The Plant Journal (2005) **42**, 95–110 doi: 10.1111/j.1365-313X.2005.02356.x

The atypical resistance gene, *RPW8*, recruits components of basal defence for powdery mildew resistance in Arabidopsis

Shunyuan Xiao[1,2], Ozer Calis[1,†], Elaine Patrick[1], Guangmin Zhang[1,‡], Piyavadee Charoenwattana[1], Paul Muskett[3,§], Jane E. Parker[3,§] and John G. Turner[1,*]

[1]*School of Biological Science, University of East Anglia, Norwich NR4 7TJ, UK,*
[2]*Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, MD 20742 4450, USA, and*
[3]*Sainsbury Laboratory, John Innes Centre, Colney, Norwich NR4 7TU, UK*

Received 1 September 2004; revised 23 November 2004; accepted 26 November 2004.
[*]For correspondence (fax +0 1603 592192; e-mail j.g.turner@uea.ac.uk).
[†]Present address: Plant Protection Department, Agricultural Faculty, Gaziosmanpasa University, Tasliciftlik-Tokat, Turkey.
[‡]Present address: Department of Plant Protection, Shangdong Agricultural University, Taian, Shangdong 271018, China.
[§]Present address: Department of Plant-Microbe Interactions, Max-Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, 50829 Cologne, Germany.

Summary

Genetic studies have identified a number of components of signal transduction pathways leading to plant disease resistance and the accompanying hypersensitive response (HR) following detection of pathogens by plant resistance (*R*) genes. In Arabidopsis, the majority of R proteins so far characterized belong to a plant superfamily that have a central nucleotide-binding site and C-terminal leucine-rich-repeats (NB-LRRs). Another much less prevalent class comprises RPW8.1 and RPW8.2, two related proteins that possess a putative N-terminal transmembrane domain and a coiled-coil motif, and confer broad-spectrum resistance to powdery mildew. Here we investigated whether RPW8.1 and RPW8.2 engage known pathway(s) for defence signalling. We show that *RPW8.1* and *RPW8.2* recruit, in addition to salicylic acid and *EDS1*, the other *NB-LRR* gene-signalling components *PAD4*, *EDS5*, *NPR1* and *SGT1b* for activation of powdery mildew resistance and HR. In contrast, *NDR1*, *RAR1* and *PBS3* that are required for function of certain *NB-LRR R* genes, and *COI1* and *EIN2* that operate, respectively, in the jasmonic acid and ethylene signalling pathways, do not contribute to *RPW8.1* and *RPW8.2*-mediated resistance. We further demonstrate that *EDR1*, a gene encoding a conserved MAPKK kinase, exerts negative regulation on HR cell death and powdery mildew resistance by limiting the transcriptional amplification of *RPW8.1* and *RPW8.2*. Our results suggest that *RPW8.1* and *RPW8.2* stimulate a conserved basal defence pathway that is negatively regulated by *EDR1*.

Keywords: RPW8, basal resistance, powdery mildew, resistance gene, salicylic acid, edr1.

Introduction

Plant disease resistance (*R*) gene-triggered defences are often associated with the hypersensitive response (HR), a form of programmed cell death (PCD) analogous to animal apoptosis (Lam *et al*., 2001; Morel and Dangl, 1997). HR is manifested at infection sites as rapid, localized death of plant cells that limits spread of the invading pathogen. In mammalian systems, apoptosis is tightly controlled by both pro- and anti-apoptotic elements (Adams and Cory, 2001; Reed *et al*., 1998). The life–death boundary of HR is also presumably defined by close interplay between positive and negative regulators in plant cells. A current challenge is to understand the mechanisms that link *R* gene-mediated pathogen recognition to the expression of resistance and the accompanying HR.

Extensive screens for mutants, mostly in Arabidopsis, have led to the identification of many genes required for defences against pathogens (reviewed by Glazebrook, 2001; Hammond-Kosack and Parker, 2003). Mutations in *NDR1*, *EDS1*, *PAD4*, *NPR1*, *EDS5*, *RAR1* or *SGT1b* suppress resistance and HR development controlled by multiple *NB-LRR* type *R* genes. *EDS1*, *PAD4*, *NPR1* and *EDS5* appear to be components of salicylic acid (SA)-dependent defence

pathway(s) that are also required for expression of basal defences against several virulent pathogen strains. Whereas *EDS1*, *PAD4* and *EDS5* participate in signal potentiation through an SA-associated amplification circuit (Falk *et al.*, 1999; Jirage *et al.*, 1999; Nawrath *et al.*, 2002), *NPR1* functions as a transcriptional regulator downstream of SA (Cao *et al.*, 1997). RAR1 and SGT1b appear to act at the level of the R protein itself and an increasing body of evidence points to roles of these proteins as molecular co-chaperones that may assist maturation and/or activation of R protein complexes (Gray *et al.*, 2003; Shirasu and Schulze-Lefert, 2003). Genes in the jasmonic acid (JA) and ethylene (ET) signalling pathways have also been implicated in plant defences. For example, mutations in *COI1* required for JA signalling (Xie *et al.*, 1998) and *EIN2* required for ET-pathway (Alonso *et al.*, 1999) compromise resistance to necrotrophic pathogens (Thomma *et al.*, 1998, 1999). Both synergistic and antagonistic interactions exist between SA-dependent processes and those engaging JA and ET, suggesting complex signal interplay in defence activation (Ellis and Turner, 2002; Feys and Parker, 2000; Glazebrook *et al.*, 2003; Turner *et al.*, 2002).

Arabidopsis mutants with constitutive expression of SA-dependent defence responses, spontaneous HR-like lesions (SHL) and enhanced disease resistance have also been isolated and some of the corresponding genes cloned as potential negative regulators in pathways leading to defence or PCD (reviewed by Hammond-Kosack and Parker, 2003; Lorrain *et al.*, 2003). However, few of these genes have been positioned genetically in *R* gene signalling pathway(s) (Brodersen *et al.*, 2002; Frye *et al.*, 2001; Rusterucci *et al.*, 2001) and little can be inferred about their precise biological functions from their protein sequences. *EDR1*, encoding a MAPKK kinase is an exception (Frye *et al.*, 2001). A loss-of-function mutation of *EDR1* results in SA- and *EDS1*-dependent enhanced disease resistance to powdery mildew in the absence of constitutive defence-related (*PR*) gene expression or formation of spontaneous lesions (Frye and Innes, 1998; Frye *et al.*, 2001).

The Arabidopsis genes *RPW8.1* and *RPW8.2* (hereafter referred to as *RPW8*, unless otherwise indicated) confer broad-spectrum resistance to powdery mildew pathogens. The two predicted RPW8 proteins bear no significant homology to other proteins (Xiao *et al.*, 2001), including the R-proteins, and thus form a unique R protein category (Dangl and Jones, 2001). Yet, *RPW8*-mediated resistance is associated with a classic HR that is SA- and *EDS1*-dependent and associated with H_2O_2 accumulation, and defence gene expression, similar to defence cascades regulated by *NB-LRR*-type *R* genes that possess an N-terminal Toll-Interleukin 1 receptor (TIR) homology domain (Aarts *et al.*, 1998; Feys *et al.*, 2001; Xiao *et al.*, 2001). Arabidopsis *RPW8* confers powdery mildew resistance in transgenic tobacco plants (Xiao *et al.*, 2003a), suggesting that although *RPW8* encodes an atypical R protein, it may regulate a conserved signalling pathway(s) leading to HR and resistance. SA and *EDS1* have been implicated in non-host resistance (Mellersh and Heath, 2003; Parker *et al.*, 1996; Yun *et al.*, 2003) and are components of basal resistance against virulent pathogens (Parker *et al.*, 1996; Reuber *et al.*, 1998). Moreover, a number of mutations have been identified that cause lesions and heightened pathogen resistance in an SA- and EDS1-dependent manner (Brodersen *et al.*, 2002; Clarke *et al.*, 2001; Pilloff *et al.*, 2002; Rate *et al.*, 1999). It is therefore unclear whether RPW8 engages processes that are specific to TIR-NB-LRR proteins or, more broadly, mechanisms of EDS1 and SA-dependent basal resistance.

Spontaneous and spreading HR-like lesions (SHL) develop in transgenic lines containing multiple copies of the *RPW8* genes under control of their native promoters (Xiao *et al.*, 2003b). SHL is associated with greatly enhanced transcriptional amplification of *RPW8* via an SA- and *EDS1*-dependent positive feedback circuit (Xiao *et al.*, 2003b). However, in Arabidopsis accession Ms-0, and in transgenic plants containing a single copy of *RPW8*, HR develops as a restricted lesion at fungal penetration sites, and there is limited transcription of the *RPW8* genes (Xiao *et al.*, 2001). These observations suggest that regulation of the transcriptional amplification of *RPW8* is involved in RPW8-mediated HR.

The *RPW8* genes challenge our understanding of *R*-gene function. Like other *R*-genes they are polymorphic, and regulate a SA-dependent HR. Unlike other *R*-genes however, they confer resistance to a range of powdery mildew pathogens, apparently not through a gene-for-gene interaction. Neither are the RPW8 proteins likely receptors of pathogen ligands. Moreover, the *RPW8* genes have evolved recently in Arabidopsis (Xiao *et al.*, 2004). This raises the significant question of whether the *RPW8* proteins regulate HR through a novel SA-dependent pathway, or recruit components of more ancient disease resistance pathways. To address these questions, we constructed Arabidopsis lines combining the *RPW8* gene with a series of characterized mutations affecting SA-dependent or JA/ET-dependent defence responses. We show that, in addition to SA and *EDS1*, four genes (*PAD4*, *EDS5*, *NPR1* and *SGT1*b) that are also recruited by *NB-LRR* genes, are required for *RPW8*-mediated resistance. In contrast, *NDR1*, *RAR1*, *PBS3*, *COI1* or *EIN2* do not contribute substantially to *RPW8* function. These data are supported by results of a screen for mutations compromising *RPW8*-conditioned powdery mildew resistance which yielded new defective alleles of *PAD4* and *NPR1*. Detailed analysis of plants expressing high levels of *RPW8* transcripts in various mutant backgrounds revealed that SHL is triggered by both *EDS1* and SA-dependent and independent processes. We also show that *EDR1* negatively regulates transcriptional amplification of *RPW8*, thus linking the MAPKKK to a conserved signalling pathway(s) shared by *RPW8* and several *NB-LRR R* genes.

