difference between the wild type and mutant plants was observed (Supplemental Fig. S3A). However, at 19-21°C, the gain-of-function mutant sr1-4D was significantly larger than wild type (Supplemental Fig. S3B, S3C). And the relative expression of defense related genes PR1, PR2 and PR5 was significantly lower in sr1-4D than in wild type at 19-21 °C (Supplemental Fig. S3D, S3E, S3F). To investigate whether sr1-4D has defects in SA accumulation, we measured the SA levels of five-week-old wild type, sr1-4D and sr1-1 plants grown at 19-21°C. Consistent with previous finding, the sr1-1 mutant accumulated higher levels of SA (Du et al., 2009), while the sr1-4D mutant accumulated significantly lower levels of SA, compared to wild type (Supplemental Fig. S4A, S4B). Consistent with this observation, the relative expression of SID2, PAD4, EDS1 and EDS5, was significantly lower in sr1-4D than in wild type (Supplemental Fig. S4C, S4D, S4E, S4F).
SR1 Directly Binds to the NDR1 and EIN3 Promoters

SR1 is a transcription factor that binds to promoters that contain a CGCG box (Yang and Poovaiah, 2002). Previously, it was shown that SR1 binds to the promoter of EDS1, a key regulator of plant defense responses, and represses EDS1 expression (Du et al., 2009). EDS1 is required by TIR-NBS-LRR type R proteins, such as RPS4, which recognizes the bacterial effector avrRPS4. In contrast, NDR1, a membrane associated protein, is required for several CC-NBS-LRR type R proteins, including RPS2 (Century et al., 1995), which is responsible for resistance to Pto DC3000 carrying avrRpt2 (Aarts et al., 1998). Since the loss-of-function sr1-1 mutant displayed enhanced disease resistance and the gain-of-function sr1-4D mutant displayed enhanced disease susceptibility to Pto DC3000 (avrRpt2), we hypothesized that NDR1 may be another direct target of SR1. Consistent with this hypothesis, NDR1 is up-regulated in sr1-1 according to microarray data (Galon et al., 2008). In addition, analysis of the NDR1 promoter sequence revealed a CGCG box (Supplemental Fig. S5), which could be a potential SR1 binding site.

To investigate whether SR1 regulates NDR1, we first examined NDR1 expression levels in sr1-1 and sr1-4D. Interestingly, the level of NDR1 transcript was higher in sr1-1 but lower in sr1-4D, compared to wild type (Figure 4A), indicating that mutations in SR1 do affect NDR1 expression. To examine whether SR1 directly binds to the NDR1 promoter, we expressed and purified recombinant SR1-N terminal truncated protein (1-146aa), which contained the DNA binding domain fused with a Glutathione S-transferase (GST) tag and performed DNA Electrophoretic Mobility-Shift Assays (EMSA). SR1-N was able to bind to the radiolabeled NDR1 promoter fragment in vitro, and the binding was blocked by addition of unlabeled NDR1 promoter fragment, but not by an NDR1 promoter fragment with mutation in the core binding sequence (CGCG box) (Figure 4B). To further confirm that SR1 binds to the NDR1 promoter, we performed chromatin immunoprecipitation (ChIP) assays. We first constructed transgenic plants that contained SR1-GFP with the
dexamethasone (DEX) inducible promoter. We then conducted ChIP assays with this transgenic line to examine whether SR1-GFP binds to the \textit{NDR1} promoter. The promoter of \textit{NDR1} was enriched in the chromatin immunoprecipitated DNA with the anti-GFP antibody; as a control, an \textit{ACTIN2} promoter sequence was not enriched in the same assay (Figure 4C), indicating that SR1-GFP binds to the promoter of \textit{NDR1} \textit{in vivo}, and thus that \textit{NDR1} is a direct target of SR1.

\textit{SR1} was first reported as an ethylene-induced gene (\textit{EICBP1}); also, \textit{SR1} was reported to bind to the promoter of \textit{EIN3}, a key component of ethylene signaling, \textit{in vitro} (Reddy et al., 2000; Yang and Poovaiah, 2002). However, to date, whether \textit{SR1} is involved in ethylene signaling has not been determined. To gain insight into the role of \textit{SR1} in ethylene signaling, we treated four-week-old \textit{sr1-1} and \textit{sr1-4D} plants with ethylene for 3 days and evaluated their leaf senescence phenotypes. We found that \textit{sr1-1} showed enhanced ethylene-induced senescence, but \textit{sr1-4D} was insensitive to ethylene (Supplemental Fig. S6), indicating that \textit{SR1} may indeed regulate ethylene-induced senescence. To test whether \textit{SR1} binds to the \textit{EIN3} promoter, we performed ChIP assays, as described above. The \textit{EIN3} promoter was also enriched in the pool of sequences immunoprecipitated with the anti-GFP antibody (Figure 4C), indicating that \textit{SR1} binds to the \textit{EIN3} promoter \textit{in vivo}; thus, \textit{EIN3} is also a direct target of \textit{SR1}.

