# Functions of *EDS1-like* and *PAD4* genes in grapevine defenses against powdery mildew

**Fei Gao[1], Ru Dai[1,2], Sharon M. Pike[1], Wenping Qiu[2], Walter Gassmann[1*]**

[1]Division of Plant Sciences, C.S. Bond Life Sciences Center and Interdisciplinary Plant Group, University of Missouri, Columbia, MO 65211-7310, USA

[2]Center for Grapevine Biotechnology, W. H. Darr School of Agriculture, Missouri State University, Mountain Grove, MO 65711

*E-mail: gassmannw@missouri.edu

Address: 371C Life Sciences Center, Columbia, MO 65211-7310, USA

Phone: 573-884-7703

Fax: 573-884-9676

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**Abstract**

The molecular interactions between grapevine and the obligate biotrophic fungus *Erysiphe necator* are not understood in depth. One reason for this is the recalcitrance of grapevine to genetic modifications. Using defense-related Arabidopsis mutants that are susceptible to pathogens, we were able to analyze key components in grapevine defense responses. We have examined the functions of defense genes associated with the salicylic acid (SA) pathway, including *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *EDS1-LIKE 2 (EDL2), EDL5* and *PHYTOALEXIN DEFICIENT 4 (PAD4)* of two grapevine species, *V. vinifera* cv. Cabernet Sauvignon, which is susceptible to *E. necator*, and *V. aestivalis* cv. Norton, which is resistant. Both *VaEDS1* and *VvEDS1* were previously found to functionally complement the Arabidopsis *eds1-1* mutant. Here we show that the promoters of both *VaEDS1* and *VvEDS1* were induced by SA, indicating that the heightened defense of Norton is related to its high SA level. Other than *Va/VvEDS1*, only *VaEDL2* complemented Arabidopsis *eds1-1*, whereas *Va/VvPAD4* did not complement Arabidopsis *pad4-1*. Bimolecular fluorescence complementation results indicated that *Vitis* EDS1 and EDL2 proteins interact with *Vitis* PAD4 and AtPAD4, suggesting that *Vitis* EDS1/EDL2 forms a complex with PAD4 to confer resistance, as is known from Arabidopsis. However, *Vitis* EDL5 and PAD4 did not interact with Arabidopsis EDS1 or PAD4, correlating with their inability to function in Arabidopsis. Together, our study suggests a more complicated EDS1/PAD4 module in grapevine and provides insight into molecular mechanisms that determine disease resistance levels in *Vitis* species native to the North American continent.

**Keywords:**

*Arabidopsis thaliana*; *Erysiphe necator*; EDS1; powdery mildew; *Vitis aestivalis*; *Vitis vinifera*

## Introduction

The most commonly cultivated species of grapevine, *Vitis vinifera*, is highly susceptible to many pathogens including powdery mildew (PM) caused by the obligate biotrophic fungus *Erysiphe necator*. Resistance to this pathogen mainly occurs in other grapevine species. Thus far, in-depth understanding of molecular grapevine-*E. necator* interactions has mainly relied on identifying candidate resistance genes that encode proteins containing conserved nucleotide binding (NB) and leucine-rich repeat (LRR) motifs at resistance gene loci. *Run1* (*Resistance to Uncinula necator 1*) (Donald et al. 2002; Barker et al. 2005) and *Ren1 (Resistance to Erysiphe necator 1)* (Hoffmann et al. 2008; Coleman et al. 2009) are two such loci associated with resistance to powdery mildew in different grapevine species. Two closely related NB-LRR genes，*RUN1* and *RPV1* (*Resistance to Plasmopara viticola 1*) were cloned from the wild North American grapevine species *Muscadinia rotundifolia* and were found to confer resistance to multiple powdery and downy mildew isolates (Feechan et al. 2013). Additionally, sequencing of the *V. vinifera* Pinot Noir genome identified potential downstream defense-related genes distributed on all 19 grapevine chromosomes (Moroldo et al. 2008), with some shown to be modulated significantly at the transcript level by pathogen infection (Fung et al. 2008; Camps et al. 2010).

Grapevine species also vary in levels of defense-related gene activation in basal resistance, a branch of the plant innate immune system that does not rely on resistance genes. Norton, a cultivar which derives much of its genome from the North American grapevine species *Vitis aestivalis* (*Va*), is highly resistant to *E. necator* and other major pathogens compared to *Vitis vinifera* (*Vv*) Cabernet Sauvignon (Fung et al. 2008; Gao et al. 2010). Norton is extensively cultivated in Missouri and other regions of the US and has been used to identify defense-related genes and networks implicated in grapevine innate immunity to PM (Fung et al. 2007; Fung et al. 2008). In these studies, orthologs of one well-known key positive regulator of basal resistance, *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), were differentially expressed in Norton and Cabernet Sauvignon, with high constitutive levels in Norton and induction in Cabernet Sauvignon following infection with *E. necator*.

The PN40024 genome sequence (Jaillon et al. 2007) indicated that grapevine possesses an expanded *EDS1* gene family and that *EDS1* probes on the *Vitis* GeneChip may not be able to distinguish between paralogs. We focused on these *EDS1-LIKE* (*EDL*) paralogs because in Arabidopsis, *EDS1* has been implicated in a salicylic acid (SA)-dependent positive feedback loop that upregulates defense responses (Rustérucci et al. 2001; Shirano et al. 2002; Shah 2003; Chandra-Shekara et al. 2004) and the resistant variety Norton has a constitutively high SA content as compared to the susceptible Cabernet Sauvignon (Fung et al. 2008). In addition, expression of *EDS1* was shown to be induced by *Plasmopara viticola* (downy mildew) in *V. vinifera* cv. Chardonnay and to be responsive to SA (Chong et al. 2008). In our previous study, we detected the expression of four *EDLs* in grapevine inoculated with *E. necator*, and verified that *EDS1* is constitutively expressed to high levels in Norton but not Cabernet Sauvignon, and that in Cabernet Sauvignon *EDS1* is induced by PM, and all four EDLs are responsive to SA (Gao et al. 2010). Both *EDS1* from Norton (*VaEDS1)* and Cabernet Sauvignon (*VvEDS1*) were able to restore resistance to the bacterial pathogen *Pseudomonas syringae* in an Arabidopsis *eds1* mutant (Gao et al. 2010).

For fungal pathogens, a critical pathogenesis step is the penetration of the epidermal cuticle and cell wall to establish a haustorium. Previous work with the Arabidopsis penetration (*pen*) mutants showed that the Arabidopsis *pen2* mutant allows increased penetration by conidiospores of *Blumeria graminis* f. sp. *hordei* (barley PM), and permits *Erysiphe pisi* (pea PM) to penetrate at a frequency similar to that of the Arabidopsis-adapted PM *Golovinomyces orontii* on wild-type plants; however, the barley and pea PM trigger hypersensitive response-like defenses in the absence of penetration defense. This second layer of defense against non-adapted pathogens is overcome by additional mutations in *EDS1* and the defense genes *PHYTOALEXIN DEFICIENT 4* (*PAD4*) and *SENESCENCE-ASSOCIATED GENE 101* (*SAG101*) (Lipka et al. 2005), which like *EDS1* encode lipase-like proteins with extensive sequence similarity to each other (Falk et al. 1999; Jirage et al. 1999; Feys et al. 2005).

In Arabidopsis, EDS1 interacts with PAD4 and SAG101 in the cytoplasm and in the nucleus in distinct complexes (Feys et al. 2005). A nuclear pool of EDS1 is needed for resistance to biotrophic and hemibiotrophic pathogens (García et al. 2010), and SA-

mediated defenses require the EDS1-PAD4 complex (Rietz et al. 2011). Whereas SAG101 and PAD4 do not interact directly, they have been reported to be co-immunoprecipitated in the presence of EDS1. This may indicate the formation of ternary complexes under certain cellular conditions (Zhu et al. 2011), although the crystal structure of EDS1 with SAG101 favors the formation of heterodimeric complexes (Wagner et al. 2013). The function of *EDS1* in pathogen resistance has been shown to be conserved in other dicotyledonous plants such as tobacco, tomato, soybean and *Medicago truncatula* (Liu et al. 2002; Peart et al. 2002; Hu et al. 2005; Tang et al. 2013; Wang et al. 2014). In addition, the observation that pathogen effectors target EDS1 in Arabidopsis and soybean highlight the significance of EDS1 in the plant defense network (Bhattacharjee et al. 2011; Heidrich et al. 2011; Wang et al. 2014). Thus, grapevine EDS1 and EDLs can be expected to play important roles in resistance to infection by *E. necator*.

In this study, we examined more closely the expanded *EDS1* gene family of Norton and Cabernet Sauvignon. To explore the possibility of sub-functionalization, we determined where *EDLs* are expressed and generated Arabidopsis mutants to further characterize Norton and Cabernet Sauvignon *EDS1* and *EDLs*. We previously used the *pen2 pad4 sag101* triple mutant to characterize a grapevine nitrate transporter that is upregulated by PM infection only in Cabernet Sauvignon (Pike et al. 2014). In the present study we employed the triple mutant and generated a *pen2 eds1* mutant to test the resistance functions of grapevine *EDLs*. We now report that *VaEDS1*, *VvEDS1* and *VaEDL2* confer resistance to powdery mildew in Arabidopsis mutants. Promoter analysis showed that *VaEDS1* and *VvEDS1* are induced by SA and have different expression patterns, which, combined with the higher constitutive SA level in Norton and the different functionality of VaEDL2 and VvEDL2, may underlie the greater resistance of Norton. Additionally, the *Vitis* EDS1, EDL2 and PAD4 interactions observed by transient expression in *Nicotiana benthamiana*, suggest a defense complex in grapevine. Our data provide a deeper insight into the expanded EDS1-PAD4 grapevine defense module.

## Methods

### Cloning of *Vitis EDS1* promoters and generation of transgenic Arabidopsis lines

The *VaEDS1* promoter (1,173 bp upstream of the start codon) and *VvEDS1* promoter (1,863 bp upstream of the start codon) were cloned into binary vector pKGWFS7.0 containing the reporter genes GUS and GFP. Transgenic *Arabidopsis* lines of Col-0 (wild type), *eds1-1*, *pen2-1*, and *pen2 pad4 sag101* mutants were generated by the floral dipping method (Clough and Bent 1998) using *Agrobacterium tumefaciens* strain GV3101. Transformants were screened on half-strength Murashige and Skoog medium (Sigma, St. Louis, MO) containing 40 μg/mL kanamycin (Sigma). Single-locus homozygous transgenic lines were selected by scoring for the segregation of kanamycin resistance in the T2 and T3 generations. At least 3 independent transgenic lines were tested and showed similar expression patterns.

### Histochemical β-glucuronidase (GUS) Assays

At the indicated timepoints, freshly excised *pVitisEDS1:GUS* tissues were infiltrated with GUS substrate buffer (0.5 mM 5-bromo-4chloro-3-indolyl glucuronide, 100 mM Tris, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 3 mM potassium ferricyanide) and incubated overnight at 37°C (Jefferson et al. 1987). Chlorophyll was cleared by incubating stained tissue in 70% ethanol.

### Cloning of *Vitis EDL2, EDL5 and PAD4* cDNAs and generation of transgenic *Arabidopsis* lines

*Vitis EDL2, EDL5* and *PAD4* cDNAs were amplified by PCR from cDNA libraries of PM-inoculated Norton and Cabernet Sauvignon, respectively, which were provided by Daniel Schachtman. The libraries were constructed using the Invitrogen CloneMiner Library Construction Kit. The primer sets used to amplify target genes (Table S1) were designed based on the predicted *EDL2*, *EDL5* and *PAD4* gene sequences of PN40024 (Jaillon et al. 2007). Different *EDL* cDNAs with the expected sizes were amplified from previously made PM-treated Cabernet Sauvignon and Norton cDNA libraries and cloned into pGEM T-easy vector (Promega, Madison, WI). Multiple individual cDNA clones

were sequenced to exclude PCR errors. Grapevine is a heterozygous species. Theoretically, by sequencing individual cDNA clones we should obtain two haplotypes for each gene. However, we only obtained one cDNA clone for each gene, possibly because the cDNA pools we sequenced were not large enough to recover both alleles. These cDNA clones were used for further functional assays.