Results

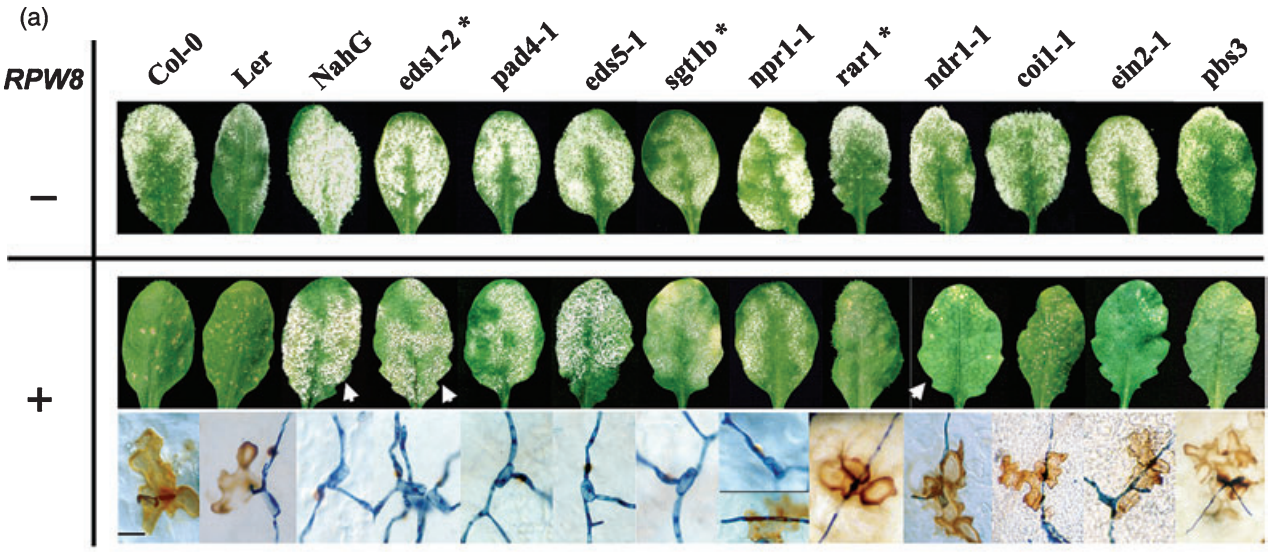
Effects of defence pathway mutants on basal powdery mildew resistance

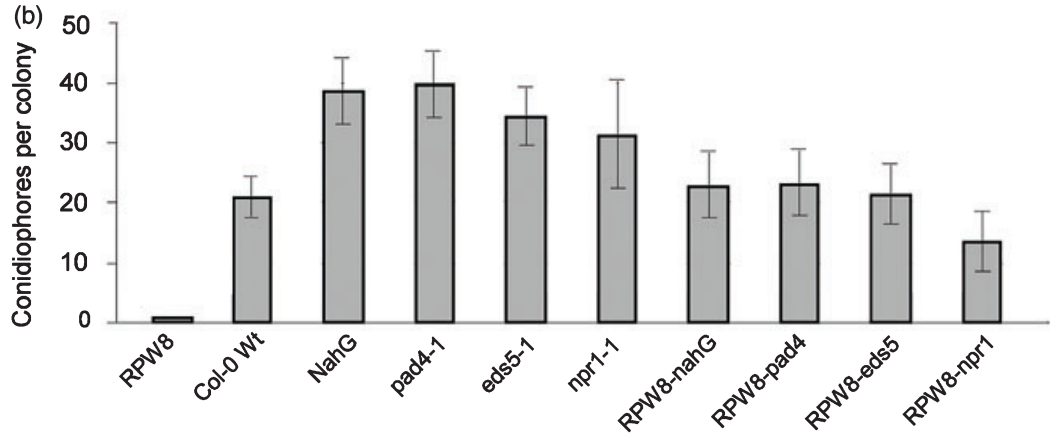
A panel of defence mutants isolated in Arabidopsis accessions Columbia (Col-0) or Landsberg-*erecta* (Ler) were tested for altered resistance to four virulent powdery mildew pathogens: *Erysiphe cruciferarum* UEA1, *E. cichoracearum* UCSC1, *E. orontii* MGH, and *Oidium lycopersici* Oxford (Xiao *et al.*, 2001). Col-0 and Ler do not have functional *RPW8.1* and *RPW8.2* genes and are susceptible to the four powdery mildew isolates (Xiao *et al.*, 2001). Disease development on mutant leaves was compared to that on Col-0 and Ler wild-type plants with the aid of a dissecting microscope. We used five scales (0, 1, 2, 3 and 4) of increasing pathogen colonization to record the disease phenotypes (see Experimental procedures); these disease reaction (DR) scores are shown in

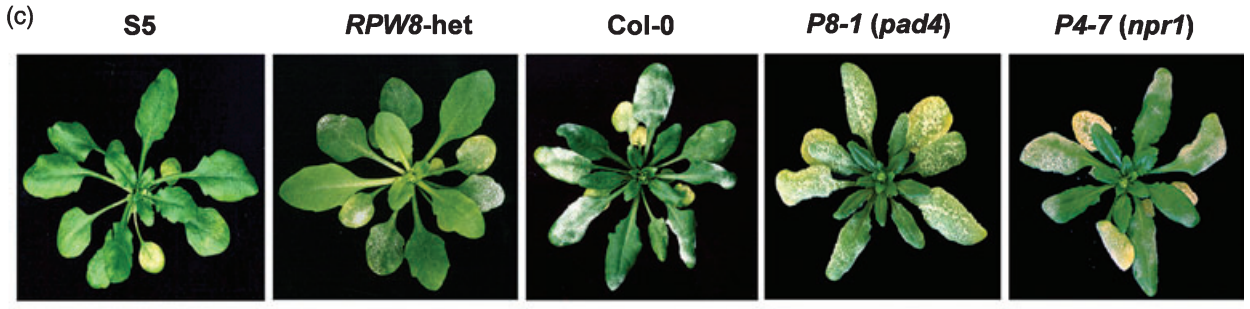
Table 1 Powdery mildew disease phenotypes of defence pathway mutants in the absence or presence of functional *RPW8*[a]

	Erysiphe isolate				
Genotypes	UEA1	UCSC1	MGH	Oxford	Reference
Col-0	3	3	3	3	
Ler	2–3	2–3	2–3	2–3	
Col*NahG*	4	4	4	4	Lawton *et al.* (1995)
Ler *eds1-2*	4	4	4	4	Falk *et al.* (1999)
Col*pad4-1*[b]	4	4	4	4	Jirage *et al.* (1999)
Ler*pad4-2*	3–4	4	4	4	Jirage *et al.* (1999)
Col *eds5-1*[b]	3–4	4	3–4	4	Nawrath *et al.* (2002)
Col*npr1-1*[b]	3	3–4	3–4	3–4	Cao *et al.* (1997)
Ler*sgt1b-1*	2–3	2–3	3	2–3	Austin *et al.* (2002)
Col*ndr1-1*[b]	3	3	3	3	Century *et al.* (1997)
Col*pbs3*	3	3	3	3	Warren *et al.* (1999)
Ler *rar1-10*	2–3	2–3	2–3	2–3	Muskett *et al.* (2002)
Col*coi1-1*	3	3–4	3–4	3–4	Xie *et al.* (1998)
Col*ein2-1*	3	3–4	3–4	3–4	Alonso *et al.* (1999)
eds1-2-ndr1-1	4	4	4	4	McDowell *et al.* (2000)
Col*RPW8* (S5)	0–1	0–1	0–1	0–1	Xiao *et al.* (2003b)
Col *RPW8*-Het[c]	1	1–2	1–2	1–2	
Ler*RPW8*[d]	0	0	0–1	0–1	
Col*edr1*	1	1–2	1–2	1–2	Frye *et al.* (2001)
Col*RPW8-NahG*[e]	2–3	3	3	2–3	
RPW8-eds1-2[e]	3	3	3	3	
Col*RPW8-pad4-1*[e]	3	3	3	3	
Col*RPW8-eds5-1*[e]	2–3	2–3	3	2–3	
Col*RPW8-npr1-1*[e]	1–2	2	2	1–2	
RPW8-sgt1b-1[e]	nt	2–3	2–3	nt	
Col*RPW8-ndr1-1*[e]	0–1	0–1	0–1	0–1	
Col*RPW8-pbs3*[f]	0–1	0–1	0–1	0–1	
RPW8-rar1-10[g]	nt	1	0–1	nt	
Col*RPW8-coi1-1*[e]	0–1	1	0–1	0–1	
Col*RPW8-ein2-1*[f]	nt	0–1	0–1	nt	
Col*RPW8-edr1*[e]	0	0	0	0	

[a]Approximately 6-week-old seedlings were inoculated with each of the *Erysiphe* isolates and the disease phenotypes were examined at 10 and 12 dpi. The criteria for the DR scoring (0 indicates no infection and 4 indicates the highest degree of susceptibility) were based on the percentage of the fungal coverage on the leaf surface at 10–12 dpi (see Experimental procedures). nt, not tested. At least three independent pathogen tests were carried for each line.
[b]Mutant lines tested with *E. orontii* MGH by Reuber *et al.* (1998).
[c]F_1 plants derived from a cross between S5 and Col-0. They are heterozygous for the *RPW8* transgene.
[d]The Ler *RPW8* line was generated by crossing S5 to Ler and backcrossing the F_1 to Ler twice. One line homozygous for *RPW8* was then selected from the selfed progeny of a BC2 individual.
[e]The single copy of *RPW8* transgene from Col-0 line S5 was combined with the mutant alleles by crossing S5 with the corresponding mutant lines and F_3 families homozygous for *RPW8* and each of the mutant alleles were used for the test.
[f]Genotypes inferred from genetic analysis (see Experimental procedures).
[g]Transgenes from two different Col-0 transgenic lines (T-B75 and T-B6L) were individually introduced in *rar1-10*.


