EDS1 in tomato is required for resistance mediated by TIR-class *R* genes and the receptor-like *R* gene *Ve*

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Summary

In tobacco and other *Solanaceae* species, the tobacco *N* gene confers resistance to tobacco mosaic virus (TMV), and leads to induction of standard defense and resistance responses. Here, we report the use of *N*-transgenic tomato to identify a fast-neutron mutant, *sun1-1* (suppressor of *N*), that is defective in *N*-mediated resistance. Induction of salicylic acid (SA) and expression of pathogenesis-related (*PR*) genes, each signatures of systemic acquired resistance, are both dramatically suppressed in *sun1-1* plants after TMV treatment compared to wild-type plants. Application of exogenous SA restores *PR* gene expression, indicating that *SUN1* acts upstream of SA. Upon challenge with additional pathogens, we found that the *sun1-1* mutation impairs resistance mediated by certain resistance (*R*) genes, (*Bs4*, *I*, and *Ve*), but not others (*Mi-1*). In addition, *sun1-1* plants exhibit enhanced susceptibility to TMV, as well as to virulent pathogens. *sun1-1* has been identified as an *EDS1* homolog present on chromosome 6 of tomato. The discovery of enhanced susceptibility in the *sun1-1* (Le_eds1-1) mutant plant, which contrasts to reports in *Nicotiana benthamiana* using virus-induced gene silencing, provides evidence that the intersection of *R* gene-mediated pathways with general resistance pathways is conserved in a Solanaceous species. In tomato, *EDS1* is important for mediating resistance to a broad range of pathogens (viral, bacterial, and fungal pathogens), yet shows specificity in the class of *R* genes that it affects (TIR-NBS-LRR as opposed to CC-NBS-LRR). In addition, a requirement for *EDS1* for *Ve*-mediated resistance in tomato exposes that the receptor-like *R* gene class may also require *EDS1*.

Keywords: disease resistance, *EDS1*, tobacco mosaic virus, enhanced susceptibility, salicylic acid.

Introduction

Plants fight pathogen infection by using basal defenses and classical, gene-for-gene resistance responses. Plants have evolved specific, induced defenses that are initiated upon recognition of pathogen-derived avirulence (Avr) gene products via structurally and functionally conserved resistance (R) proteins (reviewed in Baker *et al.*, 1997; Dangl and Jones, 2001). This recognition event generally leads to induction of a standard set of defense and resistance responses: programmed cell death at the site of pathogen exposure (the hypersensitive response or HR); induction of pathogenesis-related (*PR*) gene expression; various physiological events such as calcium ion influx, a burst of reactive oxygen production, cell wall biosynthesis and callose deposition; inhibition of the pathogen's ability to replicate and move systemically; and systemic acquired resistance (SAR) to future pathogen attack (Dangl and Jones, 2001; Hammond-Kosack and Jones, 1996; Ryals *et al.*, 1996).

Most of the *R* gene products thus far identified contain a central nucleotide-binding site (NBS) and C-terminal leucine-rich repeats (LRRs). These NBS-LRR R proteins belong to two subclasses according to the N-terminal sequence: one subclass contains a coiled coil (CC) domain; the other

subclass contains a TIR domain, which has homology with the Toll protein in *Drosophila* and the Interleukin 1-Receptor (IL-1R) in mammals, both of which are involved in immune response and signal transduction during development (Whitham *et al.*, 1994, and reviewed in Baker *et al.*, 1997; Dangl and Jones, 2001; Martin *et al.*, 2003) This trans-kingdom homology and similarity in function of TIR-containing proteins suggests that these proteins may be involved in cell signaling pathways common to many organisms, and also suggests a linkage between fundamental cellular processes and resistance pathways. Three Arabidopsis genes, *EDS1*, *PAD4*, and *NDR1*, have been identified as important components of the NBS-LRR *R* gene-mediated response to specific pathogens (Century *et al.*, 1995; Glazebrook *et al.*, 1996; Martin *et al.*, 2003; Parker *et al.*, 1996). *EDS1* and *PAD4* are typically required for resistance mediated by a different set of R genes than is *NDR1*. Though some exceptions have been observed, *EDS1*, and to a lesser extent *PAD4*, are generally necessary for resistance conferred by TIR-NBS-LRR proteins, whereas *NDR1* functions in concert with members of the CC-NBS-LRR subclass (Aarts *et al.*, 1998; Chandra-Shekara *et al.*, 2004; Feys *et al.*, 2001; Martin *et al.*, 2003). *EDS1* additionally functions in non-host resistance (Yun *et al.*, 2003; Zimmerli *et al.*, 2004), and is also critical for basal defenses in Arabidopsis, as mutations in *EDS1* result in enhanced disease susceptibility to virulent pathogens (Parker *et al.*, 1996). *EDS1* functions in resistance pathways in the Solanaceous species *Nicotiana benthamiana* (Liu *et al.*, 2002; Peart *et al.*, 2002; Schornack *et al.*, 2004), where it also showed a specificity for the TIR-class *R* genes *N* and *Bs4*, but not the CC-class *R* gene *Rx* or the kinase *Pto*. In contrast to its role in Arabidopsis, however, no enhanced disease susceptibility was detected in the *EDS1*-silenced *N. benthamiana* (Peart *et al.*, 2002). Additional proteins identified in Arabidopsis, including *SGT1* and *RAR1*, are required for multiple resistance pathways mediated by multiple classes of *R* genes, and therefore are presumed to function downstream of *EDS1*, *PAD4*, and *NDR1* (Austin *et al.*, 2002; Azevedo *et al.*, 2002).

Although several signaling components have been identified, many *R* gene-mediated pathways still remain poorly characterized. One such pathway is mediated by the tobacco *N* gene, which confers resistance to tobacco mosaic virus (TMV). The *N* gene product belongs to the TIR-NBS-LRR subclass of R proteins. TMV was the first virus shown to have infectious RNA (Fraenkel-Conrat, 1956; Fraenkel-Conrat and Williams, 1955), and it infects more than 200 plant species including most members of *Solanaceae* (Holmes, 1946; Watterson, 1993), a family that encompasses important crop plants such as potato, tomato, pepper, and eggplant. In common with other *R* gene-mediated pathways, the resistance response induced by *N* involves an HR and the induction of *PR* genes. The *N* gene was originally isolated from the wild tobacco (*N. glutinosa*), but it also confers

resistance to TMV in transgenic plants of other *Solanaceae* species such as tomato (Whitham *et al.*, 1996). It is therefore presumed that any signal transduction machinery required for *N*-mediated resistance in tobacco is also present in tomato. This enabled us to employ tomato, *Lycopersicon esculentum*, as a model genetic system in our studies of this pathway.

Seeds from an *N*-transgenic tomato line were subjected to fast-neutron mutagenesis, and a mutant was identified in which a critical component of the *N*-mediated resistance pathway is disrupted. This *sun1-1* (suppressor of N) mutant impairs resistance mediated by multiple *R* genes in addition to *N*, such as *Bs4*, *I*, and *Ve*, displays enhanced susceptibility to virulent pathogens, suppresses *PR* gene expression, and is involved in the salicylic acid (SA) pathway. However, the *sun1-1* mutation does not impair resistance imparted by *Mi-1*, a CC-NBS-LRR *R* gene. Positional cloning of *sun1-1* identified it as an *EDS1* homolog, revealing a role for *EDS1* in tomato in conferring basal resistance, resistance mediated by the TIR-class of R proteins, as well as at least one receptor-like R protein.