To further investigate the regulation of \textit{EIN3} by \textit{SR1}, We examined relative expression of \textit{EIN3} in ethylene treated or untreated wild type, \textit{sr1-4D} and \textit{sr1-1} plants. As shown in Supplemental Fig. S7A and S7B, relative expression of \textit{EIN3} is higher in \textit{sr1-1}, but lower in \textit{sr1-4D} than in wild type, which is consistent with the negative role of \textit{SR1} in \textit{EIN3} expression.

The \textit{nrd1} Mutation Suppresses \textit{sr1-1} Mediated Resistance to \textit{Pto DC3000 (avrRpt2)} and \textit{G. cichoracearum}

Since \textit{NDR1} is a direct target of \textit{SR1}, and \textit{NDR1} expression increased in the
loss-of-function sr1-1 mutant, SR1 likely regulates plant defense by repressing NDR1 expression. Therefore, the enhanced disease resistance phenotype of the sr1-1 mutant is at least partially due to high expression of NDR1. To test this hypothesis, we examined whether the ndr1 mutation can suppress the sr1-1 phenotype of enhanced resistance to Pto DC3000 (avrRpt2). As shown in Figure 5, the ndr1-3 mutation suppressed the resistance phenotype of sr1-1 to Pto DC3000 (avrRpt2), indicating that NDR1 was required for sr1-1 resistance to Pto DC3000 (avrRpt2). This is consistent with our hypothesis that the responses of sr1-1 and sr1-4D to Pto DC3000 (avrRpt2) are due to higher or lower expression of NDR1, respectively. These observations are consistent with previous findings that overexpression of NDR1 enhances resistance to Pto DC3000 (avrRpt2) (Coppinger et al., 2004). However, the ndr1 sr1-1 double mutant was less susceptible than the ndr1-3 single mutant, suggesting the modulation of Pto DC3000 (avrRpt2) resistance by SR1 is only partially dependent on NDR1 function.

To further study the role of NDR1 in sr1-1 mediated powdery mildew resistance, we infected wild type, sr1-1, ndr1-3 and the ndr1-3 sr1-1 double mutant with G. cichoracearum. The ndr1 mutant displayed a wild type like susceptible phenotype to powdery mildew, and did not show enhanced susceptibility; however, ndr1 fully suppressed sr1-1 mediated mildew-induced cell death, and partially suppressed powdery mildew resistance in sr1-1 (Figure 6A, 6B and 6C), indicating that NDR1 participated in sr1-1 mediated resistance to powdery mildew.

The ein3 Mutation Suppressed sr1-1 Mediated Ethylene-Induced Senescence

Previously, it has been shown by EMSA that SR1 binds to the EIN3 promoter in vitro. Here we show by ChIP that SR1 binds to the EIN3 promoter in vivo. However, the biological significance of SR1 binding to EIN3 has not yet been defined. Our observation that sr1-1 displayed enhanced ethylene-induced senescence, and that sr1-4D displayed delayed ethylene-induced senescence may provide genetic evidence for the role of SR1 in ethylene signaling and in the regulation of EIN3 expression. In
this scenario, the ethylene phenotypes of sr1-1 and sr1-4D might be due to the misregulation of EIN3 in these mutants. To test this hypothesis, we examined whether ein3 suppresses the enhanced ethylene-induced senescence in sr1-1. We treated wild type, sr1-1, ein3-3 and ein3-3 sr1-1 mutants with 100 μl l−1 ethylene for 3 days, and found that the ein3-3 sr1-1 double mutant displayed insensitivity to ethylene, showing delayed senescence (Figure 7A and 7B), indicating that EIN3 is required for ethylene-induced senescence in sr1-1. However, ein3-3 had no effects on the sr1-1 resistance to powdery mildew (Figure 6A, 6B), indicating that defense responses and ethylene senescence are regulated by two distinct pathways.

To further investigate the role of SR1 in ethylene signaling, we tested the response of sr1-1 and sr1-4D seedlings to 1-aminoacyclopropane-1-carboxylic acid (ACC). Both sr1-1 and sr1-4D displayed the typical triple response, which was indistinguishable from the wild type seedlings (Supplemental Fig. 6C), suggesting that ethylene-induced senescence is different from the classic ethylene signaling pathway.

To gain more insight into the function of SR1 in ethylene-induced senescence, we examined relative expression of SRI in response to ethylene treatment. As shown in Supplemental Fig. S7C, the transcript accumulation of SRI was increased after ethylene treatment. We then examined relative expression of two senescence associated genes, SAG12 and SAG24 in ethylene treated wild type, sr1-1 and sr1-4D plants. As shown in Supplemental Fig. S7D and S7E, the transcript accumulation of SAG12 and SAG24 was significantly higher in sr1-1, but much lower in sr1-4D than wild type. These data indicate that SR1 negatively regulates the expression of senescence associated genes SAG12 and SAG24.