(a)
RPW8
Col-0
Ler
NahG
eds1-2 *
pad4-1
eds5-1
sgt1b *
npr1-1
rar1 *
ndr1-1
coi1-1
ein2-1
pbs3
–
+


(b)
Conidiophores per colony
50
40
30
20
10
0
RPW8
Col-0 Wt
NahG
pad4-1
eds5-1
npr1-1
RPW8-nahG
RPW8-pad4
RPW8-eds5
RPW8-npr1


(c)
S5
RPW8-het
Col-0
P8-1 (pad4)
P4-7 (npr1)

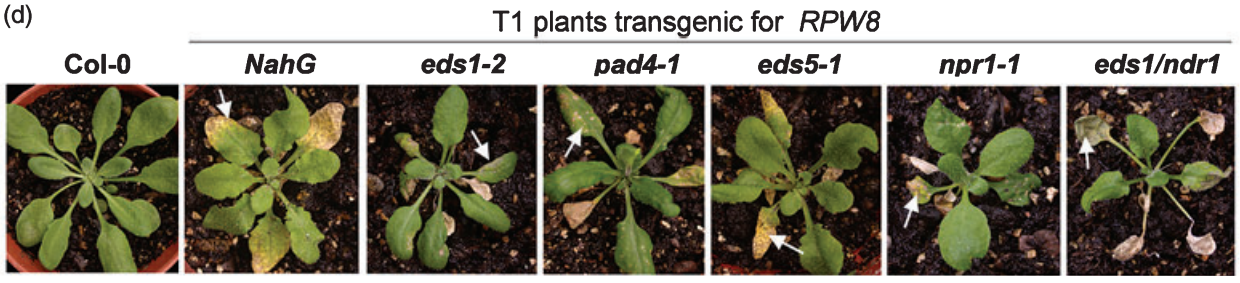

(d)
T1 plants transgenic for RPW8
Col-0
NahG
eds1-2
pad4-1
eds5-1
npr1-1
eds1/ndr1

Table 1. We found that the disease phenotypes of Col-0 (DR score 3) and Ler (DR score 2–3) to the four powdery mildew isolates were similar, with Col-0 being slightly more susceptible. Representative infected leaves from each genotype are shown in the upper panel of Figure 1(a). Col-*NahG* plants consistently supported the highest degree of fungal growth (DR score 4): inoculated leaves were covered by fungal mycelia and conidia. Plants carrying mutant alleles of *eds1-2*, *pad4-1*, *eds5-1* or *eds1-2*/*ndr1* double, and to a lesser degree, *npr1-1*, displayed enhanced susceptibility (DR score 4, or 3–4) to these isolates compared to Ler or Col-0. Plants carrying the *coi1-1* allele were also more susceptible than Col-0. *Ndr1-1*, *pbs3*, *rar1-10* and *sgt1b-1* plants did not exhibit enhanced susceptibility to the fungal isolates compared to wild-type responses (Table 1; Figure 1a). These results show that *EDS1*, *PAD4*, *EDS5* and *NPR1* play positive roles in basal resistance of Arabidopsis to multiple powdery mildew pathogens. Significantly, *COI1* also contributes to basal resistance, presumably via the JA–ET pathway. The remaining genes *NDR1*, *PBS3*, *RAR1* and *SGT1b* appear to be dispensable for expression of basal resistance to these powdery mildew isolates.

RPW8-*mediated resistance requires* PAD4, EDS5, SGT1b *and* NPR1

We examined the requirements of defence signalling components in *RPW8*-conditioned HR cell death and resistance. For this we introduced *pad4-1*, *eds5-1*, *npr1-1*, *sgt1b-1*, *rar1-10*, *pbs3*, *coi1-1* and *ein2-1* and *NahG* into an *RPW8* background (Xiao *et al*., 2001).

The results shown in Table 1 and the lower panel of Figure 1(a) indicate that plants containing *RPW8* and either *eds1-2* or the *NahG* gene were as susceptible as Col-0 to powdery mildew. Plants containing *RPW8* and *pad4-1* or *eds5-1* also failed to develop HR and were as susceptible as Col-0, indicating that *PAD4* and *EDS5* are also required for *RPW8* function. Plants containing *RPW8* and *npr1-1* were only moderately susceptible to the pathogens. Therefore, *RPW8* may activate both *NPR1*-dependent and *NPR1*-independent defences. It appeared that *RPW8* partially increased the resistance of each of these mutants (Table 1). This was examined independently by counting the number of conidiophores formed per fungal colony at 5 days after inoculation with *E. cichoracearum* UCSC1. The data shown in Figure 1(b) indicate that *NahG*, *pad4-1*, *eds5-1* and *npr1-1* were significantly ($P < 0.0001$) more susceptible than Col-0 wild type, whereas the corresponding lines containing *RPW8* were as susceptible ($P > 0.01$) as Col-0. Thus, the mutations compromise the expression of basal resistance and *RPW8* resistance in an incremental fashion. We had noted previously that plants hemizygous for a single copy of the *RPW8* transgene were less resistant to powdery mildew than plants homozygous for that transgene, indicating that *RPW8* is semi-dominant. Hemizygous plants supported intermediate levels of fungal growth (DR score 1–2; Table 1; Figure 1c). In the course of identifying F_3 families homozygous for *RPW8* and for the different mutant alleles, we further noted that plants hemizygous for *RPW8* in homozygous *NahG*, *eds1-2*, *pad4-1* or *eds5-1* backgrounds were more susceptible than plants homozygous for *RPW8* in the same background. These observations reinforced the notion that *RPW8* acts in a gene dosage-dependent manner to stimulate basal defences.

Plants containing *RPW8* and *sgt1b-1* in a Col-0 and Ler mixed background also failed to develop HR but were not more susceptible than Ler, indicating that *sgt1b-1* only partially compromises *RPW8* resistance. In contrast, plants containing *RPW8* in combination with Ler *rar1-10*, or Col-0 *pbs3*, *coi1-1* or *ein2-1* produced a normal HR and were resistant to all isolates tested (Table 1; Figure 1a), indicating that *RAR1*, *PBS3*, *COI1* and *EIN2* are not required for *RPW8* function.

Localized production of H_2O_2 in whole epidermal cells is an early sign of *RPW8*-conditioned defence response to attempted pathogen penetration (Xiao *et al*., 2001). We examined whether the disabled *RPW8* resistance in *NahG*, *eds1-2*, *pad4-1*, *eds5-1*, *npr1-1* or *sgt1b-1* backgrounds was

Figure 1. Signalling requirements of *RPW8*-mediated resistance.

(a) Leaf disease phenotypes and induction of H_2O_2 (underneath the leaf pictures). Twelve 6-week-old plants of each genotype grown in 10 × 17 cm trays (six plants for each genotype under test, two for Col-0 and two for S5 as susceptible and resistance control) were inoculated with each of the four powdery mildew isolates in four separate experiments. Disease phenotypes were examined at 10 and 12 dpi. This experiment was repeated three times and one representative leaf from plants inoculated with *Erysiphe cichoracearum* UCSC1 was shown for each genotype. Pictures were taken at 10 dpi. DAB-trypan blue staining (Thordal-Christensen *et al*., 1997; Xiao *et al*., 2003b) was used to reveal H_2O_2 (reddish-brown stain) accumulation in the epidermal cells penetrated by the fungus (blue) at approximately 2 dpi. No whole-cell H_2O_2 staining was found in any of the genotypes lacking *RPW8* and pictures were not shown. '−', lack of *RPW8*; '+', presence of *RPW8*. Mutants in Ler background are marked with '*'. Bar indicates 50 μm. Arrowheads indicate pictures published in Xiao *et al.* (2001) and used here as references.

(b) Quantitative assay of the disease susceptibility. Five 6-week-old plants of each tested line were inoculated with *E. cichoracearum* UCSC1 and two mature inoculated leaves from each plant were collected at 5 dpi and examined for the number of conidiophores produced in a single fungal colony. Around 50 well-separated colonies (approximately five colonies per leaves) were counted for each lines and the average number of conidiophores per colony and the SD were presented.

(c) Disease phenotype of mutant lines *P8-1* and *P4-7* in comparison with S5, F_1 of S5 × Col-0 (*RPW8*-het) and Col-0 wild type. Six-week-old plants were inoculated with *E. cichoracearum* UCSC1 and pictures were taken at 10 dpi. Note that *P4-7* was less susceptible than *P8-1*, and *RPW8*-het is intermediate.

(d) Spontaneous HR-like lesions in T_1 plants of Col *NahG* and five mutant lines carrying *RPW8*. Arrows indicate leaves displaying SHL. Note the transgenic lines with SHL had a reduced stature compared with Col-0 wild type and their pictures were taken at shorter distances.

associated with an altered pattern of H_2O_2 production at 2 days after inoculation with *E. cichoracearum* UCSC1. H_2O_2 was detected with 3,3-diaminobenzidine (DAB), and the fungus was stained with trypan blue (TB) (Thordal-Christensen *et al.*, 1997; Xiao *et al.*, 2003b). Plants lacking *RPW8* did not show whole-cell H_2O_2 accumulation at infection sites (data not shown). Plants containing *RPW8* in wild type Col-0 or Ler or in the *ndr1-1*, *rar1-10*, *pbs3*, *coi1-1* and *ein2-1* backgrounds displayed frequent (>30–50%) whole-cell H_2O_2 staining at sites of fungal penetration. In contrast, whole-cell H_2O_2 accumulation was not detected in plants containing *RPW8* in *NahG*, *eds1-2*, *pad4-1* or *eds5-1* backgrounds, and was only rarely seen in an *sgt1b-1* background (bottom panel of Figure 1a). Consistent with partial dependence of *RPW8* on *NPR1*, *RPW8-npr1-1* plants displayed lower levels (<10%) of whole-cell H_2O_2 staining at infection sites. These results suggest a tight correlation between the extent of *RPW8*-mediated resistance in the various backgrounds and the localized generation of H_2O_2.