Results

The sun1-1 *mutant is defective in* N-*mediated resistance, SA accumulation, and SAR*

A screen was initiated in mutagenized TMV-resistant, wild-type tomato plants [VF36 (*N*)], which carry three tandem copies of genomic DNA containing the *N* gene (Whitham *et al.*, 1996), to identify TMV-susceptible plants. A putative mutant was identified: *sun1-1* (*N*). In the absence of the pathogen, *sun1-1* (*N*) plants exhibited normal development and morphology. Seedlings of *sun1-1* (*N*) failed to develop HR when infected with TMV and supported systemic spread of the virus. F_1 seedlings from crosses of *sun1-1* (*N*) with either VF36 or with VF36 (*N*) developed HR after TMV inoculation indicating that the *N* gene was functional in these progeny (Table 1). In the *sun1-1* (*N*) × VF36 (*N*) F_2 generation, resistance to TMV segregated at a ratio of 3:1, indicating that *sun1-1* is a single locus mutation (Table 1). In addition, crossing *sun1-1* (*N*) plants to the tomato VFNT line (see below) resulted in three F_3 families that were

Table 1 Ratio of TMV-resistant (R) to susceptible (S) seedlings in F_1 and F_2 generations of *sun1-1* (*N*) crosses

	F_1		F_2		
Cross	R	S	R	S	Ratio
sun1-1 × VF36	15	0	32	35	9:7[a]
sun1-1 × VF36 (*N*)	15	0	67	19	3:1[b]

[a]Chi-square test fits ratio of 9:7 (d.f. = 1, *P* > 0.1).
[b]Chi-square test fits ratio of 3:1 (d.f. = 1, *P* > 0.1).

genotyped. All the families were selected for the presence of the *N* gene, but only one has the *sun1-1* allele, indicating that *SUN1* and *N* are not linked (data not shown).

We next tested *sun1-1* (*N*) plants for induction of *PR* gene expression, a hallmark of SAR (Guo *et al.*, 2000; Ryals *et al.*, 1996). Seedlings of VF36 (*N*), VF36, and *sun1-1* (*N*) plants were inoculated with TMV, and samples of plant tissue were collected at various time points after inoculation. RNA from these samples hybridized with *PR* gene probes revealed that expression of both *PR1-b1* and *PR2* in *sun1-1* (*N*) plants is significantly lower than that in VF36 (*N*) plants. In addition, expression of these genes in *sun1-1* (*N*) plants is even slightly reduced compared to that of VF36 plants (Figure 1a). Quantitative RT-PCR confirmed these findings. Four days after TMV infection, *PR1* expression is induced 10- to 12-fold

more in VF36 (*N*) plants compared to either VF36 or *sun1-1* (*N*) plants (Figure 1b). These results indicate that induction of *PR1* gene expression, a hallmark of SAR, is suppressed in the *sun1-1* (*N*) mutant.

To address whether SA induction resulting from *N*-mediated TMV recognition is impaired in the *sun1-1* (*N*) mutant, seedlings of VF36 (*N*), VF36, and *sun1-1* (*N*) were inoculated with TMV, and the levels of SA were monitored using HPLC. The results demonstrated that SA induction in *sun1-1* (*N*) plants was significantly reduced compared to VF36 (*N*) plants (Figure 1c). Total SA levels in VF36 (*N*) seedlings showed a strong increase by 4 days after TMV inoculation, and continued to rise throughout the 8 days of observation, at which point they were 29 times the level before infection. In contrast, the amount of SA in VF36 plants
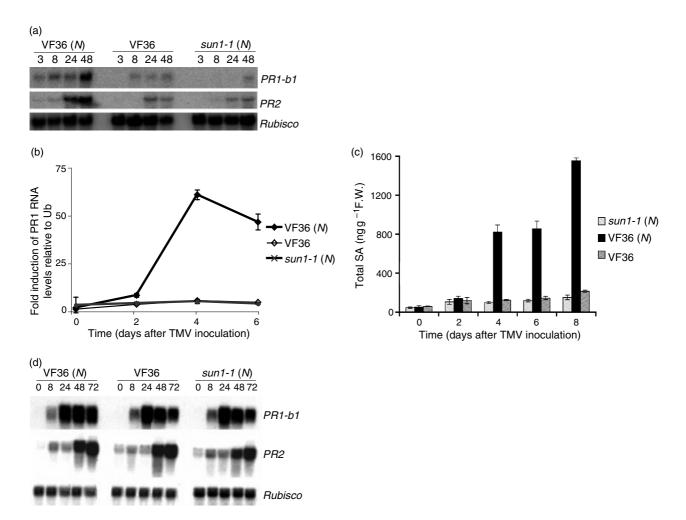

Figure 1. Salicylic acid (SA) and systemic acquired resistance are suppressed in the *sun1-1* (*N*) mutant.
(a) Analysis of *PR* gene expression in tobacco mosaic virus (TMV)-infected plants. Total RNA was isolated and hybridized with *PR1b1* and *PR2* probes. Rubisco was used as a loading control.
(b) Quantitative RT-PCR showing relative levels of *PR1b* in VF36 (*N*), VF36, and *sun1-1* (*N*) plants relative to ubiquitin.
(c) Analysis of SA induction of VF36 (*N*), VF36, and *sun1-1* (*N*) plants upon TMV inoculation. Tissue samples from three genotypes were collected at the time points indicated after TMV inoculation. HPLC analysis on each sample was performed, and the results are plotted as total SA levels (free and bound SA).
(d) Analysis of *PR* gene expression after spraying a 5-mM SA in 0.02% Silwet solution on plants. Total RNA from tissue harvested at the time points indicated hours after SA application was isolated and hybridized with *PR1b1* and *PR2* probes. Rubisco was used as a loading control.

rose to only 3.6 times pre-infection levels, and to only 3.3 times pre-infection levels in *sun1-1* (*N*) plants. This result indicates that SA induction following pathogen exposure in *sun1-1* (*N*) seedlings is impaired compared to VF36 (*N*), and is suppressed even compared to VF36 seedlings.

SA is known to induce SAR and lead to increased expression of PR proteins, possibly by activating specific components of a signal transduction pathway leading from pathogen recognition to induced basal defenses associated with SAR (Malamy *et al.*, 1990; Van Loon and Antoniw,
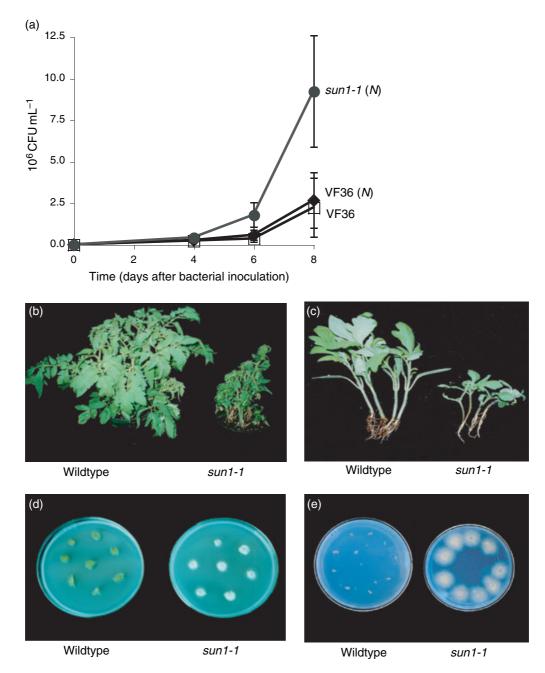

Figure 2. *sun1-1* impairs other *R* gene-mediated pathways.
(a) Growth curve of *Xanthomonas campestris* pv. *vesicatoria* strain 82-8 containing *AvrBs4* on VF36 (*N*), VF36, or *sun1-1* (*N*) plants. Each point represents the mean and standard deviation over three independent experiments.
(b) VF36 (left) and *sun1-1* (right) plants 3 weeks after inoculation with *Verticillium dahliae* race 1.
(c) Seedlings of VF36 (left) and *sun1-1* (right) 2 weeks after inoculation with *Fusarium oxysporum* f. sp. *lycopersici* race 1.
(d) Upper stem cultures of infected seedlings demonstrating recovery of *Verticillium* from *sun1-1* (right) but not VF36 (left).
(e) Upper stem cultures of infected seedlings demonstrating recovery of *Fusarium* from *sun1-1* (right) but not VF36 (left).