Using designed *Sal*I and *Sma*I sites at the ends, the cDNAs were sub-cloned into the binary plant transformation vector pMD1 containing a cauliflower mosaic virus (CaMV) 35S promoter. Transgenic *Arabidopsis* lines were generated and selected as described above. Expression of the transgenes was verified in multiple lines for each construct by semi-quantitative reverse transcription PCR.

**Quantitative RT-PCR**

To obtain fresh tissue samples for quantitative reverse transcription PCR (qRT-PCR) assays, hardwood cuttings of Cabernet Sauvignon and Norton were collected in the vineyard at the Missouri State Fruit Experiment Station. Hardwood cuttings with 2-3 buds were propagated and placed in the greenhouse. Fresh shoots were allowed to grow to a length of 20-25 cm before harvesting shoot samples (phloem, tendril, shoot tip, young leaf and mature leaf), while root tips were harvested from fresh roots on the cuttings. Three replicates for each grapevine variety and 5 plants for each replicate were used.

Total RNA was extracted from grapevine vegetative tissues using a modified CTAB extraction buffer (Fung et al., 2008). Total RNA was treated with DNase I in TURBO DNA-free reagents (Ambion, Inc. Austin, TX) and purified using an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). RNA quantity and quality were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Purified RNA was converted to cDNA using SuperScript® III Reverse Transcriptase (Life Technologies, New York, USA), and qRT-PCR was performed with the MX3005P system (Stratagene) following the manufacturer's manual, using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, California, USA). Each sample was run in triplicate in a 20 µl reaction volume. Thermal cycling conditions were as follows: 95°C for 10 min, 65 cycles of 95°C for 15 sec, 60°C for 30 sec and 1 cycle of 95°C for 1 min,

60°C for 30 sec and 95°C for 30 sec. The identity of PCR products was confirmed by sequencing the DNA fragments that were purified from an agarose gel. Data were analyzed using DART-PCR software. PCR efficiency (E) was calculated from the exponential phase of each individual amplification plot with the equation $(1+E) = 10^{slope}$, based on a previously described method (Peirson et al. 2003). Expression levels of genes of interest (GOI) were normalized to that of *60SRP* by dividing the $C_T$ value of GOI by the $C_T$ value of *60SRP* (Gamm et al. 2011). Primers used in qPCR experiments are listed in Table S1.

**Bacterial disease assays**

For disease assays, *Arabidopsis* accessions Wassilewskija-0 (Ws-0), the mutant *eds1-1* (Ws background), transgenic *eds1-1* expressing *VvEDLs* and *VaEDLs*, *pen2-1*, *pen2 eds1*, and transgenic *pen2 eds1* expressing *VvEDLs* and *VaEDLs*, were grown in an E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) programmed with an 8-h light/16-h dark cycle at 24°C, 70% humidity and a light intensity of 90-140 μmol photons $m^{-2}$ $s^{-1}$. *Pseudomonas syringae* pv *tomato* strain DC3000 (DC3000) containing the empty vector pVSP61 or expressing *avrRps4* from the vector pV316-1A (Hinsch and Staskawicz 1996) were grown overnight at 28°C on *Pseudomonas* agar (Beckton Dickinson, Sparks, MD). Bacteria were re-suspended to an $OD_{600}$ of 0.001 ($\approx 10^6$ colony-forming units/mL) in 10 mM $MgCl_2$ and infiltrated into leaves of 5-week-old plants with a needleless syringe. Disease symptoms appeared as chlorosis in the inoculated leaves at 3-5 days after inoculation.

**Powdery mildew disease assays**

Grapevine powdery mildew was grown on *V. vinifera* Cabernet Sauvignon leaves in a growth chamber with a 16-h light/8-h dark cycle at 24°C, 70% humidity and a light intensity of 90-140 μmol photons $m^{-2}$ $s^{-1}$. Grape leaves were sterilized with 1% NaClO, rinsed with sterilized water, and maintained on 1% agar plates in petri dishes. Ten to 12 days after infection, powdery mildew conidiospores were directly transferred to Arabidopsis leaves with a brush, or were collected into a 15-mL conical tube by rinsing powdery mildew colonies with 10 ml of 0.05% (v/v) Tween-20 solution and shaking.

Spores were pelleted by centrifugation at 5000g for 5 minutes and resuspended in 1ml of 0.05% (v/v) Tween-20 solution. Spores were counted and the concentration of the spore suspension was adjusted to $10^5$ spores per ml. The spore suspension was sprayed onto Arabidopsis plants with an atomizer. Arabidopsis leaves were dissected, stained, and observed at indicated time points.

**Fungal spores and hyphae staining**

Powdery mildew infected Arabidopsis leaves were detached and put onto filter paper moistened with ethanol: glacial acetic acid (3:1, v/v) for 24 to 48 hours until the chlorophyll was removed, and leaves became completely bleached. After transferring to a water-soaked paper towel for 1-2 hours, the bleached leaves were put onto paper towels soaked with lacto-glycerol solution (1:1:1, lactic acid: glycerol: water, v/v) for 4 to 24 hours. Fungal spores and hyphae were stained by 0.05% aniline blue (Sigma, St. Louis, MO) in a lacto-glycerol solution (Vanacker et al. 2000). Stained leaves were placed on glass slides and visualized with a Nikon Eclipse TS100 inverted microscope.

**Agrobacterium-mediated transient expression**

The split YFP binary constructs were electroporated into the *Agrobacterium tumefaciens* strain GV3101. Bacteria cultured overnight were harvested by centrifugation and resuspended in 10 mM $MgCl_2$ with 100 μM acetosyringone (Sigma-Aldrich, St. Louis, MO, USA) and adjusted to an OD600 of 0.25~0.3. Agrobacterium was incubated for 2 hours at room temperature and infiltrated into *N. benthamiana* leaves with a 1-ml needleless syringe. *N. benthamiana* plants were placed in a E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under a 16 h light/8 h dark cycle at 25°C, 70% relative humidity. Tissues were collected three days after infiltration for western blot and confocal microscopy.

**Bimolecular Fluorescence Complementation *in planta***

The BiFC vectors were tagged with the C-terminal portion of CFP (cCFP) or N-terminal portions of Venus (nVenus), and expression was driven by an 35S promoter. The cDNA clones of *Va/VvEDS1*, *Va/VvEDL2*, *Va/VvEDL5 and Va/VvPAD4* were sub-cloned into

pDNOR201 vectors and sequenced. Finally, all *Vitis EDS1*, *EDL2*, *EDL5* and *PAD4* clones were cloned into cCFP and nVenus vectors, respectively, using GATEWAY LR reactions. Agroinfiltration was performed as described above. For co-infiltrations, each strain was adjusted to an optical density of 0.3 at 600 nm and they were then mixed. Agrobacterium containing the HcPro silencing suppressor was also co-infiltrated into *N. benthamiana* with the same density as *Vitis* constructs. Observations were performed 3 days after infiltration.

**Confocal fluorescence microscopy**

Plant tissues were viewed directly under a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a 40/1.2 C-Apochromat water immersion. YFP fluorescence was excited by a 514-nm argon laser. Samples were detected using a 500- to 550-nm band-pass emission filter.

**Western blot analysis**

Total protein was extracted from transgenic Arabidopsis and from transiently transformed *N. benthamiana* leaves as previously described (Kwon et al. 2009). Briefly, three discs of leaf tissue were ground in 100 µl of 8M urea buffer. Twenty-five µl of 5X loading dye was added to the extracted protein and the samples were boiled for 5 min. The samples were centrifuged for 5 min at 13,200 rpm and the collected supernatant was used for immunoblotting. Forty µl of protein samples were separated on an 8-10% SDS-polyacrylamide gel and were transferred onto immune-blot PVDF membranes (Bio-Rad, Hercules, CA, USA). Immunodetection was performed as described previously (Moffett et al. 2002). GFP tag was detected with 1:5000 diluted rabbit anti-GFP primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1:5000 diluted goat anti-rabbit secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA). Detected proteins were visualized with an ECL Plus chemiluminescent kit (GE Healthcare, Buckinghamshire, UK).

## Results

### Differential tissue expression of *EDLs* in grapevine vegetative tissues

Previously we showed that differential susceptibility to grapevine PM in resistant Norton and susceptible Cabernet Sauvignon correlated with differences in the expression of *EDS1*, but not differences in *EDS1* functionality (Gao et al. 2010). We also showed that the *Vitis EDS1-like* gene family consists of six members, and detected four of these, *EDS1*, *EDL1*, *EDL2* and *EDL5*, by qRT-PCR (Gao et al. 2010). We hypothesized that subfunctionalization may contribute to differences in resistance. Therefore, we tested the expression of these *EDLs* in different tissues.

Triplicate samples from root tip, phloem, tendril, shoot tip, young leaf and mature leaf were collected from Norton and Cabernet Sauvignon, and the constitutive *EDL* mRNA levels were measured. As shown in Fig. 1, Norton *EDS1* expression on average was higher than that of Cabernet Sauvignon in young and old leaves, consistent with our previous results (Gao et al. 2010), even though variability in the samples here did not allow differences to be statistically significant. In phloem and tendril, *EDS1* expressed much higher than the other *EDLs*, suggesting that *EDS1* plays a major role in vascular tissue. Although not significantly higher, the higher expression of Cabernet Sauvignon *EDS1* in phloem as compared to Norton could suggest that it may be limited to veins, a disadvantage if a pathogen colonizes the entire leaf surface. In young leaves and root tips, *EDL2* expression was significantly higher in Cabernet Sauvignon than in Norton. *EDL1* was not detectable in any tissue samples other than root tips of Cabernet Sauvignon where it was expressed at a significantly greater level than in Norton, indicating a potential role in below-ground tissues. *EDL5* showed very low expression for both grapevine cultivars.

### *VvEDS1* and *VaEDS1* promoters are induced by SA

Previously we showed that *EDS1* from Norton and Cabernet Sauvignon are equally functional for bacterial resistance in an Arabidopsis *eds1* mutant (Gao et al. 2010). We used Arabidopsis to investigate whether the *EDS1* promoter can be activated by SA, and whether the Norton *EDS1* promoter ($VaEDS1_{pro}$) responds differently to SA than the Cabernet Sauvignon *EDS1* promoter ($VvEDS1_{pro}$).

Transgenic Arabidopsis Col-0 plants were generated that contain a 1,863 bp fragment of *VvEDS1*$_{pro}$ or a 1,173 bp fragment of *VaEDS1*$_{pro}$ upstream of a β-glucuronidase (GUS) reporter gene. Transgenic plants were treated with SA by spraying 1 mM SA solution or 0.05% Tween alone, and the leaves were collected at 48 hours post treatment. As shown in Fig. 2a, only SA-treated leaves showed GUS activity after staining. Both *VvEDS1*$_{pro}$*:GUS* and *VaEDS1*$_{pro}$*:GUS* transgenic lines were responsive to SA. Interestingly, *VvEDS1*$_{pro}$*:GUS* mainly expressed along the veins. In contrast, *VaEDS1*$_{pro}$*:GUS* expressed in the entire leaf tissue. Thus, if this expression pattern reflects *EDS1* expression in native tissues, *VvEDS1* may not be highly expressed in cells directly in contact with PM even though both promoters were very responsive to SA.