Identification of additional mutant alleles of PAD4 and NPR1 compromising RPW8 function

To identify components required for *RPW8*-mediated resistance we screened for mutants that suppressed this resistance. Seeds of the Col-0 line S5, which contains a single copy of the RPW8 transgene and is resistant to powdery mildews, were treated with ethyl methanesulfonate (EMS). Approximately 35 000 M2 generation seedlings were screened for reduced resistance to *E. cichoracearum* UCSC1. Over 40 susceptible mutants were isolated and these fell into five complementation groups. Two mutants, *P8-1* and *P4-7*, which belonged to a different complementation group, were genetically mapped. Mutants in the other three complementation groups will be described separately. The *P8-1* mutation was mapped to the lower arm of chromosome 3 in the region of *EDS1* and *PAD4* (see Experimental procedures). The *P4-7* mutation was mapped to chromosome 1 in the region of *NPR1*. Allelism tests between *P8-1* and *eds1-2* or *pad4-1*, and between *P4-7* and *npr1-1* indicated that *P8-1* is a defective allele of *PAD4* and *P4-7* a defective allele of *NPR1*. DNA sequencing revealed that *P8-1* has a C_{681} to A point mutation and *P6-1* (another mutant in the same complementation group as *P8-1*), a G_{567} to A mutation, both resulting in a premature stop codon in *PAD4. P4-7* contains a C_{1027} to T point mutation that also results in a premature stop codon in *NPR1*. Disease tests showed that *P8-1* and *P6-1* (*pad4*) were marginally more susceptible than Col-0 to *E. cichoracearum* UCSC1, whereas *P4-7* (*npr1*) was slightly less susceptible than Col-0 to the pathogen (Figure 1b). These data were consistent with our phenotypic analysis of Col *RPW8-pad4-1* and Col *RPW8-npr1-1* lines (Table 1; Figure 1a).

Transcription of RPW8.1, RPW8.2 and PR1 is attenuated by NahG, pad4-1, eds5-1 and npr1-1

Previous studies showed that inoculation of plants containing the *RPW8* transgene with powdery mildew induced transcription of the *RPW8* genes at 8 dpi (Xiao *et al.*, 2001). The transcriptional amplification of *RPW8* was also associated with activation of HR and SHL, and was positively regulated via an SA-dependent feedback loop (Xiao *et al.*, 2003b). Here, we tested whether the suppression of *RPW8*-mediated resistance in different mutant backgrounds also suppressed transcription of the *RPW8* genes. The time course of *RPW8* transcription was determined in the transgenic line S5 (Col-0 containing a single copy of *RPW8*; Xiao *et al.*, 2003b) inoculated with *E. cichoracearum* UCSC1. Samples were taken at 0, 1, 2, 4 and 7 dpi and mRNA of *RPW8.1*, *RPW8.2* and of the SA-responsive defence marker, *PR1*, were quantified by real-time quantitative reverse transcriptase-PCR (RT-PCR). Levels of *RPW8.1* and *RPW8.2* mRNA decreased slightly at 1 dpi, at the stage when fungal

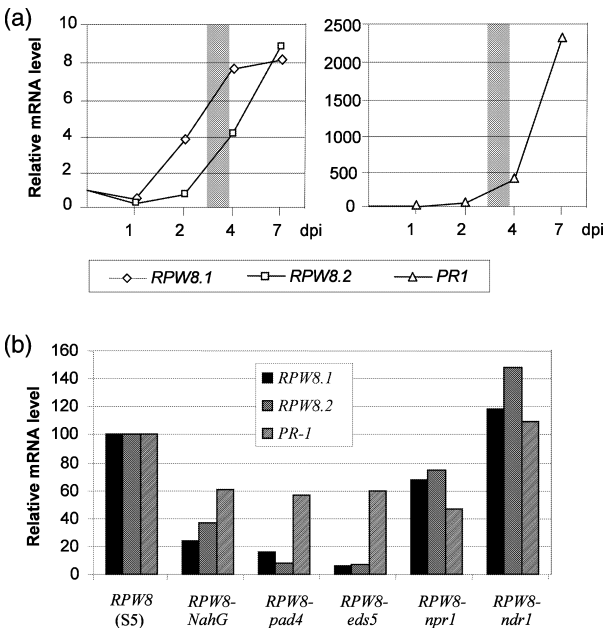


Figure 2. Induction of *RPW8.1*, *RPW8.2* and *PR1* by powdery mildew.
(a) Total RNA was prepared from 6-week-old plants of Col-0 transgenic line S5 carrying a single copy of *RPW8* inoculated with *Erysiphe cichoracearum* UCSC1 at 0, 1, 2, 4 and 7 dpi. Real-time quantitative PCR was performed from the cDNA prepared from the total RNA samples according to Xiao *et al.* (2003b). Grey-shaded bars indicate the approximate time when HR cell death was visible to the naked eye. Data represent results from one of two duplicated experiments.
(b) Six lines containing *RPW8* in Col-0 wild type (S5), *NahG*, *pad4-1*, *eds5-1*, *npr1-1* and *ndr1-1* backgrounds were inoculated with *E. cichoracearum* UCSC1 and total RNA was extracted from inoculated leaves at 0 and 7 dpi. Relative mRNA levels of *RPW8.1*, *RPW8.2* and *PR1* of the six genotypes were measured (Xiao *et al.*, 2003b) and calculated by setting those of S5 to 100 at the same time point. The data from 7 dpi are presented. This experiment was repeated once with similar results.

conidia produce germ tubes, develop appressoria and penetrate host epidermal cells (Adam and Somerville, 1996). Thereafter, *RPW8.1* and *RPW8.2* mRNAs increased from 2 to 7 dpi, reaching approximately eight times the levels measured at 0 dpi (Figure 2a). H_2O_2 production and HR at sites of fungal penetration, respectively, was first detected at 2 and 3 dpi (Figures 1a and 2a). *PR1* expression in the inoculated leaves increased dramatically between 4 and 7 dpi, reaching 377 and 2290 times of the levels at 0 dpi (Figure 2a).

RPW8 and *PR1* mRNA was measured in leaves of the Col-0 transgenic line S5 and in S5 plants containing *RPW8* in *NahG*, *pad4-1*, *eds5-1*, *npr1-1* and *ndr1-1* (all in the Col-0 background) at 0 and 7 days after inoculation with *E. cichoracearum* UCSC1. Ler contains and expresses recessive alleles *rpw8* that do not confer obvious mildew resistance (Xiao *et al.*, 2004). Because these Ler *rpw8* alleles could also be amplified during quantitative RT-PCR by the primers designed for *RPW8* (data not shown) and could thus affect the detection of the *RPW8* alleles, we did not therefore attempt to measure *RPW8* mRNA abundance in *RPW8*/*eds1-2* and *RPW8*/*sgt1b-1* lines. Levels of *RPW8.1*, *RPW8.2* and *PR1* mRNAs in the *NahG*, *pad4-1*, *eds5-1*, *npr1-1* and *ndr1-1* mutant backgrounds were calculated relative to those in S5. At 0 dpi, mRNAs of all three genes were expressed at a low level and did not differ significantly among the six tested genotypes (data not shown). At 7 dpi, *RPW8.1*, *RPW8.2* and *PR1* mRNAs were depleted in the *NahG*, *pad4-1*, *eds5-1* and *npr1-1* backgrounds compared with S5 (Figure 2b). Defects in *PAD4* and *EDS5* caused the strongest suppression of *RPW8* expression. The *npr1* mutant had a lesser effect on *RPW8* mRNA levels but strongly reduced *PR1* mRNAs. In contrast, *ndr1-1* caused a slight stimulation of expression of all three genes compared to the control line, S5. We conclude that depletion of SA or impairment of *PAD4*, *EDS5* or *NPR1* attenuates the transcription of *RPW8* and *PR1*, and this correlates with the suppression of *RPW8*-conditioned HR and resistance.

Overexpression of RPW8 activates spontaneous HR-like lesions via SA-dependent and independent pathways

A Col-0 transgenic line (S24) containing at least four copies of the *RPW8* genes driven by their native promoters displayed SHL, and this symptom was suppressed by the *NahG* transgene or the *eds1-2* mutation for 30 days after germination. This suggested that SHL was SA- and *EDS1*-dependent (Xiao *et al.*, 2003b). We have since observed that S24 plants in an *NahG* or *eds1-2* background do develop SHL at 5–6 weeks old, if they are grown under short days (8 h light/16 h dark). Moreover, SHL appeared in 3–4-week-old S24 plants grown under long-day conditions (16 h light/8 h dark) (data not shown). These data suggest that light stimulates SHL in S24, and that this is partially independent of SA and *EDS1*.

edr1 *triggers spontaneous HR-like lesions in a background containing a single copy of* RPW8.1 *and* RPW8.2

We proposed a feedback amplification of *RPW8* transcription for activation of both HR and SHL (Xiao *et al.*, 2003a). Plants of natural accessions and transgenic lines containing a single copy of *RPW8* normally lack SHL and develop strictly delimited HR lesions at powdery mildew infection sites. This suggests that suppression of the *RPW8* feedback amplification may normally prevent SHL and exaggeration of HR.

The Arabidopsis *edr1* mutant in Col-0 exhibits SA- and *EDS1*-dependent enhanced powdery mildew resistance (Frye and Innes, 1998). EDR1 is therefore a candidate for a suppressor of powdery mildew-induced cell death. We have examined whether EDR1 suppresses *RPW8*-mediated HR. The *edr1* mutation was crossed into the single-copy *RPW8* background (Col-0 transgenic line S5). Neither S5 nor *edr1* plants developed visible SHL, and both had normal stature at 6 weeks under short-day growth conditions (Figure 3a). In contrast, when plants homozygous for *RPW8* and *edr1* (S5-*edr1*) were germinated on soil, all developed SHL on cotyledons and true leaves, and these symptoms were only marginally less severe than those observed in line S24 (Figure 3a). In S5-*edr1* plants, SHL developed on newly emerged leaves and the lesions continued to spread and the leaves were killed. Interestingly, *S5-edr1* plants survived to form inflorescences that were completely free of lesions, and plants set seed, despite being 5–10% of the fresh weight of either S5 or *edr1* (measured with 6-week-old plants).