1982). Exogenous application of SA induces *PR* gene expression (Dempsey *et al.*, 1999; Ryals *et al.*, 1996), and in susceptible tomato plants, application of exogenous SA reduces accumulation of TMV after infection (White, 1979). Experiments using plants expressing the bacterial salicylate hydroxylase gene *nahG*, which converts SA to catechol, confirmed a role for SA in *N*-mediated resistance to TMV and induction of SAR (Gaffney *et al.*, 1993). To help us ascertain where SUN1 is involved in the signal transduction pathway, we investigated the effect of exogenous SA application on the *sun1-1* (*N*) mutant. As before, we measured expression of *PR* genes to assess the SAR response. We applied a 5-mM SA solution to VF36 (*N*), VF36, and *sun1-1* (*N*) plants and collected tissue samples at several time points after exposure. Total RNA from those samples was subjected to Northern blot analysis using *PR* gene probes. *PR1-b1* and *PR2* expression appeared to be induced to the same level in all three genotypes throughout the 3-day time course tested (Figure 1d), indicating that the application of exogenous SA restores *PR1* gene expression in *sun1-1* (*N*) mutants. These results show that *sun1-1* (*N*) plants are still competent to sense and respond to SA, and together with the results of reduced SA accumulation in *sun1-1* (*N*) plants presented in Figure 1(c), suggest that *SUN1* is required for the generation and/or accumulation of SA in response to pathogen.

The sun1-1 mutant impairs other R gene-mediated pathways

The VF36 line expresses other characterized *R* genes that confer upon it resistance to a variety of pathogens. These *R* genes include *Bs4*, a TIR-NBS-LRR class *R* gene mediating resistance to the bacterium *Xanthomonas campestris* pv. *vesicatoria* (*X.c.* pv. *vesicatoria*) strain 82-8 expressing *AvrBs4* (Schornack *et al.*, 2004); the two, inverted genes *Ve1* and *Ve2*, each of which encodes a membrane-spanning, LRR domain-containing protein that confers resistance to *Verticillium dahliae* race 1 (Kawchuk *et al.*, 2001); and *I*, an un-cloned gene that confers resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 1 (Sela-Buurlage *et al.*, 2001). This permitted us to test whether *SUN1* is involved in resistance responses mediated by these *R* genes. We first examined the response of *sun1-1* (*N*) plants to infection by *X.c.* pv. *vesicatoria* strain 82-8, expressing *AvrBs4*. Two days after infection, both VF36 (*N*) and VF36 plants developed strong HRs. In contrast, *sun1-1* (*N*) plants developed either very weak HR or no HR at all (data not shown). A growth curve of *X.c.* pv. *vesicatoria* strain 82-8 in VF36 (*N*) and *sun1-1* (*N*) seedlings confirmed that *sun1-1* (*N*) plants are more susceptible to *X.c.* pv. *vescicatoria* expressing *AvrBs4* relative to the resistant VF36 and VF36 (*N*) seedlings (Figure 2a). The bacteria had multiplied on *sun1-1* (*N*) plants to levels more than three times higher than on the resistant plants by 8 days after inoculation.

We next tested the effect of the *sun1-1* mutation on resistance mediated by *Ve1* and *Ve2* to *Verticillium dahliae* race 1, and by *I* to *Fusarium oxysporum* f. sp. *lycopersici* race 1 by infecting *sun1-1* and VF36 seedlings with the *Verticillium* and *Fusarium* strains. In contrast to infected VF36 seedlings, which displayed no disease symptoms, *sun1-1* seedlings infected with either fungal pathogen were stunted and wilted within 2 weeks after infection (Tables 2 and 3, Figures 2b,c and 3d, race 1-infected plants), indicating that the mutation obstructs the resistance response mediated by both of these *R* genes. Stem cultures of infected seedlings further demonstrate that the pathogens successfully propagated in *sun1-1* plants but not in VF36 plants (Figure 2d,e). The disparate responses of VF36 and *sun1-1* plants to these fungal pathogens suggest that a functional

Table 2 *Ve*-mediated resistance assays. Indicated are the numbers of susceptible plants (numerator) over the total number of plants tested (denominator)

Plant line	Genotype	Water	*Verticillium* race 1
VF36	*SUN1/SUN1 Ve/Ve*	0/12	0/15
sun1-1 (*N*)	*sun1/sun1 Ve/Ve*	0/10	14/14
Advantage	*SUN1/SUN1 Ve/Ve*	0/12	0/12
Early Pak 7	*SUN1/SUN1 Ve/Ve*	0/12	0/15

Table 3 *I*-mediated resistance assays. Indicated are the numbers of susceptible plants (numerator) over the total number of plants tested (denominator)

Plant line	Genotype	Water	*Fusarium* race 1	*Fusarium* race 2
VF36	*SUN1/SUN1 I/I i2/i2*	0/10	0/10	9/10
sun1-1 (*N*)	*sun1/sun1 I/I i2/i2*	0/10	6/10	10/10[a]
Advantage	*SUN1/SUN1 I/I i2/i2*	0/10	0/10	10/10
Early Pak 7	*SUN1/SUN1 i/i i2/i2*	0/10	9/10	10/10

[a]Severe susceptibility.

Table 4 *Mi-1*-mediated nematode resistance assays

Plant line	Genotype	Response of *Meloidogyne javanica*[a]
VFNT	*SUN1/SUN1 Mi-1/Mi-1*	Resistant
VF36	*SUN1/SUN1 mi-1/mi-1*	Susceptible
sun-1-1 (*N*)	*sun1/sun1 mi-1/mi-1*	Susceptible
VFNT × *sun1-1* (*N*), F$_2$ line no. 13	*sun1/sun1 Mi-1/Mi-1*	Resistant
Motelle	*SUN1/SUN1 Mi-1/Mi-1*	Resistant

[a]Six seedlings of each plant line were assessed. Resistant indicates that no development of nematodes inside the root was observed after 2 weeks. Susceptible indicates that nematodes developed inside the roots. Responses within each plant type were uniform for the six seedlings tested.

SUN1 gene is required for resistance conferred by *Ve1*, *Ve2*, and *I*.

The *Mi-1* gene confers resistance in tomato to the root-knot nematode *Meloidogyne javanica*, and encodes a CC-NBS-LRR protein (Milligan *et al.*, 1998). To address whether *SUN1* is required for *Mi-1*-mediated resistance to the nematode, we identified F_2 plants homozygous for *Mi-1* and *sun1-1* derived from a cross between *sun1-1* plants and the tomato VFNT line, which carries *Mi-1* (Milligan *et al.*, 1998). Roots from the F_2 seedlings were infected with nematode juveniles in a petri dish assay, and development of nematodes was assessed after 2 weeks. No nematode development (resistant phenotype) was seen when *Mi-1* was present, regardless of which *SUN1* allele was present (Table 4). Greenhouse assays in which egg mass production was assessed 6 weeks after nematode infection also showed that *sun1-1* and wild-type plants were equally resistant when *Mi-1* was present (results not shown). Based on these assays, we conclude that, in contrast to the result shown above for *N*, *Bs4*, *Ve*, and *I*, the *sun1-1* mutation has no effect on *Mi-1*-mediated resistance to *M. javanica*.

The sun1-1 (N) mutant displays enhanced susceptibility to TMV and to virulent pathogens

Interestingly, there was a clear morphological difference between the response of *sun1-1* (*N*) plants to TMV and that of isogenic, susceptible VF36 plants, which lack the *N* gene. Although both VF36 and mutant plants exhibited mosaic symptoms, the leaf surface of *sun1-1* (*N*) plants became more curled and the leaflets were stunted (Figure 3a). Occasionally, small lesions developed on the curled leaves. We wished to explore the possibility of enhanced susceptibility in the *sun1-1* (*N*) mutant. We measured the spread on TMV by quantitative RT-PCR for TMV coat protein (*CP*) RNA, over a 6-day period after TMV infection. Whereas there was no significant accumulation of TMV *CP* RNA in resistant VF36 (*N*) plants, the TMV *CP* RNA accumulated considerably in both susceptible VF36 and *sun1-1* (*N*) plants. The levels of TMV *CP* RNA rose more rapidly in *sun1-1* (*N*) plants than in VF36, such that 4 days after inoculation, the amount of accumulated TMV *CP* RNA was about threefold higher in *sun1-1* (*N*) plants than in VF36 plants (Figure 3b). These results support the conclusion that mutation of *sun1-1* causes an enhanced susceptibility to TMV.