To verify whether the vein expression pattern of *VvEDS1*$_{pro}$*:GUS* observed in Arabidopsis is similar to the expression in grapevine, we sprayed SA solutions on Cabernet Sauvignon leaves and dissected the veins from the leaves. Quantitative RT-PCR results showed that after SA treatment, *VvEDS1* mRNA levels were elevated in both veins and leaves, and accumulated to higher levels in the veins than leaves at 48 hrs (Fig. 2b), confirming the Arabidopsis results. Therefore, while the constitutively higher level of SA in Norton explains the higher expression level of *VaEDS1* in Norton compared to *VvEDS1* in Cabernet Sauvignon (Fig. 2a), the veinal expression pattern (Fig. 2b) might indicate tissue specialization of the *Vitis EDS1* promoter.

Using the Plant Cis-Acting Regulatory DNA Element database, we compared the sequences of *VvEDS1*$_{pro}$ and *VaEDS1*$_{pro}$. *VaEDS1*$_{pro}$ (1173 bp) is shorter than *VvEDS1*$_{pro}$, and with the insertion of gaps the *VaEDS1*$_{pro}$ aligned well with 1232 bp of *VvEDS1*$_{pro}$, where *VaEDS1*$_{pro}$ lacks one W box motif (TGAC or TTGAC). In the extended 641 bp distal region of *VvEDS1*$_{pro}$, we identified 4 additional W box motifs. We were not able to extend the *VaEDS1*$_{pro}$ sequence using the PN40042 genome sequence (Jaillon et al. 2007) or the *VvEDS1*$_{pro}$ sequence, indicating that *V. aestivalis* and *V. vinifera* sequences are diverged in this region. W boxes positively or negatively regulate the transcriptional plant defense response by binding WRKY transcription factors (Eulgem and Somssich 2007) and are not known to drive tissue-specific expression. The basis for the differential expression patterns driven by *VvEDS1*$_{pro}$ and *VaEDS1*$_{pro}$ will require further analysis.

**Arabidopsis mutants as hosts for *E. necator***

To further investigate the grapevine defense system, we generated Arabidopsis mutants that can be infected with *E. necator* for analyzing grapevine defense components. Previously we reported that grapevine powdery mildew could severely infect Arabidopsis *pen2 pad4 sag101* mutant plants and reproduce asexually on them (Pike et al. 2014), as had been shown for *Blumeria graminis* f. sp. *hordei* and *Erysiphe pisi* (Lipka et al. 2005). These Arabidopsis triple mutants lack both penetration defense and the second layer of defense against nonhost pathogens (Lipka et al. 2005). Mutations in *PEN2* led to increased penetration and cell death, but little hyphal growth by 10 days after inoculation with *E. necator*, whereas *pen2-1 pad4-1* double mutants supported hyphal growth at an intermediate level, and *pen2-1 pad4-1 sag101-2* triple mutants were completely susceptible to the grapevine PM (Fig. 3). Because EDS1 plays a central role in forming protein complexes with PAD4 and SAG101 (Feys et al. 2005), we generated *pen2 eds1* double mutants by crossing Arabidopsis *pen2-1* and *eds1-1* mutants. Infected with grapevine PM, *pen2-1 eds1-1* supported hyphal growth at an intermediate level resembling the phenotype of *pen2-1 pad4-1* (Fig. 3).

We transformed the *pen2-1 pad4-1 sag101-2* triple mutant with the $VvEDS1_{pro}$*:GUS* or $VaEDS1_{pro}$*:GUS* constructs and treated homozygous T3 transgenics with *E. necator* spores. As shown in Fig. 4, inoculated Arabidopsis leaves showed enhanced GUS expression as infection progressed. $VvEDS1_{pro}$*:GUS* mainly expressed along the veins; however, $VaEDS1_{pro}$*:GUS* expressed all over the leaf, consistent with our previous finding of induction by SA in transgenic Arabidopsis Col-0 plants expressing $VvEDS1_{pro}$*:GUS* or $VaEDS1_{pro}$*:GUS* (Fig. 2a). Taken together with SA induction of *EDS1* and high constitutive SA levels in Norton, these results show that SA and location of *EDS1* expression correlate with grapevine PM resistance, and demonstrate that Arabidopsis is a good model to test the function and regulation of grapevine *EDS1* genes.

**Characterization of *Vitis EDL2, EDL5 and PAD4* function in resistance to *E. necator* using Arabidopsis**

As shown in Fig. 1, the *EDLs*, particularly *EDL2*, are other components of the grapevine defense machinery besides *EDS1* that could play a role in resistance to PM. *EDL2* and

*EDL5* cDNA clones, *VvEDL2* and *VvEDL5* or *VaEDL2* and *VaEDL5*, were amplified from Cabernet Sauvignon or Norton, respectively. Comparison of the predicted amino acid sequences derived from the PN40024 genome (Jaillon et al. 2007), which we designated with "Pinot Noir", with those from Cabernet Sauvignon and Norton showed that they are highly similar to each other (Fig. S1a, b). VvEDL1 (Pinot Noir) and VvEDL2 (Pinot Noir) are very similar to each other, and our predicted VvEDL2 (Cabernet) and VaEDL2 (Norton) amino acid sequences show similarity to both EDL1 (Pinot Noire) and EDL2 (Pinot Noir). However, the cDNA sequences of our clones contained a perfect match to those of the qRT-PCR products of *EDL2*. Therefore, we named the Norton clone *VaEDL2* and the Cabernet Sauvignon clone *VvEDL2*, and further studied their functions. EDS1, EDL2 and EDL5 belong to one gene family; however, EDL2 and EDL5 are diverged from EDS1 (Gao et al. 2010). Thus it was necessary to test whether *Vitis EDL2* and *EDL5* from Cabernet Sauvignon and Norton have the same functions as *Vitis* or Arabidopsis *EDS1*.

We generated transgenic *pen2-1 eds1-1* expressing *Vv/VaEDS1*, *Vv/VaEDL2* and *Vv/VaEDL5* driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. T3 lines were used to test the gene functions in *E. necator* resistance. As shown in Fig 5, both *VaEDS1* and *VvEDS1* complemented *pen2 eds1* double mutants, consistent with these genes complementing Arabidopsis *eds1-1* susceptibility to DC3000(*avrRps4*) (Gao et al. 2010). In addition, the transgenic lines allowed the fungus to penetrate but did not support hyphal growth, resembling the phenotype of the *pen2* single mutant. Although *VaEDL2* and *VvEDL2* expressed to similar levels (Figs. S2b, S3b), only the *VaEDL2* transgene other than *Va/VvEDS1* complemented the *pen2 eds1* mutant, showing that *VaEDL2* is a functional ortholog of *AtEDS1*. Complementation was seen in 3 out of 5 *VaEDL2* transgenic lines, with low expression accounting for noncomplementation in one line and dwarfism making it impossible to test the other line. *VvEDL2* did not complement any of the 6 lines tested, even though expression levels were similar to those of the representative lines shown in Figs. S2b and S3b. Transgenic Arabidopsis *eds1-1* or *pen2-1 eds1-1* mutants expressing *Vitis EDS1, EDL2* or *EDL5* were also tested for resistance to DC3000(*avrRps4*), and only *VaEDS1*, *VvEDS1* and *VaEDL2* conferred resistance (Figs. S2a, S3a).

The *VvEDL2* transgene did not complement the *pen2 eds1* mutation, suggesting that Norton and Cabernet Sauvignon *EDL2* genes have different functions, which might be important for PM resistance. Alternatively, the *VvEDL2* allele we have cloned might have a polymorphism in a crucial residue. There is no marked difference in sequence similarity between VvEDL2 and VaEDL2 compared to Va/VvEDS1 (Table S2), and amino acid polymorphisms are distributed throughout both VaEDL2 and VvEDL2 (Fig. S1). However, it is potentially significant that VvEDL2 shows a polymorphism in a highly conserved predicted SUMOylation site that is found in EDS1 from Arabidopsis and other plant species (479AKDK482 instead of AKDE in VaEDL2 and LKNE in AtEDS1 and Va/VvEDS1). Neither the *VaEDL5* nor *VvEDL5* transgene could complement the *pen2 eds1* mutation, suggesting that *Vitis EDL5* is not a functional ortholog of *Arabidopsis* or *Vitis EDS1*. Both encode a predicted protein with an N-terminal extension that could serve as a chloroplast transit peptide. Alternatively, it is possible that *EDL5* does not perform the same function in Arabidopsis as in grapevine because it is constitutively expressed at a very low level in grapevine leaves (Fig. 1). In addition, it is likely that complementation requires interactions with additional components of the defense machinery that may not be identical in Arabidopsis and grapevine.

Although *Vitis EDS1* is a functional ortholog of *AtEDS1* in Arabidopsis (Fig. 5), *EDS1* was not functional in *pen2 pad4 sag101* (Fig. S4), indicating that *Vitis EDS1* or *EDL* genes may need these components in the Arabidopsis model system. PAD4 is a related protein from the key regulatory module EDS1/PAD4/SAG101 in pathogen resistance in Arabidopsis (Jirage et al. 1999; Feys et al. 2001; Feys et al. 2005). In the previous Affymetrix *Vitis* GeneChip data, *Vitis PAD4* mRNA levels in Norton and Cabernet Sauvignon were not significantly different after inoculation with PM (Fung et al. 2008). However, it is likely that *Vitis PAD4* nevertheless functions in grapevine PM resistance. We examined the relatedness of *Vitis* PAD4 to AtPAD4. Only one predicted *PAD4* homolog was found in the PN40024 genome sequence on chromosome 7 with a BLAST E value of $2e^{-61}$. Using the PN40024 *PAD4* sequence as a reference, we designed gene specific primers and sequenced multiple cDNA clones to determine the haplotype of *VvPAD4* and *VaPAD4* alleles. Comparison of the predicted VvPAD4 and VaPAD4

amino acid sequences show that they are almost identical to each other and to the predicted PN40024 PAD4 sequence (Fig. S1c).

As a result of non-host resistance, an Arabidopsis *pad4* single mutant is not susceptible to grapevine PM (Lipka et al. 2005). To study the function of *Vitis PAD4*, we chose the *pen2 pad4* double mutants which can support fungal growth at an intermediate level (Lipka et al. 2005). We confirmed the susceptibility of these mutants to *E. necator* (Figs. 3, S4). Driven by a CaMV 35S promoter, *VvPAD4* and *VaPAD4* cDNAs were expressed in the double or triple mutants. Stable homozygous lines were selected and tested for resistance to *E. necator*. We did not see any improved disease resistance to the pathogen (Fig. S4). *Vitis PAD4* may not complement because it is not functional in Arabidopsis or it may need other interactors to form complexes for full function.

To test whether *Vitis EDS1* and *PAD4* together can confer resistance to powdery mildew, we generated transgenic Arabidopsis *pen2 pad4 sag101* mutants containing both *VaEDS1* and *VaPAD4* by crossing the single transgenic plants. Neither *VaEDS1* nor *VaPAD4* alone were able to confer resistance to powdery mildew in the triple mutants. Expressing *VaEDS1* and *VaPAD4* together was also not sufficient to render plants more resistant (Fig. S4). Therefore, the defense-related function of the putative *PAD4* gene in the grapevine genome remains unknown.