Col-0 lacks *RPW8.1* and *RPW8.2* (Xiao *et al.*, 2001). To test whether *edr1* triggers SHL in a background containing recessive *rpw8* alleles, we examined 50 F_2 plants derived from a cross between *edr1* and Ler which contains *rpw8.1*-Ler and *rpw8.2*-Ler alleles that do not confer resistance to powdery mildew. None of these progeny displayed SHL. An F_2 individual homozygous for *edr1* and *rpw8*-Ler was identified (see Experimental procedures) and backcrossed to *edr1*. None of the 60 progeny examined developed SHL over 35 days growth under short days. These results indicate that the functional *RPW8* genes are required for the *edr1*-triggered SHL.

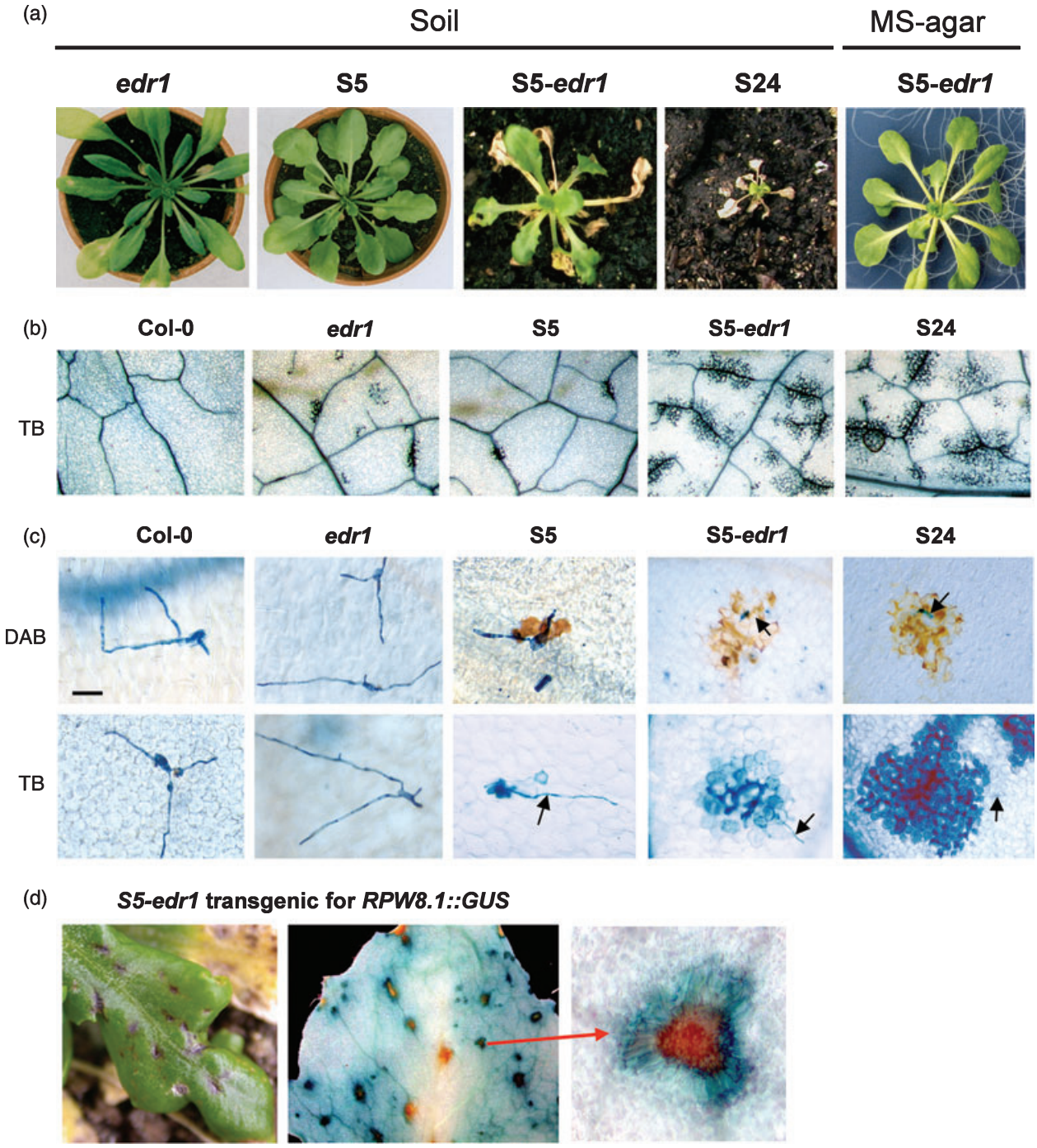
SHL is suppressed in S24 by certain environmental conditions (Xiao *et al.*, 2003b). To examine if SHL in S5-*edr1* plants is suppressed by environmental conditions, we grew S5-*edr1* plants on MS-agar medium or perlite irrigated with 1/2 strength of MS salt solution under light, temperature and humidity conditions that suppress SHL in S24 (Xiao *et al.*, 2003b). Conditions that suppressed SHL in S24 [growth on MS medium, low light (approximately 14 μmol m^{-2} sec^{-1}), high temperature (30°C), and high humidity (RH ≥96%)] also suppressed SHL in S5-*edr1* plants (Figure 3a).

SA enhances SHL in S24 plants. When 3-week-old S5-*edr1* seedlings were transplanted to MS-agar containing 100 μM

SA they became extremely chlorotic, as were similarly treated S24 seedlings, whereas S5 and *edr1* plants were indistinguishable from Col-0 plants (data not shown). Lactophenol-TB staining revealed clusters of dead cells around veins in leaves of S5-*edr1* plants, with an intensity and pattern similar to that observed in S24 (Figure 3b), indicating that S5-*edr1* plants are hypersensitive to SA, to a similar extent as S24 plants. We noted that both S5 and *edr1* but not Col-0 plants had sporadic necrosis revealed by TB staining, implying that S5 and *edr1* plants are slightly more sensitized to SA than are Col-0 wild type.

S5-edr1 *plants display an exaggerated HR in response to powdery mildew*

To assess whether *edr1* potentiates the *RPW8*-mediated HR to powdery mildew, plants were transferred from SHL-suppressive conditions (MS-agar) to SHL-permissive conditions



(soil), and were inoculated with *E. cichoracearum* UCSC1. Plant responses at infection sites were examined 2 dpi as whole-cell H_2O_2 production, and at 3 dpi as cell death. There was no significant difference between the Col-0 and *edr1* plant response to powdery mildew infection in the first 3 days after inoculation (Figure 3c). However, *edr1* plants were moderately resistant to the four powdery mildew isolates (Table 1), consistent with the observations made by Frye and Innes (1998), and H_2O_2 was detected after 2–3 days. The number of epidermal cells producing detectable H_2O_2 or undergoing cell death at >70% of infection sites in S5-*edr1* was at least five times greater than in S5 plants, and approached the exaggerated cell death response seen in S24 (Figure 3c). Fungal growth was arrested at earlier stages in S5-*edr1* and S24 compared to S5 (data not shown). These observations indicate that loss-of-function of *EDR1* mutations in S5 plants cause a more rapid and pronounced HR to powdery mildew. We conclude that *EDR1* negatively regulates an *RPW8*-dependent cell death pathway leading to HR and resistance.

edr1 *enhances transcriptional amplification of* RPW8 *independent of pathogen signals*

We considered that *EDR1* may exert negative control of *RPW8*-mediated HR at a particular step within the *RPW8*-transcriptional amplification loop or a stage of cell death execution downstream of the loop. To test whether *edr1* leads to increased transcriptional amplification of *RPW8*, we introduced an *RPW8.1*::*GUS* construct (Xiao *et al.*, 2003b) into S5-*edr1* by *Agrobacterium*-mediated transformation. All twenty 5-week-old soil-grown T_1 transgenic plants examined exhibited SHL with the same phenotypic characteristics and timing as in S5-*edr1* plants. Histochemical GUS staining of leaves of five T_1 lines revealed that GUS activity was localized mainly at margins of large lesions or to small spots that may represent incipient lesions but was also observed in small patches where there was no apparent cell death (Figure 3d). GUS activity was not detected in leaves of 3-week-old T_2 plants grown in MS-agar plates containing appropriate antibiotics for selection of *RPW8.1*::*GUS*, but was clearly detectable as small blue spots and patches in those plants at 1–2 days after transfer from MS-agar into soil before cell death was detectable by TB staining (data not shown). These observations reveal that loss of *EDR1* function leads to locally enhanced transcriptional activation of *RPW8* that precedes the formation of SHL in S5-*edr1* plants.

To measure the extent of the transcriptional amplification of *RPW*8 during SHL development, we monitored both SHL and *RPW8.1*, *RPW8.2* and *PR1* mRNA levels in S5, *edr1*, S5-*edr1* and S24 plants prior to (0), and 3 and 5 days following transfer from SHL-suppressive (MS-agar) to SHL-permissive (soil) conditions. S5 and *edr1* plants exhibited no SHL during the whole period of experiment, whereas S5-*edr1* and S24 plants started to express visible SHL in mature leaves at 4 days and exhibited massive SHL at 5–6 days after transfer. Mature leaves of S24 plants normally died after 7 days while those of S5-*edr1* died after 10–11 days (data not shown). As a control we monitored the development of SHL and mRNA levels in plants transferred from MS-agar to MS-agar, to assess the effects of transplantation. In real-time quantitative RT-PCR experiments, mRNA levels of *RPW8.1*, *RPW8.2* and *PR1* were calculated in all genotypes by setting as 1.0 the mRNA levels of the three genes in S5 plants on MS-agar prior to transplanting. The data are shown in Figure 4(a).