**The Binding of SR1 and SR1-4D to Calmodulin Requires Calcium**

SR1 is a calmodulin binding transcription activator that contains a DNA binding domain at the N terminus and two calmodulin binding IQ motifs in the C terminal 850-896aa (Yang and Poovaiah, 2002). The sr1-4D mutation is located in the first IQ
motif, which is the calmodulin binding domain. To examine whether the \textit{SR1-4D} mutation affected its binding to calmodulin, we expressed the SR1 calmodulin binding domain with a GST tag in \textit{E. coli}, and tested the calmodulin binding activity of the wild type and mutant versions of the SR1 calmodulin binding domains. Both wild type and mutated version of SR1 proteins were able to bind to calmodulin \textit{in vitro}. We then examined whether the binding between the SR1-4D protein and calmodulin requires calcium, and found that both SR1 and SR1-4D bound to calmodulin in a calcium dependent manner, as neither protein could bind to calmodulin in the absence of CaCl$_2$ \textit{in vitro} (Supplemental Fig. S8).

\textbf{DISCUSSION}

To search for components in the EDR2 signaling pathway, we performed a mutant screen and identified an \textit{edr2} suppressor mutation, \textit{sr1-4D}, which affects a calmodulin binding transcription factor. \textit{sr1-4D} is a gain-of-function mutation that suppressed all \textit{edr2} phenotypes including powdery mildew resistance and enhanced ethylene-induced senescence. In contrast, the loss-of-function \textit{sr1-1} mutant displayed increased disease resistance and enhanced ethylene-induced senescence. We showed that SR1 negatively regulates plant immunity and leaf senescence by directly binding to the \textit{NDR1} and \textit{EIN3} promoters.

Although \textit{sr1-4D} was identified in the \textit{edr2} suppressor screen, SR1 may not directly regulate the EDR2 signaling pathway, as the \textit{sr1-4D} mutant showed enhanced susceptibility to multiple pathogens, including virulent and avirulent strains of the bacterial pathogen \textit{Pto} DC3000, while \textit{edr2} does not show an altered response to these pathogens. Recently, Jing et al (2011) identified an identical mutation (\textit{camta3-3D}) in a screen for mutants that exhibit compromised systemic acquired resistance (SAR). In addition to defects in SAR, the \textit{camta3-3D} mutant displays enhanced susceptibility to virulent bacteria \textit{Pseudomonas syringae} p.v. \textit{maculicola} (\textit{P.s.m.}) ES4326 and the oomycete pathogen \textit{Hyaloperonospora arabidopsidis} (\textit{H.a.})
Noco2 (Jing et al., 2011), which is consistent with our findings. Jing et al. also showed that the transgenic lines that express higher level of SR1 have defects in basal defense and SAR (Jing et al., 2011). These data indicate that SR1 plays an important role in both SAR and basal defense, however, how SR1 regulates SAR is not clear.

Previously, it was shown that SR1 regulates EDS1 expression through binding to the EDS1 promoter (Du et al., 2009). Also, EDS1 is a positive regulator in basal defense and R gene mediated responses. The eds1 mutation suppressed powdery mildew resistance mediated by edr1, atg2 and RPW8 (Frye et al., 2001; Xiao et al., 2005). As edr2-mediated resistance is dependent on SA signaling, one possibility is that sr1-4D suppressed the edr2 resistant phenotype to powdery mildew mainly through repression of EDS1 expression by SR1-4D, which in turn leads to inactivation of SA signaling.

sr1-1 displays resistance to Pto DC3000 and B. cinerea (Galon et al., 2008; Du et al., 2009). Also, microarray data showed that many disease resistance related genes were up-regulated in sr1-1 (Galon et al., 2008). In this work, we show that sr1-1 is resistant to a virulent powdery mildew isolate and has further tightened resistance to avirulent strains of Pto DC3000 carrying avrRpt2 or avrRPS4. In contrast to sr1-1, the gain-of-function mutant sr1-4D displays susceptibility to each of these pathogens. Consistent with our finding, the gain-of-function mutant of SR1 shows enhanced disease susceptibility phenotypes to P. s. m. ES4326 and H. a. Noco2 (Jing et al., 2011) SR1 is a calcium dependent calmodulin binding transcription factor and binds to a CGCG box in the promoter of target genes to repress their expression (Yang and Poovaiah, 2002; Du et al., 2009). The mutation in sr1-4D likely leads to enhanced repression of the target genes. One possibility is that SR1-4D binds more tightly to calmodulin, and thus constitutively binds to the target promoters, leading to reduced expression of the target genes. Alternatively, the threshold of calcium concentration required for the binding between calmodulin and SR1-4D may be lower than wild
type protein. Another possibility is that the SR1-4D protein accumulates to higher levels than wild type SR1. Intriguingly, The *sr1-4D* carries a C-to-T point mutation (causing A855 to V), which was exactly the same as described for *camta3-3D* recently (Jing et al.). These two mutants are identified from independent sources, suggesting that A855 is the only or one of the few residues that play a critical role in modulation of SR1’s activity. The interactions between calcium signaling and plant defense responses are complicated, and further analysis is needed to determine why this particular mutation causes a gain-of-function phenotype.