***Vitis* EDS1 and EDL2 proteins interact with *Vitis* and *Arabidopsis* PAD4 *in planta***

In Arabidopsis, EDS1 interacts with SAG101 in the nucleus and with PAD4 in separate complexes in the cytoplasm and nucleus (Feys et al. 2005). In grapevine, the fact that *Vitis EDS1* or *Vitis PAD4* individually cannot complement Arabidopsis *pen2 pad4 sag101* triple mutants suggests that EDS1 and EDLs may also need PAD4 for complete function and *vice versa*. To test the *in planta* interactions of *Vitis* EDS1 and EDL proteins with *Vitis* or Arabidopsis PAD4 proteins and their subcellular localization, we performed bimolecular fluorescence complementation (BiFC) experiments (Citovsky et al. 2006; Ohad et al. 2007). *Vitis* EDS1, EDL2, EDL5, and PAD4 were fused to either the N-terminal (nYFP) or C-terminal (cYFP) domain of the yellow fluorescent protein. Different combinations of *Vitis* EDS1, EDL2, EDL5 or PAD4, and Arabidopsis EDS1 and PAD4 were co-infiltrated into *N. benthamiana* using Agrobacterium-mediated transient expression, and the interactions were observed by confocal microscopy. All *Vv*

protein interactions had the same pattern as those of *Va* proteins. Western blots confirmed that the proteins are actually expressed at the same level (Fig. S5). As shown in Fig. 6 (*Vv* proteins not shown) and summarized in Table S3, we confirmed that AtEDS1 interacts with AtPAD4 in the nucleus and cytoplasm. *Vitis* EDS1 also interacted with *Vitis* PAD4 and AtPAD4 in the nucleus and cytoplasm. However, *Vitis* PAD4 did not interact with AtEDS1, which may explain its inability to complement Arabidopsis mutants. *Vitis* EDS1 interacted with AtEDS1 in punctate spots in the cytoplasm similarly to the AtEDS1 dimer, which could be another indication that *Vitis* EDS1 operates likes AtEDS1 *in planta*. Additionally, *Vitis* EDS1 interacted with itself in the nucleus and cytoplasm, and *Vitis* EDS1 interacted with *Vitis* EDL2 with the same pattern.

VaEDL2 and VvEDL2 also interacted with *Vitis* PAD4 and AtPAD4 in the nucleus and cytoplasm even though they differed in their ability to complement Arabidopsis mutants. No interaction was detected with *Vitis* EDL5, except a very weak interaction with *Vitis* EDS1 in the cytoplasm, which could be a possible explanation for the non-functionality of *Vitis* EDL5.

## Discussion

In this study, we continued the investigation of how the previously determined functional *AtEDS1* orthologs *VaEDS1* and *VvEDS1* were regulated and extended our complementation assays by including Arabidopsis mutants that are susceptible to grapevine PM. We also investigated the functions of the candidate *Vitis* defense-related genes *EDL2, EDL5* and *PAD4* of PM-susceptible *V. vinifera* variety Cabernet Sauvignon and the resistant *V. aestivalis* variety Norton. Our results suggest that underlying the difference in susceptibility are different patterns of expression and induction as well as protein interactions.

### Functional dissection of *Vitis EDS1* promoters

We tested the responsiveness of *VaEDS1* and *VvEDS1* promoters to SA and showed that both were responsive. Our results show that a difference in promoter SA-responsiveness does not underlie the observed differences in regulation of *VaEDS1* and *VvEDS1* by PM

or SA (Gao et al. 2010). Importantly, without SA treatment, none of the transgenic promoter-GUS lines showed any activity, indicating that the continuously expressed *VaEDS1* in Norton does not result from a stronger basal activity, but likely from SA inducibility coupled with constitutively high SA levels in Norton (Fung et al. 2008). Therefore, the basal defense level correlated with the heightened endogenous SA level in grapevine.

Interestingly, even under the same conditions, $VvEDS1_{pro}$ was mainly expressed along veins after SA induction, in contrast to $VaEDS1_{pro}$ in Arabidopsis and grapevine. This difference in expression correlates with Norton's heightened resistance to PM since PM is not a vascular pathogen. Dissection of veins for analyzing the expression level of *Vitis EDS1* from leaves with or without PM induction confirmed that *VvEDS1* is expressed higher in veins than in the rest of the leaf after PM infection. For these reasons, we conclude that the vein-expression pattern may be important in determining PM resistance. However, it is possible that *VvEDS1* is induced upon detecting other pathogens where veinal expression would be desirable for resistance, such as grapevine yellow vein virus detected in several varieties of *Vitis vinifera* (Gooding and Hewitt 1962). *VaEDS1* may also have a similar initial pattern but respond more rapidly or strongly throughout the whole leaf.

### *EDL* gene family in grapevine

In this study, we identified *VaEDL2* as the only other family member in addition to *VaEDS1* and *VvEDS1* that can complement the Arabidopsis *eds1-1* mutant. Expressing functional *EDL2* could be one more reason why Norton is more resistant to PM than Cabernet Sauvignon. Interestingly, constitutive *VvEDL2* mRNA levels were higher in Cabernet Sauvignon leaves than in Norton (Fig. 1). *Vitis* EDS1 and EDL2 showed strong interactions in the nucleus and cytoplasm, where EDS1 dimerized and interacted with PAD4. Since *VvEDL2* could not complement the *eds1-1* mutant, EDL2 may be a non-functional version of EDS1 which competes with EDS1 in Cabernet Sauvignon, possibly by regulating the amount of functional EDS1 dimers or competing with EDS1 when interacting with PAD4. Either of these mechanisms would correlate with the fact that Cabernet Sauvignon is more susceptible to pathogens than Norton.

In grapevine, EDS1, EDL2 and PAD4 may form a complex to regulate the defense system. To further investigate the innate mechanisms of defense system regulation by EDS1 and EDL2 in Norton, it would be advantageous to develop an efficient transient expression system in the two grapevine species that would permit tracking the actual regulation through the interactions of expressed proteins.

We could not detect a function for *Vitis EDL5* or *PAD4* from Norton or Cabernet Sauvignon by complementing Arabidopsis mutants. *Vitis EDL5* may not be a functional ortholog of *AtEDS1*. Not every member of a gene family necessarily shares the same function. Gene duplications are a major driver of evolution. Paralogs can acquire new functions, new temporal or spatial expression patterns, or a function can decay. In the *Vitis EDS1* gene family, *EDL5* was mainly detected in root tissues and weakly in phloem. However, the weak interaction between EDL5 and EDS1 observed in the cytoplasm by BiFC may suggest that it could impact EDS1/EDL2/PAD4 complexes. We conclude that EDS1 is probably the most important family member in grapevine innate immunity. As for *Vitis* PAD4, its inability to interact with AtEDS1 could explain why it does not complement an Arabidopsis *pad4* mutant. However, even Arabidopsis plants expressing both *Vitis* EDS1 and PAD4 did not show rescued resistance, even though these proteins interacted when transiently expressed in *N. benthamiana*. It is likely that additional protein interactions are necessary for resistance to occur. One obvious candidate is SAG101, which in Arabidopsis competes with PAD4 for EDS1 to form nuclear heterodimers (Feys et al. 2005; Rietz et al. 2011). The *Vitis* genome appears to possess up to five *SAG101*-like genes (Jaillon et al. 2007). Complementation of the *eds1-1* mutant by *Vitis EDS1* suggests that *Vitis* EDS1 can interact with AtSAG101. Nevertheless, the presence of *Vitis* EDS1 and PAD4 in this heterologous system may disrupt the balance between EDS1-PAD4 and EDS1-SAG101 complexes that are required for resistance (García et al. 2010; Rietz et al. 2011).

In summary, we have examined the functions of the SA pathway defense genes *EDS1*, *EDL2, EDL5*, and *PAD4* of two grapevine species, *V. aestivalis* cv. Norton and *V. vinifera* cv. Cabernet Sauvignon, that differ in their levels of resistance to a range of pathogens including PM. By characterizing tissue-level expression patterns, gene induction, protein interactions and ability to complement Arabidopsis mutants, our results

suggest a more complicated EDS1/PAD4 module in grapevine than in Arabidopsis. This complexity will only increase with the inclusion of *Vitis* SAG101 in future analyses. In addition, our study extends the observations reported in Gao et al. (2010) to clarify the role of EDS1 in grapevine defense against powdery mildew and provides a deeper insight into molecular events that determine disease resistance levels in *Vitis* species native to the North American continent. Our transgenic Arabidopsis *pen2-1 eds1-1 sag101-2* lines expressing *Vv/VaEDS1*$_{pro}$*:GUS* fusion constructs would be useful to investigate grapevine defense responses to infection by other biotrophic pathogens, such as the oomycete *Plasmopara viticola* (grapevine downey mildew) and the ascomycete *Elsinoe ampelina* (grapevine anthracnose), and to other biotic and abiotic stresses. They could also be used to study the roles of hormones other than SA in grapevine defenses. More broadly, our work suggests that *pen* mutants, which were valuable for understanding Arabidopsis non-host resistance to fungal pathogens (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006), can be developed into Arabidopsis systems to investigate resistance of difficult-to-study crops to economically important fungal pathogens.

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**References**


Barker CL, Donald T, Pauquet J, Ratnaparkhe MB, Bouquet A, Adam-Blondon AF, Thomas MR, Dry IB (2005) Genetic and physical mapping of the grapevine powdery mildew resistance gene, *Run1*, using a bacterial artificial chromosome library. Theor Appl Genet 111:370-377

Bhattacharjee S, Halane MK, Kim SH, Gassmann W (2011) Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. Science 334:1405-1408

Camps C, Kappel C, Lecomte P, Léon C, Gomès E, Coutos-Thévenot P, Delrot S (2010) A transcriptomic study of grapevine (*Vitis vinifera* cv. Cabernet-Sauvignon) interaction with the vascular ascomycete fungus *Eutypa lata*. J Exp Bot 61:1719-1737

Chandra-Shekara AC, Navarre D, Kachroo A, Kang H-G, Klessig D, Kachroo P (2004) Signaling requirements and role of salicylic acid in *HRT*- and *rrt*-mediated resistance to turnip crinkle virus in Arabidopsis. Plant J 40:647-659

Chong J, Le Henanff G, Bertsch C, Walter B (2008) Identification, expression analysis and characterization of defense and signaling genes in *Vitis vinifera*. Plant Physiol Biochem 46:469-481

Citovsky V, Lee LY, Vyas S, Glick E, Chen MH, Vainstein A, Gafni Y, Gelvin SB, Tzfira T (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation *in planta*. J Mol Biol 362:1120-1131

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735-743

Coleman C, Copetti D, Cipriani G, Hoffmann S, Kozma P, Kovács L, Morgante M, Testolin R, Di Gaspero G (2009) The powdery mildew resistance gene *REN1* co-segregates with an NBS-LRR gene cluster in two Central Asian grapevines. BMC Genet 10:89

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425:973-977

Donald TM, Pellerone F, Adam-Blondon A-F, Bouquet A, Thomas MR, Dry IB (2002) Identification of resistance analogs linked to a powdery mildew resistance locus in grapevine. Theor Appl Genet 104:610-618

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol 10:366-371

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE (1999) *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc Natl Acad Sci USA 96:3292-3297

Feechan A, Anderson C, Torregrosa L, Jermakow A, Mestre P, Wiedemann-Merdinoglu S, Merdinoglu D, Walker AR, Cadle-Davidson L, Reisch B, Aubourg S, Bentahar N, Shrestha B, Bouquet A, Adam-Blondon AF, Thomas MR, Dry IB (2013) Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadinia rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine. Plant J 76:661-674

Feys BJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. EMBO J 20:5400-5411

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A, Parker JE (2005) *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell 17:2601-2613

Fung RWM, Gonzalo M, Fekete C, Kovacs LG, He Y, Marsh E, McIntyre LM, Schachtman DP, Qiu W (2008) Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. Plant Physiol 146:236-249