Prior to transplanting, there was no significant difference in the basal mRNA levels of *RPW8.1*, *RPW8.2* and *PR1* between S5 and S5-*edr1* plants, indicating the *edr1* mutation did not affect *RPW8* transcription under SHL-suppressive conditions. Messenger RNA levels of *RPW8.1* and *RPW8.2* in S24 plants were, respectively, 18 and 50 times higher than those of S5, but there were no differences in *PR1* mRNA levels. This result, together with the fact that S24 plants grown on MS-agar rarely develop SHL, suggests that threshold levels of *RPW8.1* and *RPW8.2* mRNAs must be reached in whole-leaf tissue before *PR1* expression is induced and SHL initiated. In a control experiment, 5 days after transfer from MS-agar to MS-agar, only S24 plants showed limited SHL following transfer, and the other lines were SHL-free (data not shown). We detected a 5- to 10-fold increase of *RPW8.1* and *RPW8.2* mRNAs in both S5 and S5-*edr1*, and 160-fold increase of *RPW8.1* and 260-fold increase of *RPW8.2* in S24 (Figure 4a). Despite the enhanced expression of *RPW8* in these plants, levels of *PR1* mRNA remained low. These results show that

Figure 3. *EDR1* negatively regulates *RPW8*-mediated signalling.

(a) Plants were grown in soil or MS-agar medium under short day for 3 weeks and then shifted to long day for 2 weeks. S5 is a Col-0 transgenic line carrying a single copy of *RPW8*, while S24 is a line carrying at least four copies of *RPW8* (Xiao *et al.*, 2003b). Note the size of the S5-*edr1* was approximately one-fifth of *EDR1*, and the size of S24 was approximately one-tenth of S5.

(b) Three-week-old seedlings grown on MS-agar plates were transplanted to MS-agar containing 100 μm SA. Leaf cell death was revealed by trypan blue (TB) staining at 3 days after transplanting.

(c) Four-week-old plants were inoculated with *Erysiphe cichoracearum* UCSC1 immediately after transplanted from MS-agar medium into autoclaved soil and kept under long-day conditions. Ten inoculated leaves from each genotype were DAB stained to reveal H_2O_2 accumulation, and 10 leaves were TB stained to reveal induced cell death. Reddish-brown stain indicates H_2O_2 production; blue stain indicates fungal structure and/or dead plant cells. Arrows indicate fungal conidia or hyphae. Bar represents 50 μm.

(d) S5-*edr1* plants were transformed with *RPW8.1*::*GUS* construct and T_1 transformants were selected on MS-agar medium containing 50 mg l^{-1} kanamycin and transplanted to soil to permit the development of SHL. About two to four leaves from each of twenty 5-week-old T_1 plants were subject to GUS assay. The typical GUS expression pattern in these leaves is presented. Note the red-brown spots were necrotic lesions.

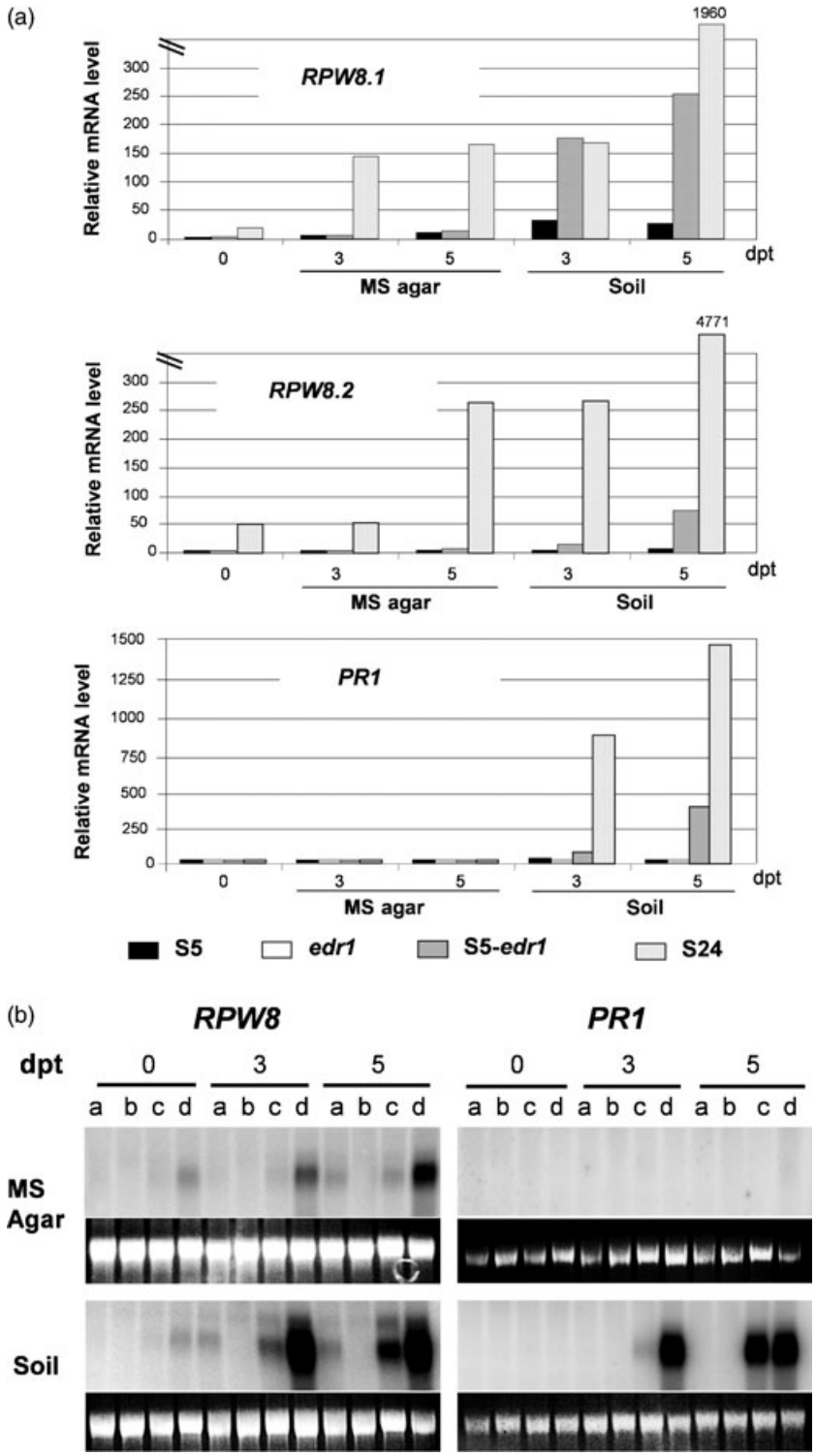


Figure 4. *edr1* mutation enhances transcriptional amplification of *RPW8*.
Four-week-old plants were transplanted from MS-agar to MS-agar or to autoclaved soil and kept under long-day conditions. Total RNA was extracted from mature leaves collected prior to, and at 3 and 5 days after transplanting for measuring mRNA of *RPW8.1*, *RPW8.2* and *PR1*.
(a) cDNA was synthesized from total RNA and used for real-time PCR using Taqman chemistry (Xiao *et al.*, 2003b). The mRNA levels of the three genes were calculated relative to those in S5 plants before transplanting (1.0). Similar results were obtained from two duplicated experiments and data from one of them was presented. dpt, days post-transplanting.
(b) About 3 μg of total RNA prepared was used for RNA gel blotting. A mixture of *RPW8.1* and *RPW8.2* cDNA was used for the detection of both *RPW8.1* and *RPW8.2* mRNA. a, S5; b, *edr1*; c, S5-*edr1*; d, S24. dpt, days post-transplanting.

disturbance associated with transfer from MS-agar to MS-agar could induce amplification of *RPW8* transcription, but this did not lead to activation of a defence pathway.

Following transfer from MS-agar to soil, expression of *RPW8.1*, *RPW8.2* and *PR1* was elevated in S5, S5-*edr1* and S24 plants at 3 and 5 days after transplanting. However, the degree of amplification of these three genes was different in the three genotypes. For example, 5 days after transfer from MS-agar to soil, S5 plants had, respectively, 27-, 7- and 3-fold increases, in *RPW8.1*, *RPW8.2* and *PR1*mRNAs, respectively,

compared with corresponding mRNAs in S5 plants in MS-agar prior to transplanting. In S5-*edr1* plants 5 days after transplanting to soil there was a 255-, 76- and 432-fold increase in *RPW8.1*, *RPW8.2* and *PR1* mRNAs respectively. In S24 plants 5 days after transplanting to soil there was a 1960-, 4771- and 1445-fold increase in *RPW8.1*, *RPW8.2* and *PR1* mRNAs respectively. Thus, loss of *EDR1* function resulted in a strong transcriptional amplification of *RPW8.1* and *RPW8.2* in S5 on transfer to SHL-inducing conditions. As S24 contained wild-type *EDR1* it appears that negative control of *RPW8* transcriptional amplification by *EDR1* can be overridden in S24 plants., which in turn led to *PR1* induction.

To validate our real-time RT-PCR data, we analysed the same RNA samples by Northern blotting. Results from this analysis supported the quantitative RT-PCR data (Figure 4b).

SHL in S5-edr1 is not abolished by eds1-2

Edr1-mediated enhanced powdery mildew resistance in Col-0 is SA- and *EDS1*-dependent, and thus *EDR1* was placed genetically upstream of *EDS1* (Frye *et al.*, 2001). We investigated if *RPW8-edr1*-mediated SHL is also *EDS1*-dependent. To test this, we constructed an *edr1-eds1-2* double mutant carrying the single copy of *RPW8* from S5. Soil-grown *edr1-eds1-2* double mutants developed SHL on both cotyledons and true leaves, although to a lesser degree than S5-*edr1* plants, and were also resistant to powdery mildew (data not shown). Thus, SHL in S5-*edr1* plants is not entirely *EDS1*-dependent. This is therefore similar to SHL in S24 plants, which also was not entirely *EDS1*-dependent.

Discussion

Genetic data presented in this analysis show that the six known signalling components, SA, *EDS1*, *PAD4*, *EDS5*, *SGT1*b and *NPR1*, which are required for function of *NB-LRR R* genes, are also required for full function of *RPW8*. Significantly, we reveal that *EDR1*, a *MAPKKK* gene, acts as a negative regulator of *RPW8*-derived signals leading to HR and resistance.