Plants recognize pathogen effectors, directly or indirectly, by R proteins (Dangl and Jones, 2001). Many Arabidopsis R proteins contain an NB-LRR domain. According to the N terminal structure, these R proteins can be divided into two classes, CC-NB-LRR and TIR-NB-LRR. In general, the resistance mediated by CC-NB-LRR proteins requires NDR1 function, but the resistance mediated by TIR-NB-LRR proteins requires EDS1 (Aarts et al., 1998; Feys and Parker, 2000). Although it has been well documented how NDR1 and EDS1 are involved in the defense response (Feys et al., 2001; Axtell and Staskawicz, 2003; Belkhadir et al., 2004; Feys et al., 2005; Day et al., 2006), it is not clear how *NDR1* and *EDS1* are regulated. Du et al. reported that SR1 directly binds to the *EDS1* promoter and represses its expression, which revealed a mechanistic link between calcium signaling and SA mediated disease resistance (Du et al., 2009). Here, we report *NDR1* is also directly regulated by SR1. This finding provides new insights into the role of SR1 in plant immunity, providing a link between *NDR1* and *EDS1* mediated resistance pathways through the co-regulator SR1.

The plant hormones SA and ethylene play important roles in plant defense responses. The cross talk between SA signaling and ET signaling in the defense response is complicated. In general, it is believed that SA signaling plays an important role in resistance to biotrophic pathogens and ET signaling plays a crucial role in resistance to necrotrophic pathogens (Glazebrook, 2005). However, there is evidence that these
two pathways may be antagonistic or agonistic to each other. For instance, Chen et al. reported that EIN3 and EIL1 bind to the SID2 promoter and repress SID2 expression (Chen et al., 2009). This is direct evidence of cross talk between SA and ET signaling, as EIN3 is one of the central components that positively regulates the ethylene signal transduction pathway (Chao et al., 1997). Also, SID2 is a key enzyme that is involved in SA synthesis and mutations in SID2 compromise pathogen-induced SA accumulation. Consequently, loss-of-function mutants of ein2 and ein3 display enhanced disease resistance to bacterial Pto DC3000 (Bent et al., 1992; Chen et al., 2009), and overaccumulation of EIN3 protein leads to enhanced susceptibility to Pto DC3000 (Chen et al., 2009). Recently, it was reported that ein2 mutants are defective in all FLS2 mediated responses, and EIN3 and EIL1 directly bind to the receptor kinase FLS2 to mediate PAMP signaling (Boutrot et al., 2010), which indicates a direct role of the ET pathway in plant immunity. SR1 may provide another link between SA and ethylene as SR1 binds to the promoters of EDS1, a positive regulator of SA signaling, and EIN3, a positive regulator of ethylene signaling; SR1 also negatively regulates the expression of EDS1, NDR1 and EIN3. This indicates that plants can up-regulate or down-regulate both SA and ET signaling pathways by modulating SR1 function. These findings indicate that the relationship among SA signaling, ET signaling and the immunity system is complicated. Negative regulation of both SA signaling and ET signaling by direct binding of SR1 to the promoter of EDS1, NDR1 and EIN3, may explain why srl-1 is more resistant to both biotrophic and necrotrophic pathogens and why srl-4D suppressed edr2 mediated resistance and ethylene induced senescence.

The cross-talk between defense responses and senescence has been discussed previously (Tang et al., 2005a; Consonni et al, 2006; Wang et al., 2011). Some mutants that display enhanced disease resistance show early senescence, such as edr1, atg2 and mlo2. However, the cross-talk between defense responses and senescence appears to be complicated. For instance, edr1-mediated resistance is SA dependent,
but senescence in edr1 is dependent on ethylene signaling, thus resistance and senescence in edr1 are regulated by separated pathways (Tang et al., 2005a). However, early senescence-like phenotype in mlo2 is suppressed by mutations in EDS5, NPR1, PAD4 and SID2, as well as by NahG transgene, indicating that SA plays an important role in mlo2-associated senescence (Consonni et al, 2006). In addition, it was shown that SA levels are higher in senescent leaves in Arabidopsis (Morris et al., 2000). Because SR1 binds to the promoter of EDS1 and EIN3, plants maybe able to control disease resistance and senescence by modulating SA signaling and ethylene signaling through their co-regulation by SR1. Further analysis of global gene expression (e.g. RNA-seq) in wild type, sr1-1 and sr1-4D may provide useful leads to identify connections between senescence and defense mediated by SR1.

In conclusion, Arabidopsis SR1 plays a critical role in plant immunity and ethylene induced senescence. Our data support a model that SR1 fine-tunes plant immunity and senescence signaling by directly regulating expression of NDR1, EDS1 and EIN3 (Supplemental Fig. S9). SR1 may represent another example of the complicated interactions between SA pathways, ethylene signaling and plant immunity.
MATERIALS AND METHODS

Plant Materials and Growth Conditions
Arabidopsis seeds were sterilized in 10% bleach and sown on 1/2 MS (Murashige and Skoog) medium containing 1% sucrose. Plates were kept in 4°C for 3 days and then moved to the greenhouse (22-24°C and 9 hour light and 15 hour dark photoperiod). Seedlings were transferred into soil after 7 days. Plants were grown in short-day conditions (9h:15h=light:dark) for phenotyping or in long-day conditions (16h:8h=light:dark) to set seeds as described previously (Nie et al., 2011), unless indicated otherwise. The sr1-1 mutant was from the Arabidopsis Biological Resource Center (ABRC) (SALK_001152). The ndr1-3 sr1-1 and ein3-3 sr1-1 double mutants were generated by standard crosses.