Fung RWM, Qiu WP, Su YC, Schachtman D, Huppert K, Fekete C, Kovacs LG (2007) Gene expression variation in grapevine species *Vitis vinifera* L. and *Vitis aestivalis* Michx. Genet Resour Crop Evol 54:1541-1553

Gamm M, Héloir MC, Kelloniemi J, Poinssot B, Wendehenne D, Adrian M (2011) Identification of reference genes suitable for qRT-PCR in grapevine and

application for the study of the expression of genes involved in pterostilbene synthesis. Mol Genet Genomics 285:273-285

Gao F, Shu X, Ali MB, Howard S, Li N, Winterhagen P, Qiu W, Gassmann W (2010) A functional *EDS1* ortholog is differentially regulated in powdery mildew-resistant and -susceptible grapevines and complements an Arabidopsis *eds1* mutant. Planta 231:1037-1047

García AV, Blanvillain-Baufumé S, Huibers RP, Wiermer M, Li G, Gobbato E, Rietz S, Parker JE (2010) Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. PLoS Pathog 6:e1000970

Gooding GV, Hewitt WB (1962) Grape yellow vein: symptomatology, identification, and the association of a mechanically transmissible virus with the disease. Am J Enol Vitic 13:196-203

Heidrich K, Wirthmueller L, Tasset C, Pouzet C, Deslandes L, Parker JE (2011) *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. Science 334:1401-1404

Hinsch M, Staskawicz BJ (1996) Identification of a new *Arabidopsis* disease resistance locus, *RPS4*, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. Mol Plant-Microbe Interact 9:55-61

Hoffmann S, Di Gaspero G, Kovacs LG, Howard S, Kiss E, Galbacs R, Testolin R, Kozma P (2008) Resistance to *Erysiphe necator* in the grapevine 'Kishmish vatkana' is controlled by a single locus through restriction of hyphal growth. Theor Appl Genet 116: 427-438

Hu GS, deHart AKA, Li YS, Ustach C, Handley V, Navarre R, Hwang CF, Aegerter BJ, Williamson VM, Baker B (2005) *EDS1* in tomato is required for resistance mediated by TIR-class *R* genes and the receptor-like *R* gene *Ve*. Plant J 42:376-391

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C, Vezzi A, Legeai F, Hugueney P, Dasilva C, Horner D, Mica E, Jublot D, Poulain J, Bruyere C, Billault A, Segurens B, Gouyvenoux M, Ugarte E, Cattonaro F, Anthouard V, Wincker P, et al. (2007) The grapevine genome

sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463-467

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc Natl Acad Sci USA 96:13583-13588

Kwon SI, Kim SH, Bhattacharjee S, Noh JJ, Gassmann W (2009) *SRFR1*, a suppressor of effector-triggered immunity, encodes a conserved tetratricopeptide repeat protein with similarity to transcriptional repressors. Plant J 57:109-119

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310:1180-1183

Liu YL, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. Plant J 30:415-429

Moffett P, Farnham G, Peart J, Baulcombe DC (2002) Interaction between domains of a plant NBS-LRR protein in disease resistance-related cell death. EMBO J 21:4511-4519

Moroldo M, Paillard S, Marconi R, Fabrice L, Canaguier A, Cruaud C, De Berardinis V, Guichard C, Brunaud V, Le Clainche I, Scalabrin S, Testolin R, Di Gaspero G, Morgante M, Adam-Blondon AF (2008) A physical map of the heterozygous grapevine 'Cabernet Sauvignon' allows mapping candidate genes for disease resistance. BMC Plant Biol 8:66

Ohad N, Shichrur K, Yalovsky S (2007) The analysis of protein-protein interactions in plants by bimolecular fluorescence complementation. Plant Physiol 145:1090-1099

Peart JR, Cook G, Feys BJ, Parker JE, Baulcombe DC (2002) An *EDS1* orthologue is required for *N*-mediated resistance against tobacco mosaic virus. Plant J 29:569-579

Peirson SN, Butler JN, Foster RG (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 31:e73

Pike S, Gao F, Kim MJ, Kim SH, Schachtman DP, Gassmann W (2014) Members of the *NPF3* transporter family encode pathogen-inducible nitrate/nitrite transporters in grapevine and Arabidopsis. Plant Cell Physiol 55:162-170

Rietz S, Stamm A, Malonek S, Wagner S, Becker D, Medina-Escobar N, Corina Vlot A, Feys BJ, Niefind K, Parker JE (2011) Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. New Phytol 191:107-119

Rustérucci C, Aviv DH, Holt BF, Dangl JL, Parker JE (2001) The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in Arabidopsis. Plant Cell 13:2211-2224

Shah J (2003) The salicylic acid loop in plant defense. Curr Opin Plant Biol 6:365-371

Shirano Y, Kachroo P, Shah J, Klessig DF (2002) A gain-of-function mutation in an Arabidopsis Toll Interleukin-1 Receptor-Nucleotide Binding Site-Leucine-Rich Repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell 14:3149-3162

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18:731-746

Tang F, Yang S, Gao M, Zhu H (2013) Alternative splicing is required for *RCT1*-mediated disease resistance in *Medicago truncatula*. Plant Mol Biol 82:367-374

Vanacker H, Carver TL, Foyer CH (2000) Early $H_2O_2$ accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction. Plant Physiol 123:1289-1300

Wagner S, Stuttmann J, Rietz S, Guerois R, Brunstein E, Bautor J, Niefind K, Parker JE (2013) Structural basis for signaling by exclusive EDS1 heteromeric complexes with SAG101 or PAD4 in plant innate immunity. Cell Host Microbe 14:619-630

Wang J, Shine MB, Gao QM, Navarre D, Jiang W, Liu C, Chen Q, Hu G, Kachroo A (2014) Enhanced Disease Susceptibility1 mediates pathogen resistance and virulence function of a bacterial effector in soybean. Plant Physiol 165:1269-1284

Zhu S, Jeong RD, Venugopal SC, Lapchyk L, Navarre D, Kachroo A, Kachroo P (2011) SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. PLoS Pathog 7:e1002318

**Fig. 1** Tissue-specific expression of *EDS1* family members. Quantitative RT-PCR analysis of *EDS1, EDL1, EDL2* and *EDL5* transcript levels in indicated tissues of Norton (N) and Cabernet Sauvignon (CS). Values represent averages from three biological replicates normalized with *60SRP* transcript levels, and error bars denote standard error. An asterisk denotes a statistically significant difference between Norton *EDS1* or *EDL* and Cabernet Sauvignon *EDS1* or *EDL* transcript levels (*P<0.05).

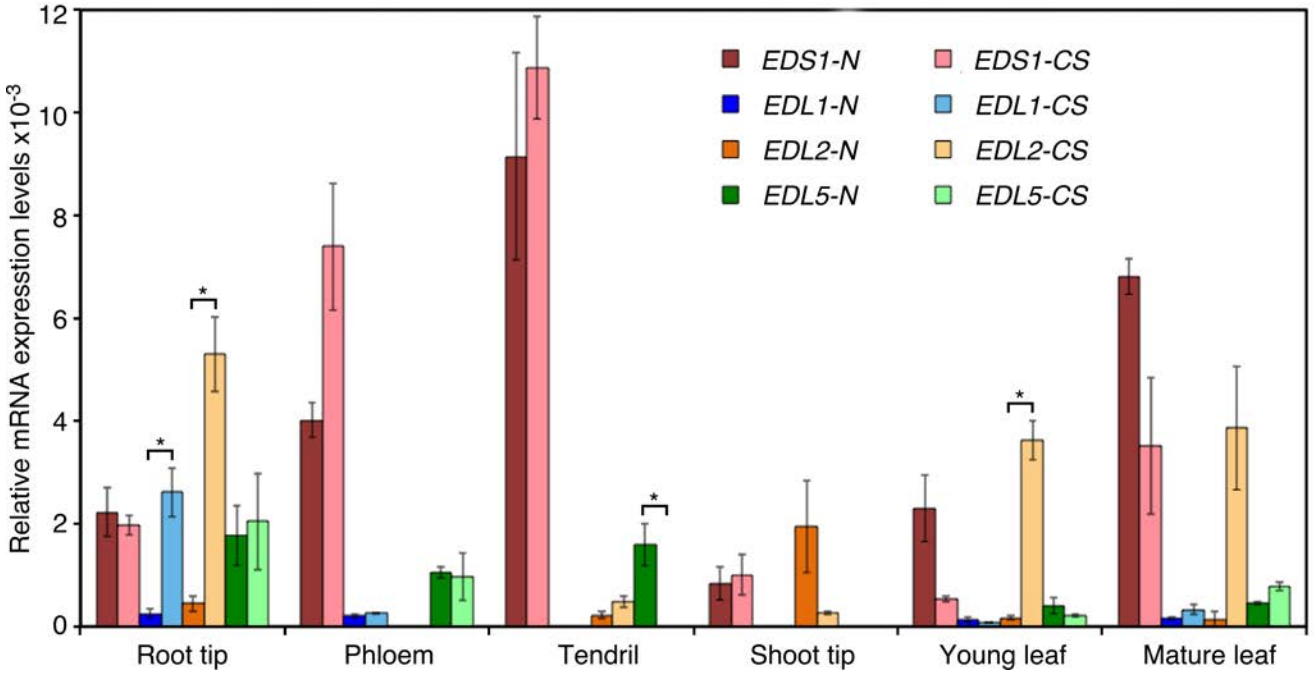
**Fig. 2** Differential activity of *Vitis EDS1* promoters. **a** SA-induced *Vitis EDS1* promoter activity in transgenic Arabidopsis. Transgenic Arabidopsis Col-0 plants containing either the *35S:GUS*, *VvEDS1*$_{pro}$*:GUS*, or *VaEDS1*$_{pro}$*:GUS* fusion construct were sprayed with a solution containing 1.5 mM SA and 0.05% Tween-20 or with 0.05% Tween-20 alone, and sampled at 48 hrs post treatment for staining for GUS activity. Images are representative of three independent transgenic lines analyzed. This experiment was repeated four times with similar results. **b** Quantitative real-time PCR analysis of transcript levels in non-treated and SA-treated veins and leaves of Cabernet Sauvignon at 0 hpi, 24 hpi and 48 hpi. Values represent averages from three biological replicates normalized with *60SRP* transcript levels, and error bars denote standard error. An asterisk denotes a statistically significant difference between *VvEDS1* levels in PM-treated veins and leaves at P<0.05.

**Fig. 3** Arabidopsis mutants as host to grapevine powdery mildew. Arabidopsis wild type and indicated mutant leaves were sampled 10 days post infection with *E. necator*. Blue color indicates spores and hyphae stained with 0.05% aniline blue. Scale bar = 0.5 mm.

**Fig. 4** Powdery mildew-induced *Vitis EDS1* promoter activity in transgenic Arabidopsis. Transgenic Arabidopsis *pen2-1 eds1-1 sag101-2* plants containing the *VvEDS1*$_{pro}$*:GUS* or *VaEDS1*$_{pro}$*:GUS* fusion construct were inoculated with powdery mildew spores on the right half of the leaf surface. Arabidopsis leaves were sampled at indicated time points for staining for GUS activity. Images are representative of three independent transgenic lines analyzed. This experiment was repeated three times with similar results.

**Fig. 5** Complementation tests for resistance to powdery mildew with *Vitis EDS1, EDL2, EDL5* and *PAD4* cDNAs in transgenic *Arabidopsis* mutants. Arabidopsis *pen2* and *pen2 eds1* mutants, and transgenic *pen2 eds1* expressing *Va/VvEDS1* or *EDLs* in the T3 generation, were inoculated with *E. necator* spores and sampled 10 days post infection. Blue color indicates spores and hyphae stained with 0.05% aniline blue. Scale bar = 0.5 mm.