We next tested whether the enhanced susceptibility phenotype of the *sun1-1* (*N*) mutant is also exhibited in response to virulent pathogens. We tested the growth of the bacterial pathogen, *X.c.* pv. *vesicatoria*, strain 85-10, which does not carry any *Avr* genes recognized by *R* genes present in the VF36 line. The growth curves demonstrated that, although all three genotypes are susceptible to the bacterial strains, the bacteria is able to propagate to levels approximately two times higher on *sun1-1* (*N*) plants than on either

VF36 (*N*) or VF36 plants by 6 days after inoculation (Figure 3c). We also tested the response of *sun1-1* (*N*) mutant plants to the virulent pathogen *F. oxysporium* f. sp. *lycopersici* race 2. VF36 plants, which do not carry the corresponding *R* gene *I2*, become wilted within 3 weeks after inoculation with *F. oxysporium* f. sp. *lycopersici* race 2, but not after mock infection or infection with *F. oxysporium* f. sp. *lycopersici* race 1 (Figure 3d, top). *sun1-1* (*N*) mutant plants, however, show a much more dramatic stunting and wilting phenotype in response to the fungus (race 2) than do the VF36 plants (Figure 3d). Stem cultures of infected seedlings demonstrate that the *F. oxysporium* f. sp. *lycopersici* race 2 successfully propagated in both *sun1-1* (*N*) and VF36 (data not shown). These results indicate that the *sun1-1* (*N*) mutation confers enhanced susceptibility to virulent pathogens.

SA application on sun1-1 (N) plants partially suppresses the enhanced disease susceptibility, but does not restore resistance to TMV

We hypothesized that the enhanced susceptibility of the *sun1-1* mutant is due to its inability to properly induce SA accumulation after pathogen attack (see Figure 1c). We therefore tested whether exogenously applied SA in the *sun1-1* (*N*) plants is sufficient to allow normal induction of *PR1* gene expression in response to TMV. Similar to that observed for SA treatment alone, exogenous application of SA followed by TMV inoculation induced *PR1* expression on all three genotypes (Figure 4a). However, in contrast to SA treatment alone, expression of *PR1* in VF36 (*N*) was induced more strongly than in *sun1-1* (*N*) plants, particularly at 4 days after TMV inoculation, where levels in VF36 (*N*) plants were four to five times higher than in VF36 or *sun1-1* (*N*) plants. These results indicate that exogenous SA is not sufficient to provide the additional burst of *PR1* expression upon TMV infection in the *sun1-1* (*N*) mutant that is observed in VF36 (*N*).

To test whether the impaired SA induction in *sun1-1* (*N*) plants contributed to their enhanced susceptibility, we exogenously applied SA to VF36 (*N*), VF36, and *sun1-1* (*N*) seedlings, and monitored TMV *CP* RNA accumulation using quantitative RT-PCR. In order to allow time for changes in gene expression of SA-mediated defense genes, such as *PR1*, to occur before pathogen challenge, TMV was inoculated on the seedlings 20 h after SA application. SA-treated *sun1-1* (*N*) plants were still susceptible to TMV, accumulating significantly more TMV *CP* RNA than resistant plants [VF36 (*N*)] by 4 days after TMV inoculation (Figure 4b), indicating that SA application does not restore the *N*-mediated resistance to *sun1-1* (*N*) plants. However, the difference in TMV *CP* RNA accumulation between SA-treated VF36 and *sun1-1* (*N*) plants was reduced when compared with the plants not treated with SA (Figure 4b).
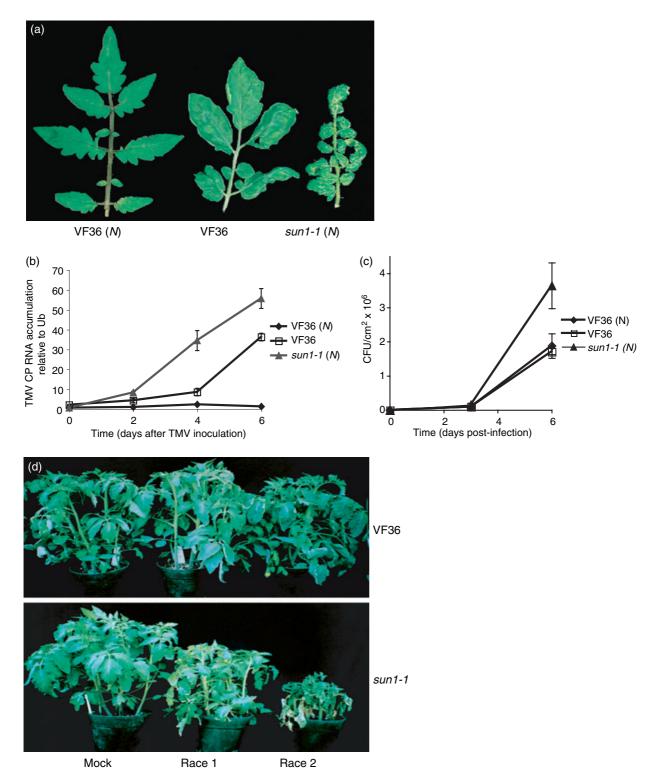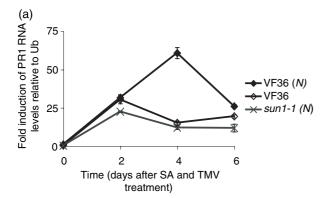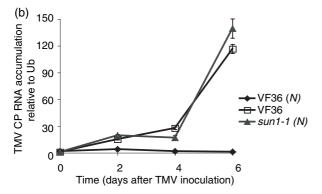
Figure 3. Enhanced susceptibility of *sun1-1* (*N*) plants.
(a) Characterization of the tobacco mosaic virus (TMV) response in newly expanded leaves of VF36 (*N*) plants (left), VF36 plants not expressing *N* (middle), and *sun1-1* (*N*) plants (right) 30 days after inoculation with TMV.
(b) Quantitative RT-PCR showing relative levels of TMV *CP* RNA in TMV-infected VF36 (*N*), VF36, and *sun1-1* (*N*) plants relative to ubiquitin.
(c) Growth curve of *Xanthomonas campestris* pv. *vesicatoria* strain 85-10 in infected VF36 (*N*), VF36, and *sun1-1* (*N*) plants.
(d) Disease phenotype of VF36 (top) and *sun1-1* (bottom) plants 3 weeks after mock infection (left) or infection with *Fusarium oxysporium* f. sp. *lycopersici* race 1 (middle) or race 2 (right).
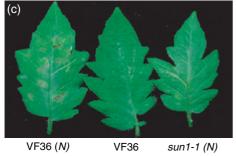
Figure 4. Salicylic acid (SA) application partially suppresses the enhanced susceptibility of *sun1-1* (*N*) plants. Seedlings were sprayed with 5 mM SA.
(a) Plants were inoculated with tobacco mosaic virus (TMV) immediately after SA treatment. Quantitative RT-PCR showing relative levels of *PR1* in SA-treated, TMV-infected VF36 (*N*), VF36, and *sun1-1* (*N*) plants relative to ubiquitin at various times after TMV inoculation.
(b) Plants were inoculated with TMV 20 h after SA treatment. Quantitative RT-PCR for TMV *CP* RNA accumulation in VF36 (*N*), VF36, and *sun1-1* (*N*) at various times after TMV infection.
(c) TMV was inoculated on leaves at various time points after SA treatment. The cell death phenotype developed on all the VF36 (*N*) plants 3 days after TMV inoculation. No cell death was detected on VF36 or *sun1-1* (*N*) plants. Shown are plants inoculated with TMV 5 h after SA application, and the pictures were taken 7 days later.