Pathogen Inoculation
Powdery mildew (G. cichoracearum UCSC1) was kept on highly susceptible pad4-1 plants. Powdery mildew infection was performed with either high-density or low-density inoculation. High-density inoculation was used for mutant screening and mapping and was achieved by gently brushing the target leaves with infected leaves to pass the fungal spores (Adam and Somerville, 1996). To quantify the number of conidiophores per colony, low-density inoculation was used to achieve an even inoculation density as described previously (Wang et al., 2011). The number of conidiophores per colony was counted at 7 dpi (Consonni et al., 2006). Infections with P. syringae pv. tomato (Pto) DC3000 virulent and avirulent strains were performed as described previously (Nie et al., 2011). Botrytis cinerea was grown on potato dextrose agar plates (PDA) (Difco, BD, USA) and the leaves of four-week-old plants were inoculated as described previously (Ferrari et al., 2003).

Staining and Microscopy
Fungal growth and host cell death were examined by staining infected leaves with
trypan blue at 8 dpi for plants infected with powdery mildew (Frye and Innes, 1998). Hydrogen peroxide was examined by staining infected leaves with 3,3′-diaminobenzidine-HCl at 2 dpi (Xiao et al., 2003). Samples were observed and photographed using an Olympus BX60 microscope.

**Ethylene-Induced Senescence Assay**

Four-week-old plants were kept in a sealed box with 100 μl l⁻¹ ethylene for 3 days. Then plants were photographed and chlorophyll was extracted using 100% ethanol. Chlorophyll content was measured with a Multiskan Spectrum spectrophotometer (Thermo Scientific) at 665nm and 649nm wavelengths (Tang et al., 2005).

**SA measurement**

SA extraction and measurement were performed as described previously (Gou et al., 2009).

**Statistical Analyses**

Statistical analyses were performed by Student t-test for samples from two genotypes or one-way ANOVA for samples from multiple genotypes (Wang et al., 2011).

**Mutant Screen and Mapping**

The edr2 srl-4D mutant was identified from an EMS-mutagenized population (Nie et al., 2011). To map the srl-4D mutation, an edr2 srl-4D plant was crossed with Landsberg erecta (Ler), and F2 homozygous edr2 plants were identified and used for rough mapping. For fine mapping, a large number of F3 plants (derived from F2 plants that displayed edr2 phenotypes) were used, and ultimately, the mutation was mapped to the region between markers T26C19 and F14M13. We then sequenced the candidate genes in this region. A nucleotide change (C2564T) in the 12th exon was found in At2g22300 (SR1); this mutation also leads to an amino acid change (A855V). Then we amplified a 7kb genomic DNA fragment from the edr2 srl-4D mutant and cloned it into pEASY-blunting (Transgen biotech). The genomic clone included 1.4kb
upstream of the ATG and 0.8kb downstream of At2g22300. This genomic DNA was digested and inserted into binary vector pBINPLUS. The construct was introduced into Agrobacterium tumefaciens strain GV3101 then transformed to the edr2 plants by the floral dip method. The transformants were screened on 1/2 MS medium with 50μg/ml Kanamycin.

**EMSA**
The SRI sequence encoding a truncated protein (amino acids 1-146aa) was constructed in pGEX4t and expressed in E. coli BL21(DE3)pLysS (TransGen Biotech) and purified by GST beads (GE Healthcare). The probe was synthesized as forward and reverse strands, and renatured to a double-stranded probe in 0.15M NaCl under 70°C for 5min. Then the probe was labeled by γ^{32}P-ATP using T4 polynucleotide kinase (NEB) and purified by G-25 spin columns (GE healthcare). The gel shift assay was performed according to the Promega gel shift assay system manual.

**Calmodulin Binding**
The SR1 calmodulin binding domain (800-900aa and 800-930aa) was cloned into in pGEX4t vector and expressed in E. coli BL21(DE3)pLysS (TransGen Biotech). An animal version of calmodulin was used in the experiments. The calmodulin binding assay was performed using AffinityH CBP Fusion Protein Detection Kit (Stratagene) according to the manufacturer’s instructions. For testing whether calmodulin binding is Ca^{2+} dependent, 1mM CaCl₂ or 5mM ethylene glycol tetraacetic acid (EGTA) was added to the reaction, respectively.