**Fig. 6** Interactions of Arabidopsis and *Vitis* EDS1 and PAD4 *in planta*. The indicated fusions to nVenus (n) and cCFP (c) were transiently expressed in *N. benthamiana* by co-infiltration. Interactions are indicated by yellow fluorescence detected by confocal microscopy three days after infiltration. No interaction was detected between VaPAD4 and AtEDS1, or VaEDL5 and VaPAD4. Scale bars = 20 μm.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Phloem
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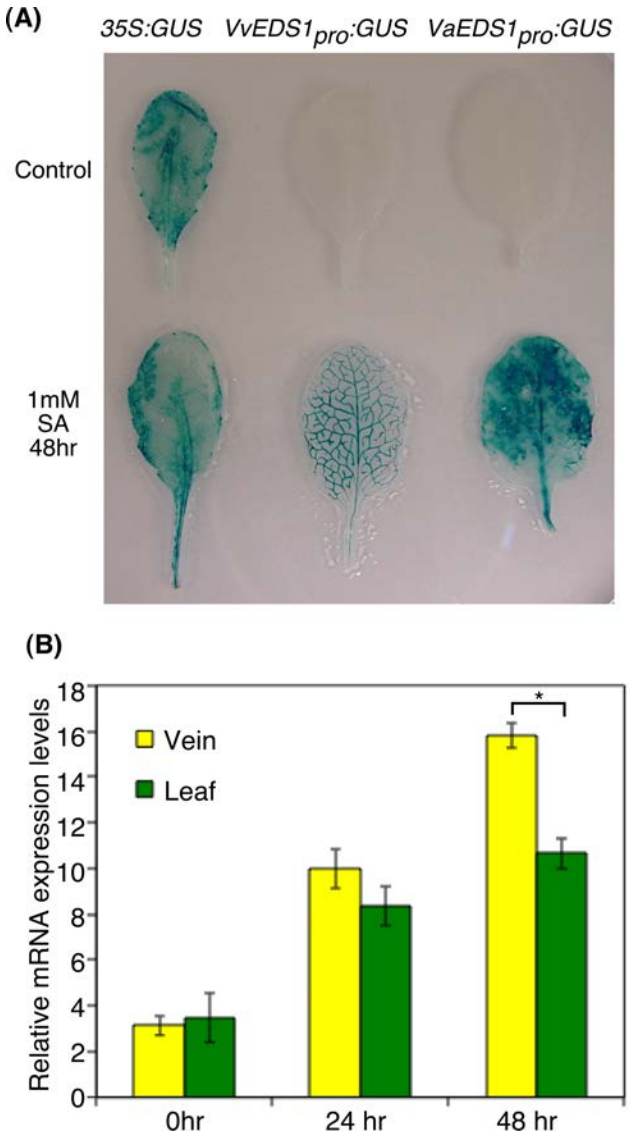
Shoot tip
Young leaf
Mature leaf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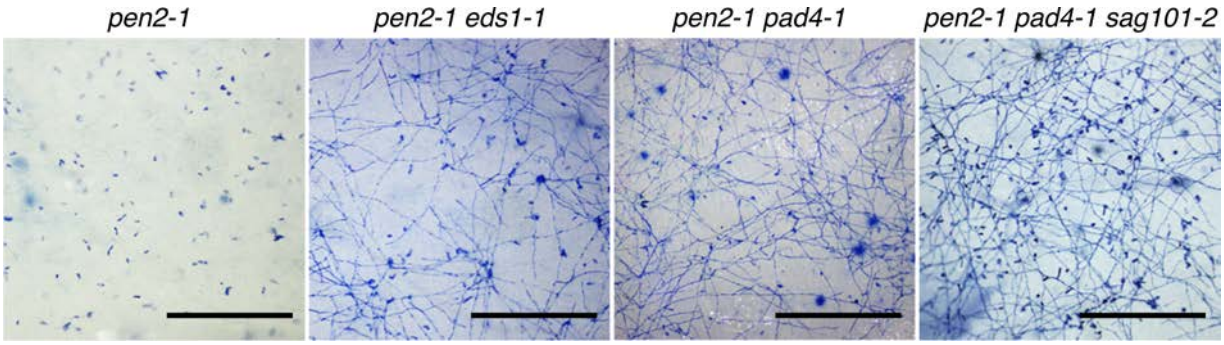
48 hr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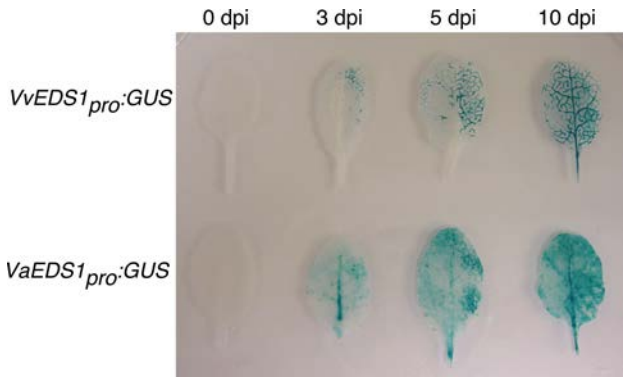
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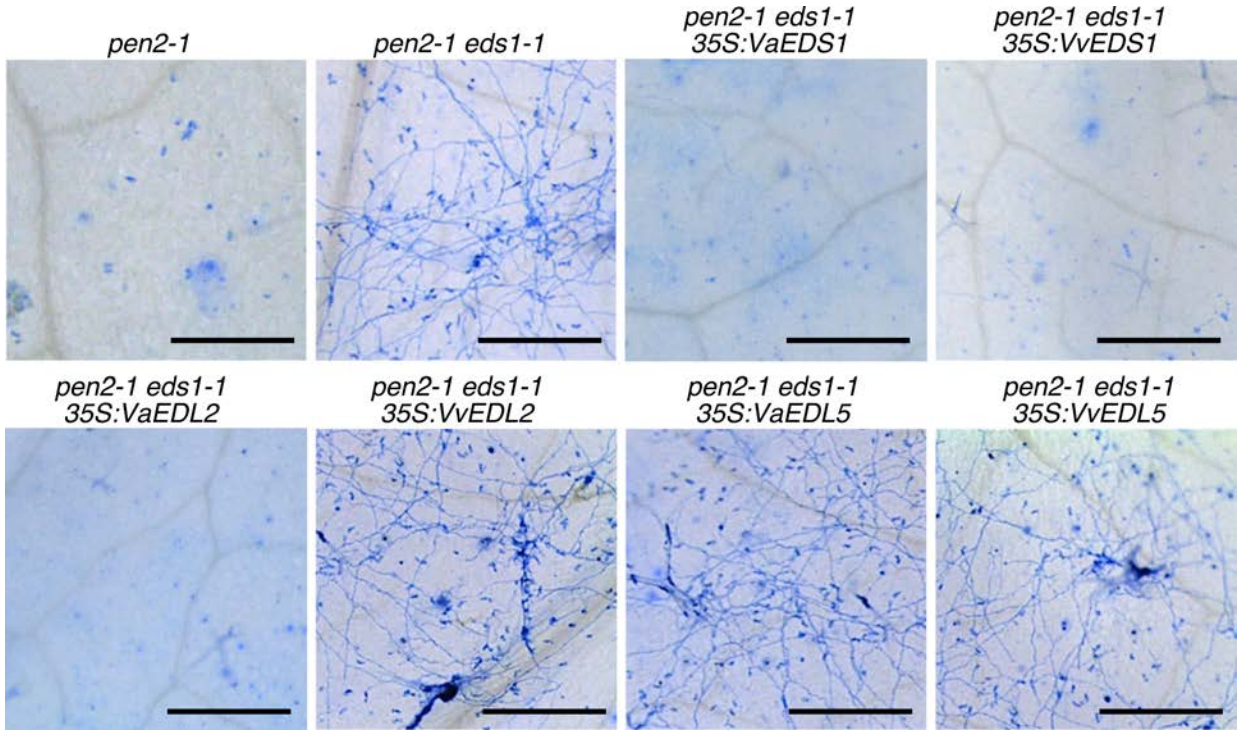
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pen2-1 eds1-1
35S:VaEDL5
pen2-1 eds1-1
35S:VvEDL5

YFP
Bright field overlay
YFP
Bright field overlay
nAtEDS1 - cAtPAD4
nVaEDS1- cVaPAD4
nVaEDL2 - cVaPAD4
nAtEDS1 - cVaPAD4
nAtEDS1 - cVaEDS1
nVaEDL2 - cVaEDS1

# Plant Molecular Biology

# Supplementary materials

**Functions of *EDS1-like* and *PAD4* genes in grapevine defenses against powdery mildew**

**Fei Gao[1], Ru Dai[1,2], Sharon M. Pike[1], Wenping Qiu[2], Walter Gassmann[1*]**

[1]Division of Plant Sciences, C.S. Bond Life Sciences Center and Interdisciplinary Plant Group, University of Missouri, Columbia, MO 65211-7310, USA

[2]Center for Grapevine Biotechnology, W. H. Darr School of Agriculture, Missouri State University, Mountain Grove, MO 65711