Over several independent experiments, we observed a range from no difference in TMV *CP* RNA accumulation between VF36 and *sun1-1* (*N*) plants to enhanced accumulation in *sun1-1* (*N*) plants compared to VF36 (as is shown in Figure 4b), but the difference between VF36 and *sun1-1* (*N*) plants was never as great as had been observed in plants not treated with SA (see Figure 3b). These results indicate that

SA application does at least partially suppress the enhanced susceptibility in *sun1-1* (*N*) plants.

Cell death is another important characteristic associated with *R* gene resistance responses. It is closely correlated with EDS1 function and SA production (Alvarez, 2000; Durrant and Dong, 2004; Feys *et al.*, 2001), and is impaired in *sun1-1* mutant plants. Even after exogenous application of SA, *sun1-1* (*N*) plants were not able to induce an HR in response to TMV infection (Figure 4c). These results indicate that SA application is also not sufficient to restore the cell death pathway in *sun1-1* mutant plants.

Identification of SUN1 *as the tomato* EDS1 *homolog*

To clone the *sun1-1* mutation, bulk segregant analysis was performed using 123 RFLP markers covering the 12 chromosomes of *L. esculentum* at an approximately 10 cM spacing. One of the probes tested, TG292, detected a clear polymorphism between the susceptible and resistant pools, thus indicating linkage to this molecular marker, which maps to the long arm of chromosome 6 in *L. esculentum* (Figure 5a). Based on this finding, we tested additional markers in this region. Two additional markers, TG444 and TG356, at chromosomal location 50.5 and 37.9 cM, respectively, were also polymorphic (data not shown). This allowed us to tentatively place *SUN1* in this region of chromosome 6. Fine mapping was performed using RFLP analysis of the individuals in our mapping population with probes in the 29–77 cM region of chromosome 6 (Figure 5b). Based on recombination events in these populations we identified two markers tightly linked to *sun1-1*, TG364 (56 cM) and TG253 (55 cM). TG364 is estimated to be linked to *sun1-1* at a genetic distance of approximately 0.2 cM (one unique recombination event in 513 individuals scored) and TG253 is estimated to be approximately 1.2 cM (six unique recombination events in 513 individuals scored) away from *sun1-1* in our population (data not shown).

A 1-kb fragment of TG364 was used to probe the tomato Heinz 1706 BAC library (http://www.genome.clemson.edu/ groups/bac/libraries_genomic.html). Four BAC clones were isolated in this screen, and one was sequenced and annotated by Robin Buell's laboratory at TIGR (Rockville, MD, USA; http://www.tigr.org/tigr-scripts/tdb/potato/BAC_ annotation/bac_display.pl) to identify *SUN1* candidate genes. Annotation by TIGR identified 16 putative genes (Figure 5c). PCR analysis of wild type and *sun1-1* genomic DNA using various primer sets across the identified sequence revealed an 8.1-kb deletion in the *sun1-1* mutant that spans from 921 bp upstream of the betaine aldehyde dehydrogenase (*BADH*) gene to the middle of the *EDS1*-like gene, 1427 bp downsteam of its START codon (Figure 5c,d).

To confirm that the phenotype we had observed in *sun1-1* plants was due to this deletion, we performed complementation experiments by transforming *sun1-1* (*N*) plants with
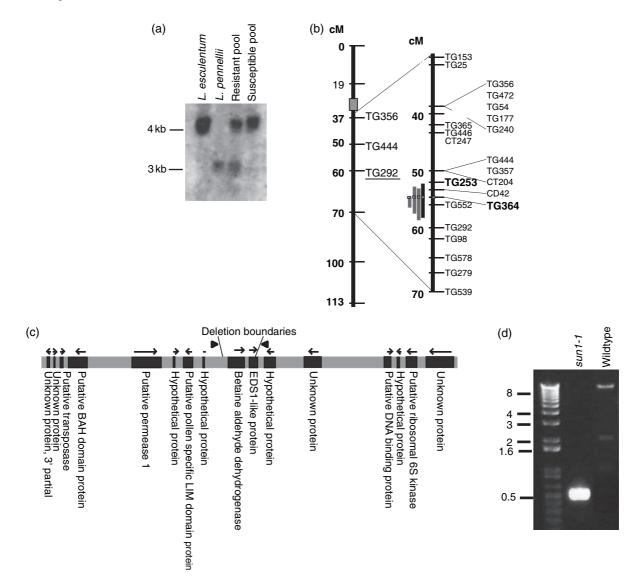
Figure 5. Cloning of *sun1-1*.
(a) Southern blot containing DNA from *Lycopersicon esculentum*, *L. pennellii*, a pool of 10 resistant BC$_1$F$_1$ plants, or a pool of ten susceptible BC$_1$F$_1$ plants digested with *Bst*NI and probed with RFLP marker TG292 (chromosome 6, position 60 cM).
(b) RFLP markers on chromosome 6 of tomato. *sun1-1* was initially landed with marker TG292 (underlined), and then additional markers in the 29–77 cM interval were used in screening the mapping population. Markers TG364 and TG253 (bold) tightly flank the *sun1* locus. Using marker TG364, four BACs were identified (noted as thick vertical bars; not drawn to scale), and one was chosen for sequencing (the black vertical bar).
(c) This sequence was annotated, and contains 16 putative genes, which are indicated by the darker sections, with arrows above denoting the direction of the open reading frame. Arrowheads mark positions of primers used in (d), and the lines denote the location of the deletion boundaries in the *sun1-1* mutant, which encompasses the two genes shown as solid black segments.
(d) Amplification of genomic DNA from wild type or *sun1-1* mutant plants reveals the 8.1 kb deletion in the *sun1-1* sequence, spanning from 921 bp upstream of the *BADH* START codon to 1427 bp downstream of the *EDS1* START codon.

Agrobacteria containing a construct of a 11.4 kb genomic region encompassing both *BADH* and the *EDS1*-like genes. Leaves from the T0 transgenic plants were infected with TMV, and the HR response was scored 5 days later, and TMV accumulation in upper, uninoculated leaves was assayed after several weeks. The HR response was restored in the *sun1-1* (*N*) plants expressing this construct (Figure 6a), and no TMV was detected in the upper, uninoculated leaves

(data not shown). The described role of *EDS1* in disease resistance in Arabidopsis (Parker *et al.*, 1996) led us to hypothesize that the phenotypes we had observed in our *sun1-1* (*N*) mutant were caused by disruption of the *EDS1*-like gene in tomato, rather than *BADH*. Therefore, we tested whether the HR response was restored in mutant plants transformed with a construct containing only the *BADH* gene. These plants did not produce an HR response
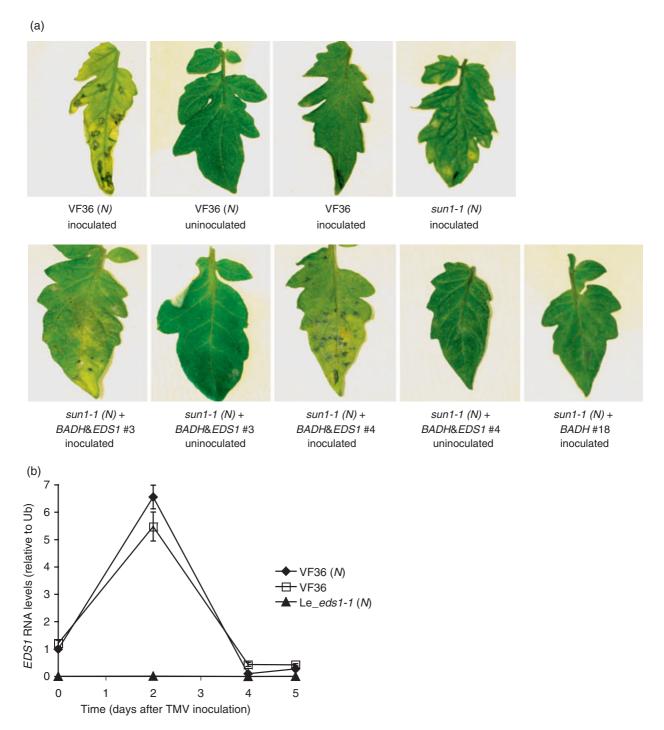
(a)

| VF36 (*N*) inoculated | VF36 (*N*) uninoculated | VF36 inoculated | *sun1-1* (*N*) inoculated |

| *sun1-1* (*N*) + *BADH*&*EDS1* #3 inoculated | *sun1-1* (*N*) + *BADH*&*EDS1* #3 uninoculated | *sun1-1* (*N*) + *BADH*&*EDS1* #4 inoculated | *sun1-1* (*N*) + *BADH*&*EDS1* #4 uninoculated | *sun1-1* (*N*) + *BADH* #18 inoculated |