**ChIP Assay**
To produce an inducibly-expressed, GFP-tagged SR1 (DEX:SR1-GFP), we cloned the full length coding sequence of SRI into pBAV150 and transformed this construct into wild type Col-0. ChIP was performed as described previously with minor modifications (Bowler et al., 2004; Saleh et al., 2008). Briefly, wild type and
DEX:SR1-GFP transgenic seeds were grown on 1/2 MS plate for 8-10 days, then transferred to 20μM DEX plates for 2 days. Roots were harvested and cross-linked by 1% formaldehyde for 15 min in vacuum and stopped by 0.125M glycine. Roots were ground in liquid nitrogen and nuclei were isolated. Chromatin was immunoprecipitated by anti-GFP (Roche) and protein G beads (Millipore). DNA was precipitated by isopropanol, washed by 70% ethanol and dissolved in 30μl water with 20μg/ml RNase. Gene specific primers (NDR1-ChIP-F, NDR1-ChIP--R; EIN3-ChIP-F, EIN3-ChIP--R, EDS1-ChIP-F, EDS1-ChIP--R, ACTIN2-ChIP-F, ACTIN2-ChIP—R, SAG12-F, SAG12R, SAG24F, SAG24R) were used (TAKARA, sybgreen kit) to quantify enrichment of each fragment. Primers used in this study are listed in Supplemental Table 1.

Gene Expression Analysis

RNA was extracted by TRIzol reagent (Invitrogen) and the first strand was synthesized using MLV reverse transcriptase (Promega). Accumulation of transcripts was examined by real time PCR using the sybgreen kit (TAKARA). Primers used in this study are listed in Supplementalgreen Table 1.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. *sr1-4D* suppressed the *edr2* phenotype of resistance to powdery mildew and ethylene induced senescence

A. Four-week-old wild type, *edr2*, *edr2 sr1-4D* plants were infected with powdery mildew *G. cichoracearum* UCSC1 and the representative leaves were removed and photographed at 8 dpi. The *edr2 sr1-4D* double mutant displayed a susceptible phenotype, showing visible powder and no necrosis, which was similar to wild type. Thirty plants were evaluated for each genotype.

B. Trypan blue staining to visualize plant cell death and fungal growth. Leaves were stained with trypan blue at 8 dpi. The *edr2* mutant displayed massive cell death and very few conidia, while *edr2 sr1-4D* supported wild type like conidia formation. Bar = 100 μm.

C. DAB staining for H2O2 at 2 dpi. Note that *edr2* accumulated more H2O2 than wild type. Bar = 100 μm.

D. Accumulation of H2O2 was quantified as described previously (Wang et al., 2011). The bars represent mean and standard deviation of intensity per area from at least 6 leaves of 3 plants for each genotype. Lower-case letters indicate a significant difference (P<0.01, one-way ANOVA). The experiments were repeated three times with similar results.

E. Quantification of the fungal growth by counting the number of conidiophores per colony at 7 dpi. The bars represent mean and standard deviation of samples (n=25). Low-case letters indicate statistical significance (P<0.01, one-way ANOVA). The experiment was repeated 3 times with similar results.

F. Accumulation of *PRI* mRNA in *edr2* was suppressed by *sr1-4D*. Four-week-old plants were inoculated with *G. cichoracearum*. Accumulation of *PRI* transcripts was examined by real-time PCR and normalized to *ACT8* as an internal control. The bars represent mean and standard deviation from three biological replicates. The asterisk indicates significant difference from wild type (P<0.01, Student’s *t*-test)
G. Ethylene-induced senescence. Four-week-old plants were treated with 100 μl l⁻¹ ethylene for 3 days.

H. Chlorophyll content of the fourth to the sixth leaves of day 0 and day 3 after 100 μl l⁻¹ ethylene treatment. The bars represent mean and standard deviation (n=4). Statistical differences are indicated by lower-case letters (P<0.01, one-way ANOVA). The experiment was repeated more than 3 times with the similar results.

**Figure 2. SRI encodes a calmodulin binding transcription factor**

A. Positional cloning of SRI. A nucleotide change (C2564T) in the 12th exon in At2g22300 (SRI) was identified, which led to a substitution (A855V) in the SR1 protein.

B. A genomic clone of SRI from edr2 sr1-4D suppressed edr2-mediated powdery mildew resistance. Wild type, edr2, edr2 sr1-4D and edr2 transformed with the genomic clone of mutated SRI (derived from the edr2 sr1-4D mutant) were inoculated with powdery mildew. The plants were photographed (upper panel) and stained with trypan blue (lower panel) at 8 dpi. Bar = 100 μm. Forty-nine independent T1 transgenic plants were evaluated and forty-five of them showed sr1-4D like susceptible phenotype.

C. The mutation site in SR1-4D is in the first IQ motif of SR1.

D. The mutation site of SR1-4D, A855, is conserved in proteins homologous to SR1 in different organisms.

SR1 protein sequence was used to perform blast searches against the NCBI database. SR1 and its homologues identified in different organisms were aligned using Megalign software (DNASTAR, Inc.) and the alignment was further edited in Genedoc software.

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- **O. sativa:** Oryza sativa accession number EEC74662.1;  
- **P. trichocarpa:** Populus trichocarpa accession number XP_002310562.1.  
- **R. communis:** Ricinus communis accession number XP_002519355.1;  
- **V. vinifera:**  

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**Figure 3. Response of wild type, sr1-4D and sr1-1 to other pathogens**

A-C. Four-week-old plants were inoculated with virulent or avirulent strains of *Pto DC3000*. A. *Pto DC3000*. B. *Pto DC3000 avrRPS4*. C. *Pto DC3000 avrRpt2*. Ten plants were used for each genotype. The bars represent mean and standard deviation of 3 biological samples. Statistical differences are indicated by lower case letters (P<0.01, one-way ANOVA). The experiment was repeated more than 3 times with similar results.