RPW8 shares signalling components with TIR-NB-LRR genes and stimulates basal resistance

RPW8 is a distinct type of *R* gene that activates resistance against powdery mildew pathogens in a non-race-specific manner (Xiao *et al.*, 2001). Our genetic data show that *RPW8* recruits components of a conserved signalling pathway that are also used by *TIR-NB-LRR* race-specific *R* genes such as *RPP4* and *RPP5* (van der Biezen *et al.*, 2002; Feys and Parker, 2000) for activation of HR cell death and resistance. A substantial body of evidence points to activities of *EDS1*, *PAD4* and *EDS5* within an SA-associated defence amplification loop that is important both for *TIR-NB-LRR*-triggered resistance against avirulent pathogens as well as for expression of basal resistance against virulent pathogens (Glazebrook *et al.*, 1996; Nawrath and Metraux, 1999; Parker *et al.*, 1996; Reuber *et al.*, 1998; this study). Thus, *EDS1*, *PAD4* and *EDS5* control SA accumulation in response to pathogen attack but are themselves upregulated by pathogens or exogenous SA application (Falk *et al.*, 1999; Jirage *et al.*, 1999; Nawrath *et al.*, 2002) and, in the case of EDS1 and PAD4, are responsive to SA-derived signals in signal potentiation beyond pathogen infection sites (Rusterucci *et al.*, 2001). Significantly, *RPW8* is also induced by powdery mildew and its transcription is amplified via an SA-dependent feedback circuit (Xiao *et al.*, 2003b). Therefore, we propose that *RPW8* engages a feedback amplification circuit consisting of *EDS1*, *PAD4*, *EDS5* and SA (Figure 5). These observations collectively suggest that *EDS1*, *PAD4*, *EDS5* and *SA*-mediated signal amplification contribute to plant basal resistance. They also raise the possibility that *RPW8* engages the basal resistance machinery to potentiate defences against biotrophic pathogens such as powdery mildew. This model could explain why *RPW8* confers broad-spectrum resistance against powdery mildew: expression of *RPW8* could be directly or indirectly induced by fungal penetrations from different pathotypes of *Erysiphe* and amplified by the feedback circuit, eventually resulting in activation of defence responses in a non-race-specific manner. Based on this hypothesis, it is anticipated that *RPW8* may condition resistance against other types of biotrophic pathogen if appropriately induced at pathogen invasion sites. Although our previous results showed no effect of *RPW8* on DR of Col-0 to a virulent isolate of *Peronospora parasitica* (Noco2), the experiments were carried out under high humidity (>95%) (Xiao *et al.*, 2001) in which *RPW8* expression is attenuated (Xiao *et al.*, 2003b). This hypothesis therefore remains to be critically tested under conditions in which *RPW8* expression is not suppressed.

Our data on partial suppression of *RPW8* resistance function by *npr1-1* (Figure 1a) and slight attenuation of transcriptional amplification of *RPW8* (Figure 1b) suggest that *RPW8*-generated signals are transmitted via *NPR1*-dependent and *NPR1*-independent processes. An *NPR1*-independent signalling pathway was implicated in the activation of defence responses by gain-of-function mutations in two *TIR-NB-LRR* genes, *SSI4* (Shirano *et al.*, 2002) and *SNC1* (Zhang *et al.*, 2003) and in overexpression of another *TIR-NB-LRR* gene, *ADR1* (Grant *et al.*, 2003). In addition, the *RPW8* alleles in *ssi4* mutant (in accession Nössen that contains *RPW8*) are strongly induced by the *ssi4* mutation (Zhou *et al.*, 2004), suggesting a further mechanistic link between *RPW8-TIR-NB-LRR*-triggered defences. Consistent with the above link, a null mutation of *NDR1* did not affect *RPW8* resistance (Xiao *et al.*, 2001), nor did it

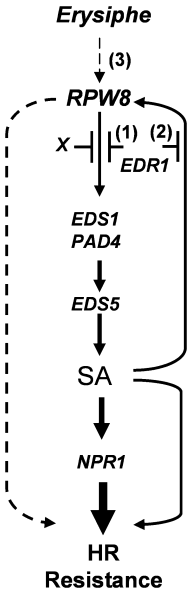


Figure 5. A model for *RPW8*-signalling.
RPW8 may be a polymorphic component of a conserved defence pathway(s). Upon challenge by powdery mildew, *RPW8* transcription is mainly amplified via the *EDS1-PAD4-EDS5*-SA feedback circuit, leading to activation of hypersensitive response (HR) and resistance through *NPR1*-dependent and independent pathways. *EDR1* acts as a negative regulator of this pathway to gauge the amplification of *RPW8* for an appropriate expression of HR and resistance. However, once the amplification of *RPW8* reaches a threshold level, the negative control of *EDR1* may be overridden or ineffective, resulting in the activation of spontaneous HR-like lesions (SHL), HR and resistance via both the default SA pathway and other independent pathway(s). *EDR1* may act in a step at the *RPW8*-SA amplification circuit (position 1 or 2) or upstream (position 3) of *RPW8*. Additional negative regulator(s) (x) such as *LSD1* may be required for preventing the HR or SHL activated by *RPW8* from runaway cell death.

compromise the SHL mediated by *RPW8*-overexpression. *NDR1* was previously identified as a necessary component of resistance triggered by a number of *CC-NB-LRR* type *R* genes but is dispensable for resistance conferred by *TIR-NB-LRR* genes (Aarts *et al.*, 1998).

An important finding in the current study was that *RPW8* resistance, but not basal resistance, depends on *SGT1b.* This is consistent with the previous finding that cell death induced by transient overexpression of *RPW8* in tobacco leaves is blocked by silencing tobacco *SGT1* (Peart *et al.*, 2002). The position of *SGT1b* within the *RPW8* signalling pathway is not known. However, the fact that SGT1b is dispensable for basal resistance to powdery mildew suggests that RPW8 is unlikely to be a core component of the basal defence machinery. A more likely scenario is that *RPW8* stimulates or lowers the threshold for activation of basal defences. This idea is supported by our finding that *RPW8* can increase defences in an incremental fashion, even if components of basal resistance are disabled (Figure 1b). SGT1 is conserved in plants, yeast and mammals and has the structural and molecular characteristics of a co-chaperone (Hubert *et al.*, 2003; Muskett and Parker, 2003; Takahashi *et al.*, 2003). In yeast, SGT1 is an essential gene required for the function of a number of signalling complexes including SCF (Skp1-Cullin-F box) E3 ligases that mediate ubiquitination of proteins that are normally then targeted for degradation by the proteasome (Muskett and Parker, 2003). In Arabidopsis, SGT1b participates in SCF-^{TIR1} and SCF-^{COI1} controlled responses, respectively, to auxin and JA (Gray *et al.*, 2003). It remains unclear whether SGT1b activity in RPW8 signalling lies at the assembly of an RPW8 complex. Other sites of action could be SCF E3 ligases involved in coupling, for example, *RPW8* to basal resistance or in the assembly of other, as yet unknown, signalling complexes.

A null mutation in *RAR1*, another component of resistance conditioned by a number of *R* genes in Arabidopsis, barley and *Nicotiana benthamiana* (Liu *et al.*, 2002; Muskett *et al.*, 2002; Shirasu *et al.*, 1999; Tornero *et al.*, 2002) did not compromise *RPW8*-induced HR and resistance. In summary, our data show that *RPW8* regulates broad-spectrum mildew resistance through a highly conserved signal transduction pathway that is also used by *TIR-NB-LRR* genes. An important implication from this study is that different types of plant disease resistance seem to have been superimposed on a common, conserved basal resistance mechanism. A future challenge is to understand how *RPW8*-derived signals are integrated with this conserved pathway.

Negative regulation of RPW8 *by* EDR1

HR involves rapid death of a few cells at pathogen infection sites. How the cell suicide programme is initiated and delimited remains unclear. Our earlier data (Xiao *et al.*, 2003b) and results presented here show that *RPW8* involves a transcriptional amplification mechanism possibly via the SA-dependent feedback circuit, and it is probably the local amplification of *RPW8* that leads to the activation of HR and resistance upon powdery mildew attack. We argued that transcription of *RPW8* must be negatively regulated in order to restrict the extent of pathogen-induced cell death. Given that *edr1* confers SA- and *EDS1*-dependent enhanced resistance to powdery mildew (therefore placing *EDR1* upstream of *EDS1* and SA), we tested whether *EDR1* acts as a negative regulator of *RPW8*-mediated HR and resistance. This was indeed found to be the case. An important implication from this analysis is that a MAP kinase cascade is associated directly or indirectly with *RPW8* signalling. However, we are unable to determine genetically whether *EDR1* acts downstream (position 1 and 2 in Figure 5) or upstream (position 3) of *RPW8*, as *RPW8* works through an amplification circuit, as described. We reasoned that *EDR1* may function to gauge signals from *RPW8* to the *EDS1*-dependent pathway, permitting cell death activation only at pathogen penetration sites where local amplification of *RPW8* reaches a threshold and overrides the negative effect

from *EDR1*. How *EDR1* exerts negative control over *RPW8* transcriptional amplification is not known. Notably, both *EDR1* (Frye *et al.*, 2001) and *RPW8* are induced to higher levels at 3 dpi by the same powdery mildew isolate *E. cichoracearum* UCSC1. We detected a temporary reduction of *RPW8* mRNA levels at 1 dpi when the fungus normally starts to penetrate the host epidermal cells by its appressorium, before an eventual upregulation appeared. This phenomenon may be attributed to an early negative control of *RPW8* transcription. It is tempting to speculate that *EDR1* may exert negative regulation of *RPW8* transcription at this stage. A more detail and earlier time-course analysis of the *RPW8* and *EDR1* (*edr1*) transcripts in S5 and S5-*edr1* after powdery mildew challenge can help to clarify this interplay.

The observation that SHL in soil-grown, S5-*edr1* plants is manifested as gradually progressing lesions indicates that there may exist additional negative control that prevents *RPW8*-mediated SHL from becoming runaway cell death. For example, *LSD1*, a negative regulator of cell death (Dietrich *et al.*, 1997), has been shown to function in an *EDS1-PAD4*-dependent manner (Rusterucci *et al.*, 2001) and thus may provide such additional negative control (x in Figure 5) of *RPW8*-mediated HR and SHL.