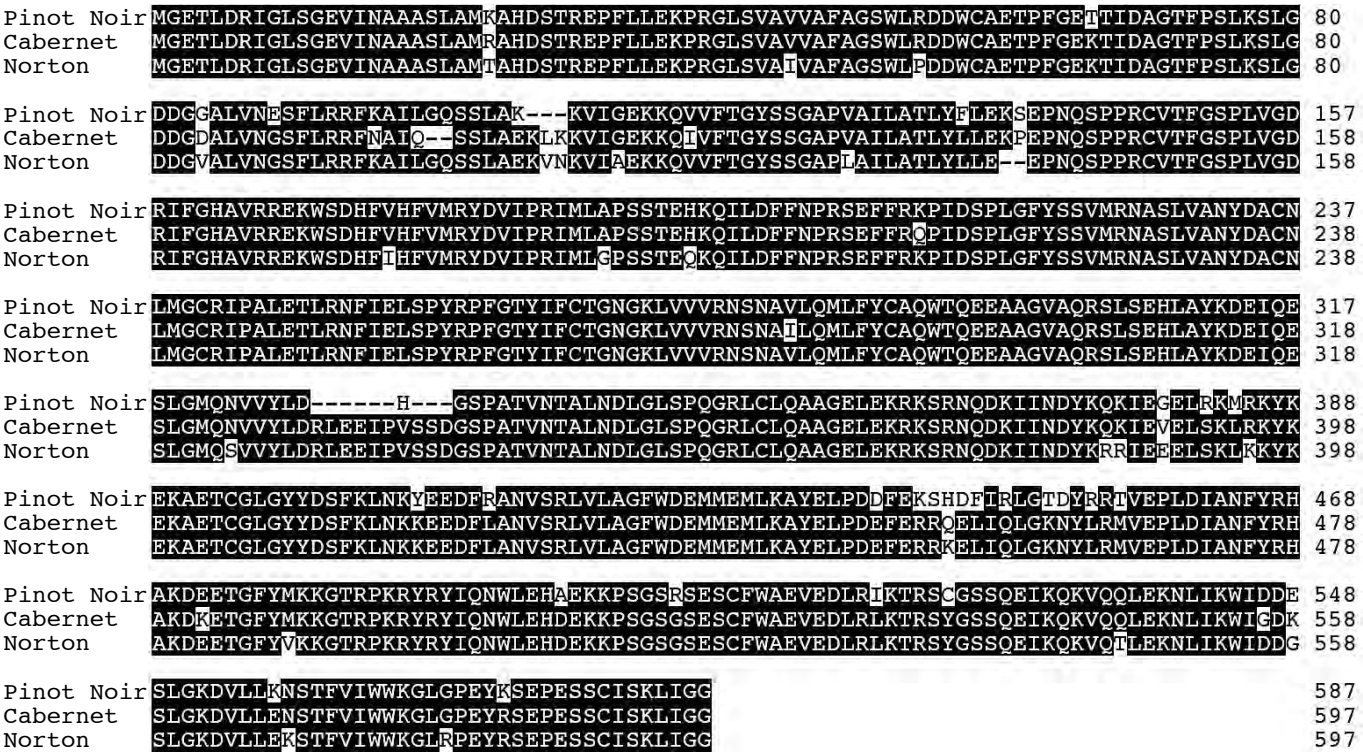
*E-mail: gassmannw@missouri.edu

**Address:** 371C Life Sciences Center, Columbia, MO 65201

**Phone:** 573-884-7703

**Fax:** 573-884-9676

## (A) EDL2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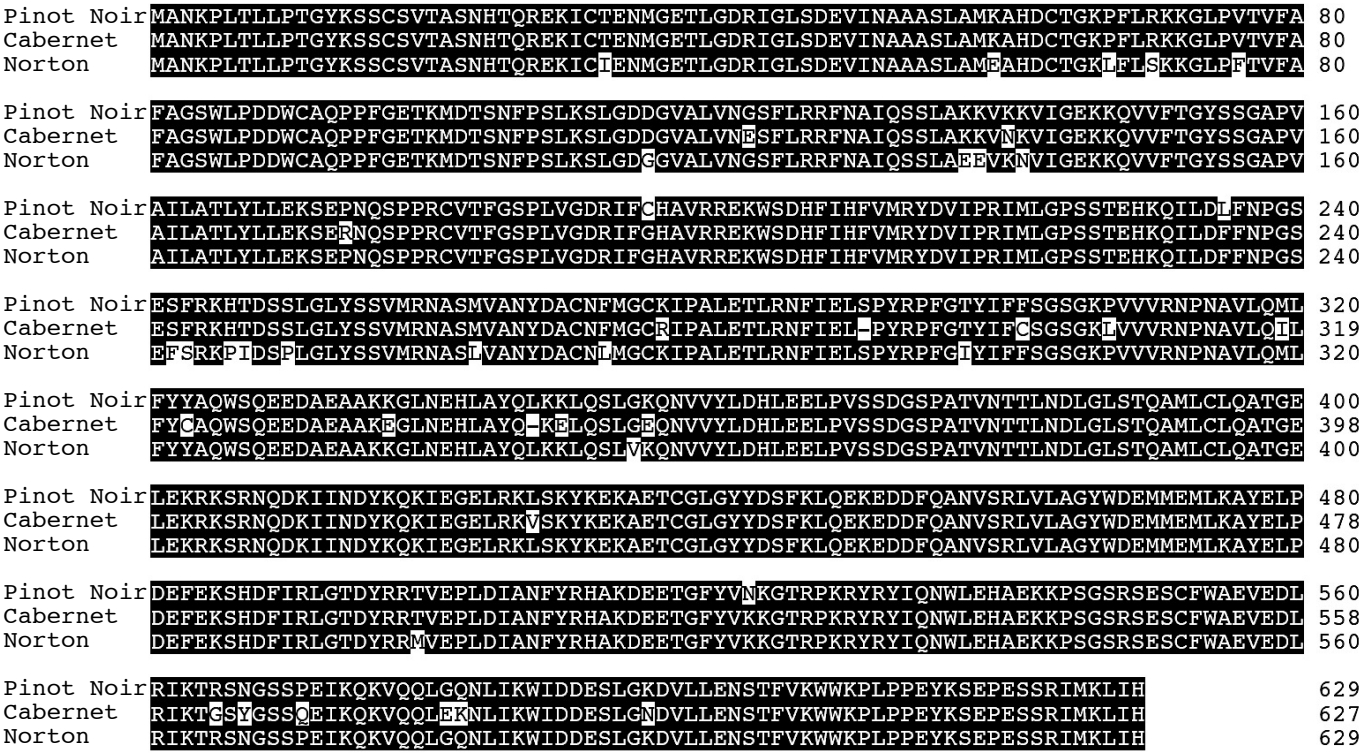
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**(C) PAD4**

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**Fig. S1** Alignment of **a** EDL2, **b** EDL5 and **c** PAD4 protein sequences from *V. vinifera* PN40024 (Pinot Noir) and Cabernet Sauvignon (Cabernet), and *V. aestivalis* Norton (Norton) using the neighbor-joining method in ClustalW. Amino acids that are identical in the majority of proteins are shaded black.

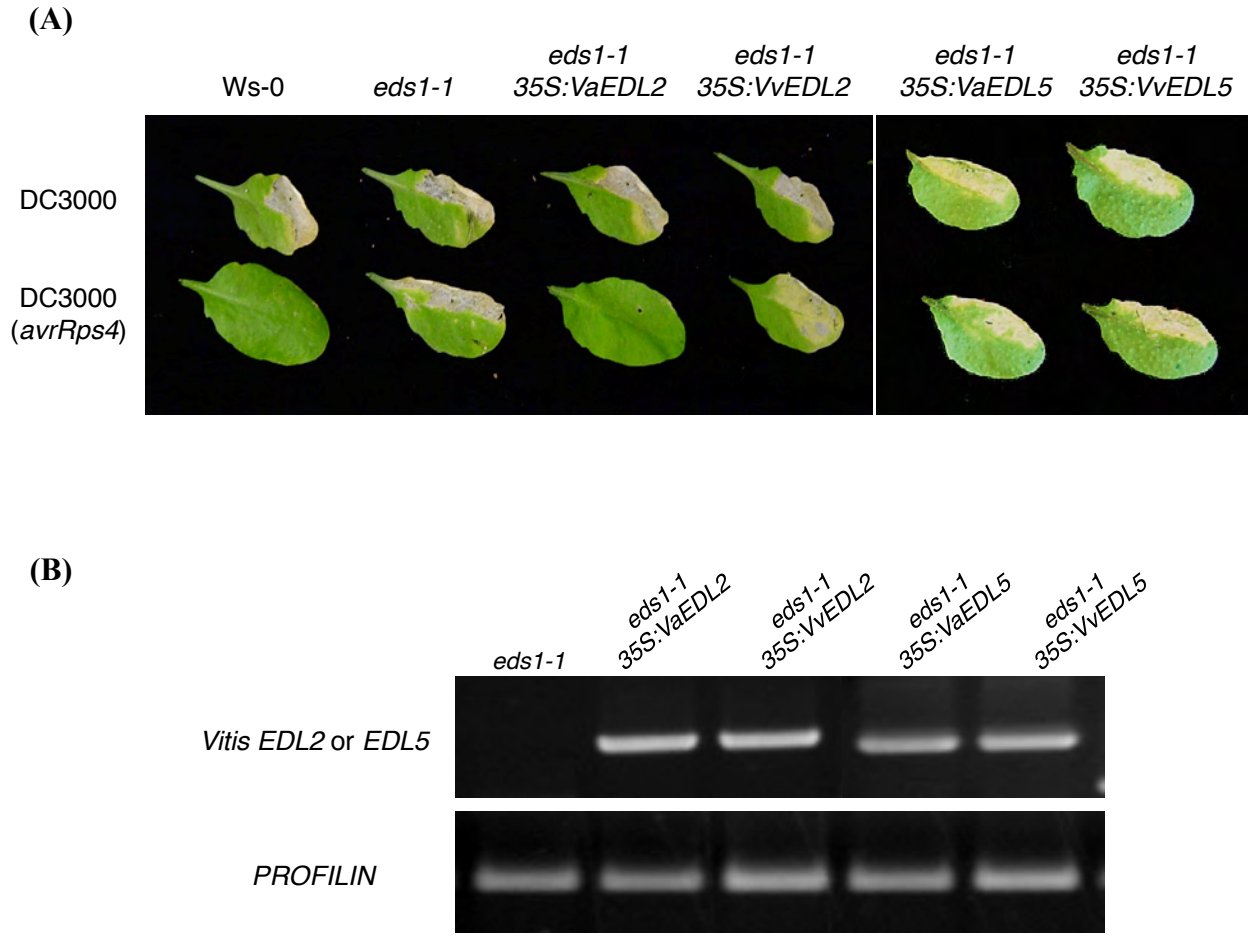


**Fig. S2** A *VaEDL2* cDNA complements the *Arabidopsis eds1-1* mutant for resistance to DC3000(*avrRps4*). **a** Wild type Ws-0, mutant *eds1-1* and transgenic *eds1-1* expressing *Va/VvEDL2* or *Va/VvEDL5* in the T3 generation were infiltrated with virulent DC3000 (top row in each panel) or DC3000(*avrRps4*) (bottom row) at a density of $1 \times 10^6$ colony-forming units/mL. Disease symptoms (chlorosis) were recorded 5 days after inoculation. **b** Expression of *Va/VvEDL2* or *Va/VvEDL5* determined by semi-quantative RT-PCR *with Vitis EDL* specific primers (top row in each panel) and *PROFILIN* as control (bottom row).