(b)

Figure 6. Complementation of *sun1-1* with the *EDS1*-like gene.
(a) One leaf of control plants (VF36 (*N*), VF36, and *sun1-1* (*N*)) or of transgenic plants (*sun1-1* (*N*) transformed with either *BADH* and *EDS1*, or *BADH* alone) was infected with TMV. Pictures of the infected leaf and an upper, uninfected leaf were taken 5 days later (or 7 days for VF36 (*N*) and VF36 plants).
(b) Quantitative RT-PCR of *EDS1* RNA levels in VF36 (*N*), VF36, and Le_*eds1-1::N* at various times following TMV infection.

(Figure 6a), and accumulated TMV in the upper, uninoculated leaves (data not shown), suggesting that the loss of *BADH* function is not responsible for the mutant phenotype. These results indicate that loss of an *EDS1*-like gene in tomato is responsible for the observed block in several resistance

pathways, and we therefore will now refer to this gene as Le_*EDS1*. The nature of the deletion suggests that the Le_*EDS1* gene is unlikely to be expressed in the mutant plants. Quantitative RT-PCR of Le_*EDS1* expression confirmed this, demonstrating that in the mutant there is little or

no measurable expression of the gene, compared to both VF36 and VF36 (*N*) plants (Figure 6b). In addition, TMV infection resulted in increased expression of Le_*EDS1* in both VF36 and VF36 (*N*) plants. Similar findings in *A. thaliana* and *N. benthamiana* that pathogen stimulates *EDS1* expression (Falk *et al.*, 1999; Peart *et al.*, 2002) support the idea that Le_*EDS1* is a functional homolog of Arabidopsis *EDS1*. Further evidence is revealed in an alignment of the predicted protein sequence of Le_EDS1 with those from *A. thaliana*, *N. benthamiana*, *N. tabacum*, and *Oryza sativa japonica*, which shows that the critical residues of the lipase domain are conserved across these plant species (Figure 7 and Falk *et al.*, 1999; Jirage *et al.*, 1999). Le_EDS1 also contains the EP domain in its C-terminus, a domain found in EDS1, PAD4, and other plant proteins (Feys *et al.*, 2001). Within the EP domain, Le_EDS1 contains the KNEDT motif, which distinguishes EDS1 proteins from PAD4 (Figure 7 and Peart *et al.*, 2002).

Discussion

We have used a genetic approach in *N*-transgenic tomato to identify a critical component of the signal transduction pathway leading from an *R* gene product to resistance responses. We have identified the mutated gene as a homolog of the *EDS1* gene, Le_*EDS1*. Based on Southern blot analysis, Le_*EDS1* appears to be the only copy of an *EDS1*-like sequence in the *L. esculentum* genome (G. Hu, A.K.A. deHart and B. Baker, unpublished data). This is in contrast to Arabidopsis accession Col-0, which contains two closely related *EDS1* genes [*EDS1*: At3G48090 and the 83/72% identical (nucleotide/amino acid) *EDS1*-like: At3g48080], and the aneuploid *N. benthamiana*, which also contains two copies of *EDS1* (Liu *et al.*, 2002; Peart *et al.*, 2002).
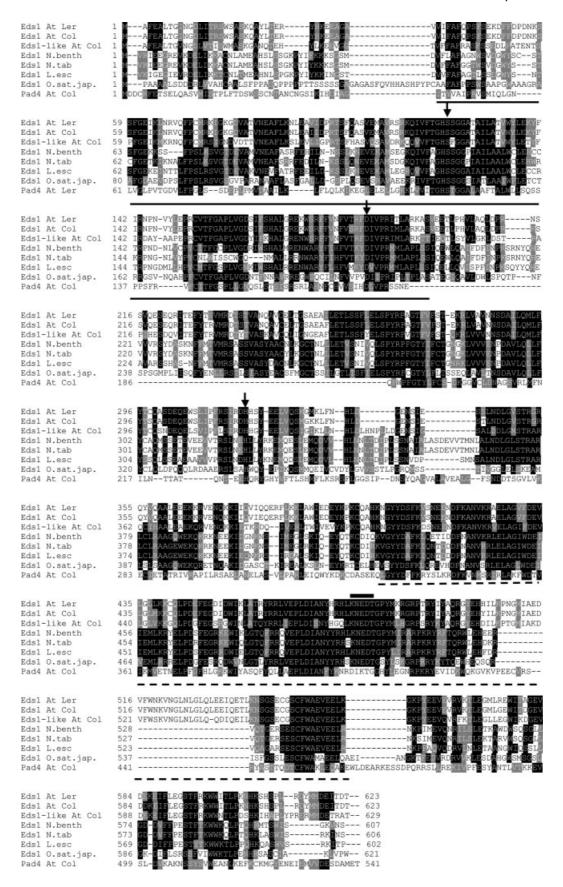
Le_*eds1-1* plants have reduced levels of SA, and display impaired *R* gene-mediated resistance to viral, bacterial, and fungal pathogens. We have shown that Le_*EDS1* functions in multiple, but not all, resistance responses. In addition to exhibiting impaired *R* gene-mediated resistance, Le_*eds1-1* mutant plants appear more susceptible to invading pathogens than plants that lack the corresponding *R* genes. This suggests a role for Le_*EDS1* in basal defense.

In Arabidopsis *EDS1* is involved in resistance primarily mediated via the TIR-class *R* genes, but not the CC-class *R* genes (Aarts *et al.*, 1998). This finding was supported by studies in *N. benthamiana*, showing that silencing of Nb_*EDS1* affected resistance by *N* and *Bs4*, but not by *Rx* or *Pto* (Peart *et al.*, 2002; Schornack *et al.*, 2004). Some exceptions have been reported, including *R* genes that require neither *EDS1* nor *NDR1* (Aarts *et al.*, 1998; and see Martin *et al.*, 2003) and a requirement for *EDS1* in mediating resistance by *HRT* (a CC-NBS-LRR *R* gene) to turnip crinkle virus in Arabidopsis (Chandra-Shekara *et al.*, 2004). The

findings presented here extend this characteristic of *EDS1* to tomato, and significantly expand the number of *R* genes tested, which strengthens the case for a bias of TIR-class *R* genes to require *EDS1*. Our Le_*eds1-1* mutant exhibits impaired *R* gene-mediated resistance by several TIR-class *R* genes, including *N* and *Bs4*, whereas resistance mediated by the CC-class *R* gene *Mi-1* is unaffected. Severe reduction of SA levels in an *nahG* plant results in a partial loss of *Mi-1*-mediated resistance to the root-knot nematode (Branch *et al.*, 2004). Though Le_*eds1-1* mutant plants have reduced levels of SA, our findings suggest that the residual levels of SA must be sufficient for full *Mi-1*-mediated resistance.

Our results in tomato have revealed that Le_*EDS1* is also required for resistance mediated by Ve1 and Ve2, receptor-like *R* proteins containing extracellular LRR domains, transmembrane domains, and putative cytoplasmic endocytic motifs (Kawchuk *et al.*, 2001), exposing a new class of *R* genes that may require *EDS1*. A role for either *EDS1* or *NDR1* has not yet been determined for other receptor-like R proteins with a similar domain structure as the Ve proteins, including the *Cf* resistance genes and the recently identified *EIX2* and *RPP27* genes (Ron and Avni, 2004; Tör *et al.*, 2004). In Arabidopsis, *EDS1* is required for resistance mediated by another, novel type of receptor-like *R* gene, *RPW8*, which confers resistance to a broad spectrum of *Erysiphe* isolates (Xiao *et al.*, 2001). *RPW8* is predicted to encode a protein with an N-terminal transmembrane domain and a CC domain (Xiao *et al.*, 2001). It will be interesting to determine whether the requirement for *EDS1* is shared among other members of the receptor-like *R* gene class.