D. Four-week-old plants were infected with *G. cichoracearum* and the representative leaves were removed and photographed at 8 dpi. Thirty plants were evaluated for each genotype.

E. Infected leaves with *G. cichoracearum* at 8 dpi were stained with trypan blue to visualize fungal growth and plant cell death. Bar = 100 μm.

F. The number of conidiophores per colony was counted at 7 dpi. The bars represent mean and standard deviation of samples (n=25). Statistical differences are indicated by lower-case letters (P<0.01, one-way ANOVA). The experiment was repeated 3 times with similar results.

G. Leaves from four-week-old plants were infected with *B. cinerea* and photographed at 3 dpi. Leaves from at least 30 plants were used for each genotype.

H. Leaves were inoculated with *B. cinerea*. The lesion size was determined by measuring the major axis of the necrotic area. The bars represent mean and standard deviation of samples. Statistical differences are indicated with lower-case letters (n=30, P<0.01, one-way ANOVA). The experiments were repeated 3 times with similar results.

**Figure 4. SR1 directly binds to the promoter of NDR1 and EIN3**

A. Levels of *NDR1* transcripts in four-week-old wild type, sr1-4D and sr1-1 plants were examined by quantitative real-time PCR and normalized to *ACT8* as an internal control. The bars represent the values of mean and standard deviation.
from three independent biological replicates. The lower-case letters indicate significant differences (P<0.01, one-way ANOVA)

B. EMSA assay for SR1 binding to the promoter fragment of \textit{NDRI} \textit{in vitro}. GST-SR1-N (1-146aa) was incubated with radiolabeled \textit{NDRI} promoter fragment. The samples were loaded and separated on a polyacrylamide gel. The NDR1m sequence contained a mutated CGCG box (CGCG to CGAT).

C. The promoter fragments of \textit{NDRI} and \textit{EIN3} were enriched in a ChIP assay. Chromatin from wild type and \textit{DEX:SR1-GFP} transgenic plants was immunoprecipitated by anti-GFP and the enrichment of the fragments was determined by quantitative real-time PCR. The \textit{ACTIN2} promoter was used as a negative control and the \textit{EDS1} promoter as a positive control. The bars represent mean and standard deviation of samples (n=3). The experiment was repeated 4 times with similar results.

\textbf{Figure 5.} \textit{ndr1} suppressed the resistant phenotype of \textit{sr1-1} to \textit{P. syringae pv. tomato} DC3000 carrying \textit{avrRpt2}.

Four-week-old plants were inoculated with \textit{Pto} DC3000 \textit{avrRpt2}. Ten plants were used for each genotype. The bars represent means and standard deviation. Statistical differences are indicated with lower-case letters (n=3, P<0.01, one-way ANOVA). The experiment was repeated more than 3 times with similar results.

\textbf{Figure 6.} \textit{ndr1}, not \textit{ein3} suppresses the resistance of \textit{sr1-1} to powdery mildew.

A. Four-week-old plants were inoculated with \textit{G. cichoracearum} and the representative leaves were removed and photographed at 8 dpi. Thirty plants were evaluated for each genotype.

B. Trypan blue staining of the leaves inoculated with \textit{G. cichoracearum} at 8 dpi. Bar = 100 \(\mu\)m. The fungal structures and dead plant cells were stained.

C. The number of conidiophores per colony was counted at 7 dpi. The bars represent means and standard deviation (n=25, P<0.01, one-way ANOVA). Different letters indicate the significant difference between genotypes. The experiment was
repeated 3 times with similar results.

**Figure 7. ein3 suppressed ethylene induced senescence of sr1-1**

A. Four-week-old plants were treated with 100 μl l⁻¹ ethylene for 3 days.

B. Decrease in chlorophyll content induced by ethylene treatment, measured by ratio of chlorophyll content at day 3 divided by content at day 0, of the fourth to the sixth leaves treated with 100 μl l⁻¹ ethylene for 0 day and 3 day. The bars represent means and standard deviation (n=4). Statistic difference is indicated by different lower-case letters (P<0.01, one-way ANOVA). The experiment was repeated 3 times with similar results.
SUPPLEMENTAL DATA

Supplemental Figure 1. SR1 was induced by powdery mildew and Pto DC3000

Supplemental Figure 2. sr1-4D suppressed edr1-mediated powdery mildew resistance

Supplemental Figure 3. Temperature dependent growth phenotype of sr1-4D.

Supplemental Figure 4. SA accumulation of sr1-4D.

Supplemental Figure 5. The NDR1 promoter sequence contains a CGCG box

Supplemental Figure 6. SR1 is involved in ethylene induced senescence, but is not involved in ACC induced triple response.

Supplemental Figure 7. Relative expression of several defense and senescence related genes.