Our current model does not explain why *edr1* conditions enhanced powdery mildew resistance in Col-0 (Frye *et al.*, 2001) in which *RPW8.1* and *RPW8.2* are absent (Xiao *et al.*, 2001), nor does it explain why *EDS1*, *PAD4* and *EDS5* participate in an SA-dependent positive feedback circuit in the absence of the functional *RPW8* genes (in Ler or Col-0 background) (Falk *et al.*, 1999; Jirage *et al.*, 1999; Nawrath *et al.*, 2002). We have supporting evidence to suggest that RPW8 engages basal resistance but may not be an intrinsic component of it. It is therefore possible that RPW8 has some intrinsic defence activity in the absence of one basal resistance layer. Alternatively, in the absence of *RPW8*, the homologues of *RPW8*, *HR1*, *HR2* and *HR3* that are present in all tested Arabidopsis accessions (Xiao *et al.*, 2001, 2004) may play a positive role in the induction of basal resistance via the conserved SA-dependent pathway. Characterization of the role of *RPW8* homologues in plant defences will clarify this possibility.

Experimental procedures

Plant materials, genotyping and cultivation

The following Arabidopsis lines were used in this analysis: *edr1* (Frye and Innes, 1998), *NahG* (Lawton *et al.*, 1995), *eds1-2* (Parker *et al.*, 1996), *ndr1-1* (Century *et al.*, 1997), *pad4-1* and *pad4-2* (Jirage *et al.*, 1999), *eds5-1* (Rogers and Ausubel, 1997), *npr1-1* (Cao *et al.*, 1997), *rar1-10* (Muskett *et al.*, 2002), *sgt1b-1*(Austin *et al.*, 2002), *pbs3* (Warren *et al.*, 1999), *coi1-1* (Xie *et al.*, 1998), *ein2-1* (Guzman and Ecker, 1990), and *eds1-2*/*ndr1-1* double mutant (McDowell *et al.*, 2000). *eds1-2*, *pad4-2*, *rar1-10* and *sgt1b-1* are in Ler background, *eds1-2*/*ndr1* probably has a Col-0 and Ler mixed background, and the remaining lines are in Col-0 background. None of these lines contains the functional *RPW8.1* and *RPW8.2* genes.

Col-0 transgenic line S5 carrying a single copy of *RPW8.1* and *RPW8.2* in an approximately 14 kb genomic fragment from Ms-0 (Xiao *et al.*, 2003b) was used for the genetic crossings with the above lines, except in the case of *rar1-10*, as the transgene was inserted to a region of chromosome 5 very close to *RAR1*. We used two other *RPW8* transgenic lines (Col-0), T-B6-L and T-B75 for the genetic analysis of *rar1-10*. In all genetic analyses, the *RPW8* transgene was initially selected by spraying, shortly after germination, herbicide 'Challenge' containing 150 g l^{-1} glufosinate-ammonium (AgrEvo UK Limited, Saffron Walden, UK) at a concentration of 0.02% (v/v) (as the *RPW8* construct contains the *BAR* gene which confers herbicide resistance) and eventually confirmed by PCR amplification using gene-specific primers (Xiao *et al.*, 2001). The mutant alleles were identified by CAPS markers developed by relevant groups, which distinguish the wild-type alleles from the mutant alleles, except in the cases of *NahG*, *edr1*, *eds5-1* and *pbs3* and *ein2-1*. The presence of *NahG* gene was selected by PCR with *NahG*-specific primers. The presence of the *edr1* or *eds5-1* allele was first inferred from phenotypes of candidate F_3 families homozygous for the *RPW8* transgene, and then confirmed by sequencing across the point mutation in *EDR1* (Frye *et al.*, 2001) and in *EDS5* (Nawrath *et al.*, 2002). The presence of the *pbs3* and *ein2-1* allele was inferred from genetic analyses described below. Over 100 F_2 individuals carrying the *RPW8* transgene (selected by Basta herbicide resistance) were resistant or moderately resistant to powdery mildew, among which a quarter should be *pbs3* or *ein2-1*, as both *PBS3* and *EIN2* are located on top of chromosome 5 (Alonso *et al.*, 1999; Warren *et al.*, 1999), >50 cM away from the *RPW8* transgene located near *RAR1*.

Unless otherwise indicated, seeds were sown in autoclaved soil and kept under short day (8 h light, 16 h dark) for 2 weeks before transplanted to fresh soil and shifted to long day (16 h light, 8 h dark) for various treatments.

Pathogen isolates, plant inoculation and disease phenotyping

Four powdery mildew isolates belonging to distinct *Erysiphe* species were used. They *are E. cruciferarum* UEA1 (Xiao *et al.*, 1997), *E. cichoracearum* UCSC1 (Adam and Somerville, 1996), *E. orontii* MGH (Reuber *et al.*, 1998), and *Odium lycopersici* Oxford (Xiao *et al.*, 2001). Method of inoculation was the same as previously reported (Adam *et al.*, 1999; Xiao *et al.*, 1997). The criteria for scoring the DR phenotypes of the inoculated leaves at 10–12 dpi with *E. cichoracearum* UCSC1 and *E. orontii* MGH were as follows: 0, no or very limited sporulation with HR. The fungal mycelia or conidia were barely visible to the naked eye; 1, low level of sporulation with weaker or delayed HR. Some white powdery mildew could be seen on the tip or edge of the inoculated leaves; 2, moderate sporulation without HR; 10–30% of the leaf surface was covered by powdery mildew; 3, heavy sporulation without HR; 30–60% of the leaf surface was covered by powdery mildew; 4, very heavy sporulation without HR. >60% of the leaf surface was covered by powdery mildew. The disease phenotypes caused by *E. cruciferarum* UEA1 and *O. lycopersici* Oxford were generally weaker than those caused by the other two isolates. The DR scores for these two isolates were adjusted to the same 0–4 scale by setting the DR score of Col-0 to 3. Quantitative assay of the susceptibility of some mutant lines were carried out by a procedure briefed below. Five 6-week-old plants for each genotype were sparsely and evenly inoculated with dislodged *E. cichoracearum* UCSC1 conidia collected from infected squash (*Cucurbita maxima*) leaves with a fine brush. Two fully

expanded mature inoculated leaves from each plant were collected and cleared in a solution containing ethanol, phenol, acetic acid and glycerol (8:1:1:1, vol:vol) and were subsequently stained with TB (250 μg ml^{-1}) in a solution of lactic acid, glycerol and water (1:1:1, vol:vol) for visualization of fungal structure under a Nikon Optiphot-2 light microscope (Nikon UK Ltd, Kingston Upon Thames, UK). About 50 well-isolated fungal colonies from each genotype were randomly chosen for counting the number of conidiophores produced. The average number of conidiophores per colony was used to indicate the degree of susceptibility.

Isolation of P4-7 (npr1) and P8-1(pad4) mutants

Seeds of Col-0 line S5 carrying a single copy of *RPW8.1* and *RPW8.2* were mutagenized with 0.5% EMS. About 25 000 M1 plants were grown to maturity and seeds from approximately 500 M1 plants were pooled. Approximately 35 000 M2 seedlings were inoculated with *E. cichoracearum* UCSC1 and their disease phenotypes were examined at 10–12 dpi. Susceptible mutants were first grouped by complementation tests between themselves. Mutants *P8-1* and *P4-7* were crossed with Ler (as the female parent), and approximately 50 F_3 families from each cross homogeneously resistant to Basta (therefore homozygous for *RPW8*) and homogeneously resistant or susceptible to *E. cichoracearum* UCSC1 were generated for initial mapping. *P8-1* and *P4-7* mutants were then subject to complementation tests with *eds1-2* and *pad4-1*, and with *npr1-1* respectively. The mutations were identified by direct sequencing of the two putative genes.

DNA construct and generation of transgenic plants

A 6.2 kb genomic fragment from Ms-0 carrying *RPW8.1* and *RPW8.2* with their native promoters was cloned in SLJ755I5 (construct EE6.2 in Xiao *et al*., 2001). Col *NahG*, *eds1-2*, *pad4-1*, *eds5-1*, *npr1-1*, *rar1-10* and *eds1-2*/*ndr1-1* mutant plants were transformed with this construct by agroinfiltration, and T_1 transgenic plants were selected by spraying herbicide 'Challenge' at a concentration of 0.02% (v/v) for three times at a 2-day interval shortly after seed germination. T_1 plants were monitored for the formation of SHL by the naked eye for a period of 3–6 weeks after seed germination. For *RPW8.1::GUS* analysis in S5-*edr1* plants, the *RPW8.1::GUS* construct (Xiao *et al*., 2003b) was introduced into S5-*edr1* by *Agrobacterium*-mediated transformation. T_1 plants were selected on MS-agar medium containing 50 mg l^{-1} kanamycin (Melford Laboratories Ltd, Chelsworth, UK). Three week-old T_1 seedlings were then transplanted into soil to allow the development of SHL. GUS staining was carried out with leaves from 5 week-old T_1 plants as previously reported (Xie *et al*., 1998).

Other analyses

Methods for quantitative measurement of the mRNA levels of *RPW8.1*, *RPW8.2*, and *PR1*, for RNA gel blot analysis of *RPW8* and *PR1* and for the detection of H_2O_2 by DAB-TB staining, and for cell death by lactophenol-TB staining were the same as reported in Xiao *et al*. (2003b).

Acknowledgements

We thank Roger Innes for seeds of *edr1* and *pbs3*, Xinnian Dong for *npr1-1* and Col-0 transgenic for *35S::NahG*, Fred Ausubel for *eds5-1*, Jane Glazebrook for *pad4-1*, Nottingham Arabidopsis Stock Center for *ein2-1*. We thank Juan Alvaro for sequencing the *pad4* allele in the *P8-1* mutant, David Alden and Geoff Eagle for technical support. This work was supported by BBSRC grant P15697 given to J.G. Turner and the start-up fund from the Center for Biosystems Research, University of Maryland Biotechnology to S. Xiao.

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