Our studies have also revealed that Le_EDS1 is important for basal defenses in tomato. Le_*eds1-1* mutant plants display enhanced susceptibility to TMV, *X.c.* pv. *vesicatoria* strain 85-10, and *Fusarium oxysporium* f. sp. *lycopersici* race 2. This role for EDS1 in basal defense in Solanaceous species was not uncovered using a reverse genetic approach (Peart *et al.*, 2002). This discrepancy could reflect a fundamental difference in the function of EDS1 in basal defenses in tomato compared to *N. benthamiana*, or more likely may be the result of residual *EDS1* expression in *EDS1*-silenced *N. benthamiana*. Several findings provide evidence that SA induction is often required for basal defenses. Transgenic tobacco, tomato, and Arabidopsis plants expressing *nahG* display enhanced susceptibility to many virulent pathogens, including TMV, *Pseudomonas syringae* pv. *tabaci*, *Phytophthora parasitica*, *Cercospora nicotianae*, *Botrytis cinerea*, and *Erysiphe orontii* (Achuo *et al.*, 2004; Delaney *et al.*, 1994; Reuber *et al.*, 1998). In addition, the *eds16* mutant in Arabidopsis, which causes enhanced susceptibility to virulent pathogens, encodes isochorismate synthase, an enzyme responsible for synthesizing SA in plants (Dewdney *et al.*, 2000; Wildermuth *et al.*, 2001). The role of Le_*EDS1* in both gene-for-gene resistance and basal resistance further indicates that both resistance pathways share parts of the

same signal transduction circuitry. It is also possible that *EDS1* does not function directly in *R* gene pathways, but rather that certain *R* gene pathways are more sensitive to the presence of an alternate pathway that *EDS1* participates in, such as a basal defense pathway. Further characterization of the Le_eds1-1 mutant will undoubtedly enhance our understanding of how these complex signaling networks converge.

In analyzing the role of Le_*EDS1* in signaling pathways triggered by *R* genes that the Le_eds1-1 line does not carry, we co-infiltrated Le_eds1-1 plants with *Agrobacterium* strains expressing *R* genes and the corresponding *Avr* genes. Preliminary results indicated that HR responses mediated by the *Pto*, *Bs2*, and *Rx* genes are suppressed in Le_eds1-1 plants (G. Hu and B. Barker, unpublished data). Tests to determine the effect on resistance mediated by these *R* genes by Le_*EDS1* are being carried out by crossing the Le_eds1-1 mutation into lines carrying *Pto* and *Bs2*. The preliminary findings indicate that Le_*EDS1* might have a broader, more minor role in all resistance pathways, perhaps through its effect on basal resistance, and also points to a need for caution in the interpretation of transient, co-infiltration assays for resistance phenotypes.

How and where Le_EDS1 acts in the defense pathway remains to be determined. Both *EDS1* and its partner *PAD4* encode proteins with a class 3 lipase domain (http://www.pfam.wustl.edu/cgi-bin/getdesc?name=Lipase_3; Falk et al., 1999; Feys et al., 2001; Jirage et al., 1999). However, lipase activity has not yet been demonstrated for either protein. In addition, no information is known about where in the cell EDS1 or PAD4 act. SA is known to activate SAR and the expression of *PR* genes via an SA amplification loop regulated by EDS1 and PAD4 (Feys et al., 2001; Jirage et al., 1999; Zhang et al., 1999). In our experiments, SA application was capable of inducing SAR, measured by *PR1* expression, in Le_eds1-1 plants. These results support the conclusion that Le_EDS1 is involved in a point upstream of SA production. Our results showing that exogenous application of SA is not sufficient to provide Le_eds1-1 mutant plants with the ability to induce *PR1* expression after TMV inoculation to the same extent as in wild type resistant tomato, to cause HR, to restore resistance to TMV, or even to fully reverse its enhanced susceptibility, suggest that the function of Le_EDS1 is not simply to stimulate SA levels. Identification of the mechanism of EDS1 action should offer clues as to how EDS1 can affect resistance in ways other than by regulating SA levels. It is also possible that our

method of exogenously applying SA, though sufficient to induce *PR1* gene expression, is not able to properly mimic the natural levels or forms of SA induced upon pathogen recognition.

We report here the identification of the *EDS1* homolog in tomato that functions in both basal and *R* gene-mediated resistance. The nature of the mutation has thus allowed us to demonstrate that this dual function of *EDS1* is conserved from Arabidopsis to Solanaceous species, a conclusion that was previously called into question. We have expanded the family of *R* genes known to require *EDS1* function for imparting resistance to include the receptor-like *Ve* genes, and extended the analysis of the role that *EDS1* plays in Solanaceous plants to include fungal pathogens. These findings confirm the important role of *EDS1* in mediating resistance in plants. Further characterization of the mechanistic function of EDS1 should provide valuable insights into how resistance pathways are regulated.

Experimental procedures

Plant materials and growth conditions

We have previously described transformation of *L. esculentum* cultivar VF36 with the *N* gene (Whitham et al., 1996) to obtain the VF36 (*N*) line, which carries three tandem copies of the *N* gene. Independent of the mutant screening regime described below, plants were grown in greenhouses or growth chambers under 12 h of light, 24°C days, and 18°C nights.

Identification of sun1-1

Seeds from VF36 (*N*) plants were pooled and subjected to fast-neutron mutagenesis (International Atomic Energy Agency, Agriculture and Biotechnology Laboratory, Vienna, Austria). Approximately 6000 M1 plants were propagated from the mutagenized seeds, and M2 seeds were screened in flats. Mutagenized seedlings were subjected to a seedling lethal screen, which relies on the temperature sensitivity of *N*-mediated resistance. Seedlings were inoculated with TMV at the restrictive temperature of 28°C. After 2 days, the temperature was shifted to 24°C, a permissive temperature at which *N* function is restored, killing plants with a functional *N*-mediated HR response. Plants with a disruption in a component of *N*-mediated resistance should be able to survive the temperature shift. As we were interested in recovering plants with mutations in loci other than *N*, screen survivors were assayed for intact *N* by Southern blot analysis. Only seedlings with apparently normal banding patterns were accepted for further screening. Approximately 100 000 M2 seeds were screened, and a putative mutant was identified and designated *sun1-1*, for <u>su</u>ppressor of <u>N</u>.

Figure 7. Sequence alignment of Le_EDS1 with EDS1 sequences from *Arabidopsis thaliana* (EDS1 from Landsberg erecta: AF128407; EDS1 from Columbia: At3G48090; EDS1-like from Columbia: At3g48080), *Nicotiana benthamiana* (AAL85347.1), *N. tabacum* (AAM62411.1), and *Oryza sativa japonica* (XP_450883.1), and with PAD4 from *A. thaliana*, Columbia (CAB41130.1). The lipase 3 domain, defined by Pfam, is indicated with a solid line underneath the sequence; the three critical residues (serine, aspartic acid, and histidine) of the lipase catalytic triad are indicated with an arrow; the EP domain, as defined by Feys et al. (2001), is indicated with a dashed line underneath the sequence; and the EDS1-specific KNEDT motif is indicated with a thick bar over the sequence. Sequences were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/) and shaded using Boxshade (http://www.ch.embnet.org/software/BOX_form.html), with identical residues shaded in black and similar residues shaded in gray.