Supplemental Figure 8. Calcium is needed for SR1-4D binding to the calmodulin in vitro. An animal version of calmodulin was used in the experiments.

Supplemental Figure 9. A model illustrating the role of SR1 in defense responses and senescence.

Supplemental Table 1. Primers used in this study.
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- **A. thaliana**: Arabidopsis thaliana SR1; **B. napus**: Brassica napus accession number AAM10969.1; **N. tabacum**: Nicotiana tabacum accession number AAG39222.1; **O. sativa**: Oryza sativa accession number EEC74662.1; **P. trichocarpa**: Populus trichocarpa accession number XP_002310562.1; **R. communis**: Ricinus communis accession number XP_002519355.1; **V. vinifera**: Vitis vinifera accession number CBI35638.3
Figure 3. Response of wild type, sr1-4D and sr1-1 to other pathogens
A–C. Four-week-old plants were inoculated with virulent or avirulent strains of Pto DC3000. A. Pto DC3000. B. Pto DC3000 avrRPS4 C. Pto DC3000 avrRpt2. Ten plants were used for each genotype. The bars represent mean and standard deviation of 3 biological samples. Statistical differences are indicated by lower case letters (P<0.01, one-way ANOVA). The experiment was repeated more than 3 times with similar results.

D. Four-week-old plants were infected with G. cichoracearum and the representative leaves were removed and photographed at 8 dpi. Thirty plants were evaluated for each genotype.

E. Infected leaves with G. cichoracearum at 8 dpi were stained with trypan blue to visualize fungal growth and plant cell death. Bar = 100 μm.

F. The number of conidiophores per colony was counted at 7 dpi. The bars represent mean and standard deviation of samples (n=25). Statistical differences are indicated by lower-case letters (P<0.01, one-way ANOVA). The experiment was repeated 3 times with similar results.

G. Leaves from four-week-old plants were infected with B. cinerea and photographed at 3 dpi. Leaves from at least 30 plants were used for each genotype.

H. Leaves were inoculated with B. cinerea. The lesion size was determined by measuring the major axis of the necrotic area. The bars represent mean and standard deviation of samples. Statistical differences are indicated with lower-case letters (n=30, P<0.01, one-way ANOVA). The experiments were repeated 3 times with similar results.
Figure 4. SR1 directly binds to the promoter of NDR1 and EIN3

A. Levels of NDR1 transcripts in four-week-old wild type sr1-4D and sr1-1 plants were examined by quantitative real-time PCR and normalized to ACT8 as an internal control. The bars represent the values of mean and standard deviation from three independent biological replicates. The lower-case letters indicate significant differences (P<0.01, one-way ANOVA)

B. EMSA assay for SR1 binding to the promoter fragment of NDR1 in vitro. GST-SR1 was incubated with radiolabeled NDR1 promoter fragment. The samples were loaded and separated on a polyacrylamide gel. The NDR1m sequence contained a mutated CGCG box (CGCG to CGAT).

C. The promoter fragments of NDR1 and EIN3 were enriched in a ChIP assay. Chromatin from wild type and DEX:SR1-GFP transgenic plants was immunoprecipitated by anti-GFP and the enrichment of the fragments was determined by quantitative real-time PCR. The ACTIN2 promoter was used as a negative control and the EDS1 promoter as a positive control. The bars represent mean and standard deviation of samples (n=3). The experiment was repeated 4 times with similar results.
Figure 5. *ndr1* suppressed the resistant phenotype of *sr1-1* to *P. syringae pv. tomato* DC3000 carrying *avrRpt2*. Four-week-old plants were inoculated with *Pto* DC3000 *avrRpt2*. Ten plants were used for each genotype. The bars represent means and standard deviation. Statistical differences are indicated with lower-case letters (n=3, P<0.01, one-way ANOVA). The experiment was repeated more than 3 times with similar results.
Figure 6. *ndr1*, not *ein3* suppresses the resistance of *sr1-1* to powdery mildew.

A. Four-week-old plants were inoculated with *G. cichoracearum* and the representative leaves were removed and photographed at 8 dpi. Thirty plants were evaluated for each genotype.

B. Trypan blue staining of the leaves inoculated with *G. cichoracearum* at 8 dpi. Bar = 100 μm. The fungal structures and dead plant cells were stained.

C. The number of conidiophores per colony was counted at 7 dpi. The bars represent means and standard deviation (n=25, P<0.01, one-way ANOVA). Different letters indicate the significant difference between genotypes. The experiment was repeated 3 times with similar results.
Figure 7. *ein3* suppressed ethylene induced senescence of *sr1-1*

A. Four-week-old plants were treated with 100 μl l⁻¹ ethylene for 3 days.

B. Decrease in chlorophyll content induced by ethylene treatment, measured by ratio of chlorophyll content at day 3 divided by content at day 0, of the fourth to the sixth leaves treated with 100 μl l⁻¹ ethylene for 0 day and 3 day. The bars represent means and standard deviation (n=4). Statistic difference is indicated by different lower-case letters (P<0.01, one-way ANOVA). The experiment was repeated 3 times with similar results.