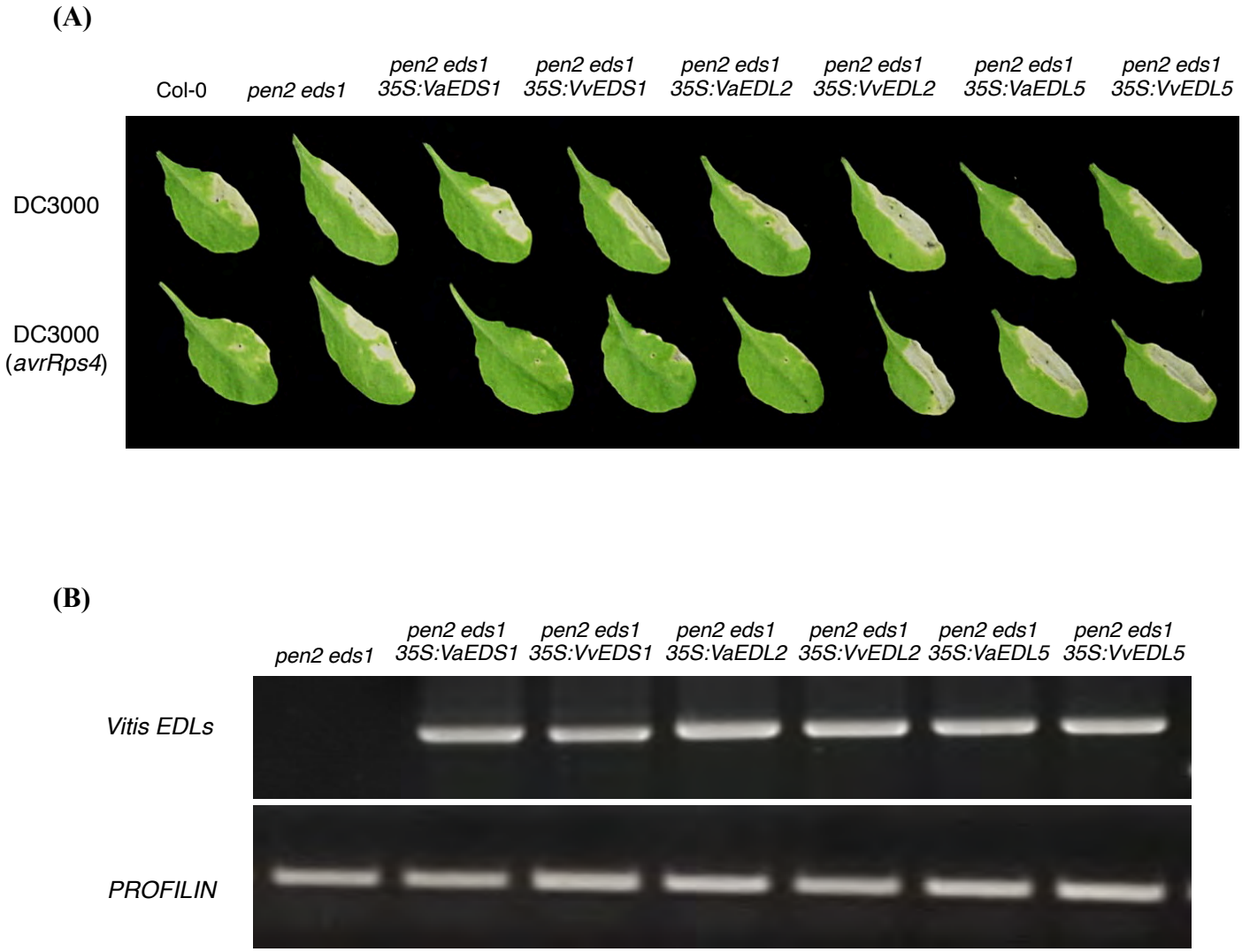


**Fig. S3** *Vitis EDS1* and *VaEDL2* cDNAs complement an *Arabidopsis pen2 eds1* mutant for resistance to DC3000(*avrRps4*). **a** Wild type Ws-0, mutant *pen2-1 eds1-1* and transgenic *pen2-1 eds1-1* expressing *Va/VvEDS1, Va/VvEDL2* or *Va/VvEDL5* in the T3 generation were infiltrated with virulent DC3000 (top row in each panel) or DC3000(*avrRps4*) (bottom row) at a density of $1 \times 10^6$ colony-forming units/mL. Disease symptoms (chlorosis) were recorded 5 days after inoculation. **b** Expression of *Va/VvEDS1, Va/VvEDL2* or *Va/VvEDL5* determined by semi-quantative RT-PCR *with Vitis EDL* specific primers (top row in each panel) and *PROFILIN* as control (bottom row).

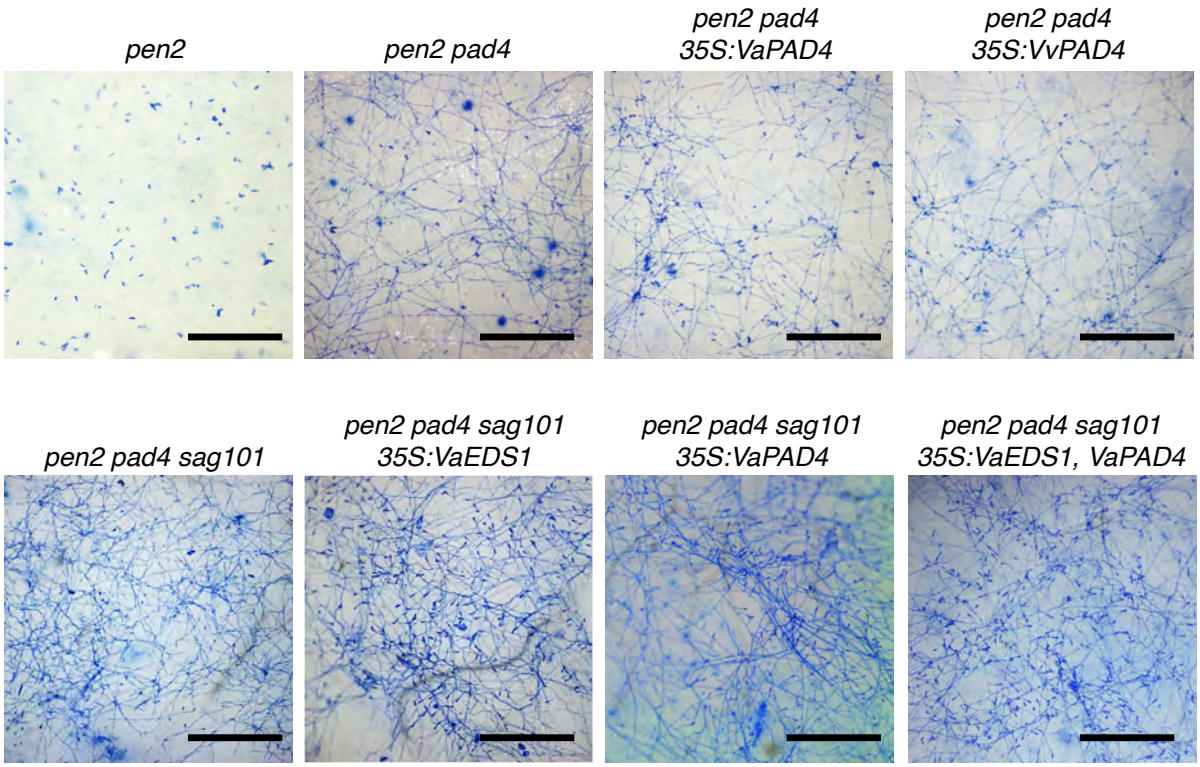


**Fig. S4** Testing *Vitis EDS1 and Vitis PAD4* cDNA transgenic Arabidopsis for resistance to powdery mildew. Arabidopsis *pen2-1, pen2-1 pad4-1* double mutant and transgenic *pen2-1 pad4-1* expressing *Va/VvPAD4* in the T3 generation, or Arabidopsis *pen2-1 pad4-1 sag101-2* mutants and their indicated mutants expressing *VaEDS1* or *VaPAD4* in the T3 generation leaves were sampled 10 days post infection with *E. necator*. *pen2-1 pad4-1 sag101-2* mutants expressing both *VaEDS1* and *VaPAD4* are F1 progeny from the cross of *pen2-1 pad4-1 sag101-2 35S:VaEDS1 and pen2-1 pad4-1 sag101-2 35S:VaPAD4.* Blue color indicates spores and hyphae stained with 0.05% aniline blue. Scale bar = 0.5 mm.

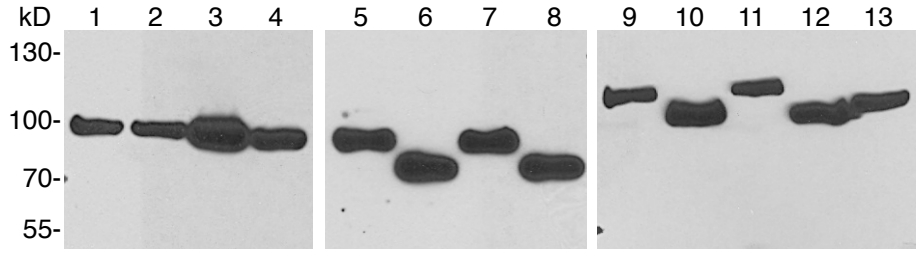


**Fig. S5.** Expression of tagged proteins for BiFC. (1) VaEDS1-nVenus, (2) VvEDS1-nVenus, (3) VaEDL2-nVenus, (4) VvEDL2-nVenus, (5) VaEDS1-nVenus, (6) VaEDS1-cCFP, (7) VvEDS1-nVenus, (8) VvEDS1-cCFP, (9)VaEDL5-nVenus, (10)VaEDL5-cCFP, (11)VvEDL5-nVenus, (12)VvEDL5-cCFP, (13)VaPAD4-nVenus. Proteins were detected by western blot. Immunodetection was performed as described previously (Moffett et al. 2002). GFP tag was detected with 1:5000 diluted rabbit anti-GFP primary antibodies and 1:5000 diluted goat anti-rabbit secondary antibodies.

**Table S1.** Primers used in this study

| **Primers** | **Forward primer** |
|---|---|
| *VEDS1-qPCR-F* | 5'-AGGGTTTTATATTGTTATCTCAAGGC-3' |
| *VEDS1-qPCR-R* | 5'-GGAAGAAAATATCTTATTACTACATAATGTTTCA-3' |
| *VEDL1-qPCR-F* | 5'-GAGCTTCCGGTGTCTTCTG-3' |
| *VEDL1-qPCR-R* | 5'-CTCGGCCTTTTCCTTATACTCA-3' |
| *VEDL2-qPCR-F* | 5'-GCTGGATCTTGGCTTCGC-3' |
| *VEDL2-qPCR-R* | 5'-AACTTGCTTCTTCTCTCCAATTAC-3' |
| *VEDL5-qPCR-F* | 5'-CACTTGGCCTACCAATTAAAGA-3' |
| *VEDL5-qPCR-R* | 5'-GCTTGAAGGCATAGCATGG-3' |
| *VitisACTIN3-F* | 5'-TCCATTGTCCACAGGAAGTGC-3' |
| *VitisACTIN3-R* | 5'-CCCCACCTCAACACATCTCC-3' |
| *Vitis60SRP-F* | 5'-ATCTACCTCAAGCTCCTAGTC-3' |
| *Vitis60SRP-R* | 5'-CAATCTTGTCCTCCTTTCCT-3' |
| *VvEDS1pro-F* | 5'-CACCAAGGTACACATCCTTTTTCATC-3' |
| *VvEDS1pro-R* | 5'-ATTCTCTCTCCCTCTCCTG-3' |
| *VaEDS1pro-F* | 5'-CACCGTAGAATAAATCAACACTC-3' |
| *VaEDS1pro-R* | 5'-CCAAGTGTTTCTCCCAT-3' |
| *VEDL1-clone-F* | 5'-AAATCACAGAGAGAAAATTTGCATGAGAGA-3' |
| *VEDL1-clone-R* | 5'-TCAATCTTTCTCATGGATGAGTTTCAT-3' |
| *VEDL2-clone-F* | 5'-ATGGGAGAAACACTTGATCGTA-3' |
| *VEDL2-clone-R* | 5'-TTACCCACCGATGAGTTTCGAAATGC-3' |
| *VEDL5-clone -F* | 5'-ATGGCCAATAAACCACTCACTCT-3' |
| *VEDL5-clone -R* | 5'-CTAATGGATGAGTTTCATAATGCG-3' |
| *VPAD4-clone-F* | 5'-ATGGATGCAGAAACTTCATTG-3' |
| *VPAD4-clone-R* | 5'-TTAAGGAACCATCTCTTCAT-3' |

**Table S2.** Percent predicted amino acid sequence identity between EDS1 and EDL2 proteins

| | VaEDS1 | VvEDS1 | VaEDL2 | VvEDL2 |
|---|---|---|---|---|
| AtEDS1 | 43.9 | 43.4 | 42.1 | 42.2 |
| VaEDS1 | 100 | 98.7 | 70.2 | 68.5 |
| VvEDS1 | | 100 | 69.6 | 68.1 |
| VaEDL2 | | | 100 | 95.0 |

**Table S3.** BiFC interactions between Arabidopsis and grapevine defense proteins

| | AtEDS1 | AtPAD4 | Va/ VvEDS1 | Va/ VvEDL2 | Va/ VvEDL5 | Va/ VvPAD4 |
|---|---|---|---|---|---|---|
| AtEDS1 | punctate spots | Nucleus & cytoplasm | punctate spots | punctate spots | No | No |
| AtPAD4 | Nucleus & cytoplasm | No | Nucleus & cytoplasm | Nucleus & cytoplasm | No | No |
| Va/ VvEDS1 | punctate spots | Nucleus & cytoplasm | Nucleus & cytoplasm | Nucleus & cytoplasm | cytoplasm | Nucleus & cytoplasm |
| Va/ VvEDL2 | punctate spots | Nucleus & cytoplasm | Nucleus & cytoplasm | Nucleus & cytoplasm | cytoplasm | Nucleus & cytoplasm |
| Va/ VvEDL5 | No | No | cytoplasm | cytoplasm | No | No |
| Va/ VvPAD4 | No | No | Nucleus & cytoplasm | Nucleus & cytoplasm | No | No |