Plant DNA and RNA extraction and analyses

Tomato DNA was isolated using 2X CTAB buffer. Total RNA was extracted from seedlings at the four- to five-leaf stage using the protocol described (Lagrimini *et al.*, 1987), and 10 μg of RNA from each sample was separated on formaldehyde-agarose gels. After transfer to nylon membrane (MSI), hybridizations were performed with ^{32}P-labeled (Amersham, Piscataway, NJ, USA) probes. When more than one probe was used, blots were stripped after the first hybridization and re-probed. *PR1b1* and *PR2* probes were kindly provided by the Staskawicz Laboratory (UC Berkeley, CA, USA). The probe for tomato ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was amplified through RT-PCR (forward primer 5′-CTACGTCTGGAAGATCTGCGAATC-3′; reverse primer 5′-CCACTGCTGCAAAATTAAATACGATC-3′) from tomato RNA.

Four-leaf stage seedlings were inoculated with TMV. For SA treatment, a 5-mM aqueous solution of SA (Sigma, St Louis, MO, USA) plus 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA), or 0.02% Silwet L-77 alone for mock treatment, was sprayed on leaf surfaces of seedlings at the four-leaf stage prior to TMV infection. RNA from tissue collected at various time points after TMV inoculation was DNase-treated and reverse transcribed using oligo dT primer (Invitrogen, Carlsbad, CA, USA) plus TMV *CP* reverse primer (5′-TCCGGTTCCTCTGATCAATTCT-3′) in the same tube. Quantitative RT-PCR reactions were labeled with SYBR® GREEN and performed on ABI 7000 Sequencing Detection System (ABI, Foster City, CA, USA). Ubiquitin was used as the reference gene. Sequences of primers for quantitative RT-PCR are: TMV *CP* forward: 5′-CGTGTTCTTGTCATCAGCGTG-3′; TMV *CP* reverse: 5′-CGACAGTTCGAGCTTGTTGTGT-3′; ubiquitin forward: 5′-ACAAAACACCAACAGCAACAGA-3′; ubiquitin reverse: 5′-AAGGACTCTGGCGGACTACAA-3′; *PR1* forward: 5′-TGCGGTTCATAACGATGC-3′; *PR1* reverse: 5′-CAAGACATAGGCCCGACTCC-3′. Le_*EDS1* forward: 5′-GAATGACCTTGGCCTGAGTACAAG-3′; Le_*EDS1* reverse: 5′-CCTGCTGCACGAAGACACAG-3′. The fold expression level changes varied between experiments, so only values obtained within the same experiment are compared.

SA analyses

SA levels from tomato leaf tissue were analyzed from four- to five-leaf stage seedlings that were inoculated with TMV. Three seedlings from each genotype were pooled as one time point. Tissue samples were ground in liquid nitrogen and extracted as described by Gaffney *et al.* (1993), with minor modifications. The samples were then subjected to HPLC analyses using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) with a Novapak C18 (Waters Corporation, Milford, MA, USA) column and detected fluorometrically using excitation and emission wavelengths of 296 and 405 nm respectively. The isocratic mobile phase was 23% methanol and 77% 20 mM sodium acetate, pH 5.5 at a flow rate of 1 ml min^{-1} at 25°C. Average recovery rates were about 70% using *o*-anisic acid as an internal standard.

Pathogen assays

Three leaves per plant were infiltrated with a suspension of bacterial strains at OD600 = 0.5 (approximately 10^5 colony forming units ml^{-1}) in 10 mM MgSO$_4$. Bacterial growth was monitored as described previously (Ronald *et al.*, 1992). Bacterial strains of *X.c.* pv. *vesicatoria* strain 82-8 and *X. c.* pv. *vesicatoria* strain 85-10 were kindly provided by the Staskawicz laboratory.

Conidia of *Fusarium oxysporum* f. sp. *lycopersici* race 1, race 2, and *Verticillium dahliae* race 1 were harvested from 2-week-old

cultures, filtered through a cheesecloth, and adjusted to 10^6 (or 10^7 for *V. dahliae*) conidia ml^{-1}. Two to three-week-old seedlings (first true-leaf stage) were gently uprooted and inoculated by dipping the roots into conidial suspension for 1 min. Control plants were dipped in water. Seedlings were then re-planted in soil in 12-inch pots. Reaction to the pathogens was scored 3 weeks after inoculation. Plants were rated as susceptible if they exhibited necrotic streaks in the stem xylem. Replication of pathogens in susceptible plants was confirmed by culturing surface-sterilized stem sections of all plants. Pathogens were cultured on potato dextrose agar (Difco, Detroit, MI, USA).

The presence of *Mi-1* was assessed in progeny of a *sun1-1* (*N*) × VFNT cross using the *Mi-1*-linked PCR marker *Rex-1* (Williamson *et al.*, 1994). Line no. 13 was produced from an F$_2$ plant determined to be homozygous for both *Mi-1* and *sun1-1*. A week after germination, the green parts of the seedlings were discarded and roots were transferred onto 1X Murashige and Skoog medium containing 2% sucrose and 2% gelrite. Surface-sterilized juveniles of *M. javanica* strain VW4 (250 per plate) were pipetted onto each plate, and, after 2 weeks, roots were scored as resistant or susceptible based on nematode development (Branch *et al.*, 2004).

Cloning of sun1

sun1-1 (*N*) (pollen donor) was crossed to *L. pennellii* and the resultant F$_1$ hybrid was used as the pollen donor in a *sun1-1* (*N*) × F$_1$ backcross. Seed collected from the backcross was designated BC$_1$F$_1$. In the BC$_1$F$_1$ population all individuals carried the *N* transgene making all individuals (either *NN* or *Nn*) useful in our mapping project. The population was scored for TMV resistance by monitoring HR development following TMV inoculation. We performed bulk segregant analysis using 123 RFLP markers. Markers were chosen from those catalogued on the Solanaceae Genomics Network database (http://www.soldb.cit.cornell.edu/) to provide coverage of the 12 chromosomes of *L. esculentum* at an approximately 10 cM spacing. These probes were hybridized with blots containing parental (*L. esculentum* and *L. pennellii*) DNA, resistant pooled DNA from 10 HR-positive BC$_1$F$_1$ individuals, and susceptible pooled DNA from 10 HR-negative BC$_1$F$_1$ individuals.

An internal 1050 bp TG364 probe was prepared by PCR amplification of TG364 sequence with the primers: 5′-AGGATGACGTTGCAGGATGT-3′ and 5′-AACAGGCAAACCTTGTCCAC-3′ and gel-purified. An *L. esculentum* BAC library (Le-HBa Heinz 1706; provided by Steven Tanksley, Cornell University, Ithaca, NY, USA) was screened according to standard protocols. Four BAC clones were isolated in this screen. The presence of the TG364 sequence was confirmed by PCR and Southern blot analysis, and a preliminary contig was assembled based on restriction digest analysis. One of the BACs was sequenced and annotated by TIGR. The BAC contained 16 putative genes, and the sequence data have been deposited with the EMBL/GenBank data libraries under accession number AY796114. The deletion in *sun1-1* mutant genomic DNA, spanning from 921 bp upstream of the *BADH* START codon to 1427 bp downstream of the *EDS1*-like START codon, was identified by amplification with the primers: 5′-AGTAGGGGTGTTCGTGATTCGATTTG-3′ and 5′-CATCAGGGTTCTCAACGACAACC-3′, which span the deletion.

Genomic sequences of either *BADH* alone (from 1775 bp upstream to 3280 bp downstream) or *BADH* and *EDS1*-like (from 1775 bp upstream of *BADH* to 1155 bp downstream of *EDS1*-like) were amplified from the BAC DNA and inserted into pGreen II 0179 (http://www.pgreen.ac.uk/pGreenII/pGreenII.htm). *sun1-1* (*N*) plants were transformed with the constructs at the Ralph M. Parsons Foundation Plant Transformation Facility (UC Davis, Davis, CA,

USA). T0 plants expressing both the desired transgene and the hygromycin resistance gene that was used as selection were identified. These plants were inoculated with TMV to score for complementation of the mutation by recovery of HR (observed phenotypically) and by accumulation of TMV in the upper, uninoculated leaves, assayed with the ImmunoStrip Test Kit for TMV (Agdia, Elkhart, IN, USA).

Acknowledgements

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