Characterization of *RPW8*-Mediated Mildew Resistance in *Arabidopsis* and Tobacco

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Plant disease resistance (*R*) genes control the recognition of specific pathogens and activate subsequent defense responses. The *Arabidopsis* locus *RPW8* contains two naturally polymorphic, dominant *R* genes, *RPW8.1* and *RPW8.2*, which individually control resistance to a broad range of powdery mildew pathogens (Xiao et al. 2001). The predicted RPW8 proteins are small (~20 kDa) and basic (pI>9) and differ from previously characterised R-proteins. However, *RPW8.1* and *RPW8.2* (referred to hereafter as *RPW8.1/2*) induce salicylic-acid-dependent defense responses similar to those regulated by other types of *R*-gene. Here we report the signalling components required by *RPW8.1/2*; the regulation of *RPW8.1*expression; and we describe spontaneous cell death conferred by *RPW8.1/2*-overexpression. We also characterize *RPW8* homologues from *Brassica* species and report that the *Arabidopsis RPW8.1/2* confers resistance to a powdery mildew disease of *Nicotiana tabacum*.

Signalling Components Required by *RPW8.1/2*

Previously we showed that a mutation in *EDS1* (*eds1-2*), or SA-depletion by the *nahG* transgene, compromised *RPW8*-mediated resistance, but that a mutation in *NDR1* (*ndr1-1*) had no effect (Xiao et al. 2001). To extend our analysis of *RPW8*-mediated signalling, we introduced *RPW8* by genetic crossing into a number of other characterised mutant backgrounds. These mutants included *pad4-1, eds5-1, npr1-1 pbs3,* and *coi1-1.* We report that mutations in *EDS5* and*PAD4* completely, and in *NPR1* partially,

compromise *RPW8.1/2*-mediated resistance to the 4 *Erysiphe* species tested. However, mutations in *PBS3*, and *COI1* do not affect resistance.

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

RPW8 genotype	phenotype*	mutant background							
		nahG	*eds1-2*	*pad4-1*	*eds 5-1*	*npr1-1*	*ndr1-1*	*pbs3*	*coi1-1*
-/-	H_2O_2 2dpi	-	-	-	-	-	-	-	-
	disease, 8 dpi	ES	ES	ES	ES	S	S	S	S
RPW8/ RPW8	H_2O_2 2dpi	-	-	-	-	+/-	+	+	+
	disease, 8 dpi	S	S	S	S	I	R	R	R

* "ES", enhanced susceptibility; "S", Susceptible; "I", Intermediate or partially susceptible; "R", Resistant; "- ", No H_2O_2 accumulation detected by diaminobenzidine staining; "+", H_2O_2 accumulation detected; "+/-", little H_2O_2 accumulation detected.

These conclusions are based on observations that plants containing *RPW8.1/2* in *eds1-2*, *nahG* transgene, *pad4-1* and *eds5-1* backgrounds lacked a hypersensitive response (HR) which can normally be revealed at cellular level as a brown stain from the reaction of applied diaminobenzidine (DAB) with H_2O_2 formed at the sites of fungal penetration (Table 1), and they were susceptible to the powdery mildew pathogens. By contrast plants containing *RPW8.1/2* in *ndr1-1*, *pbs3*, and *coi1-1* had localised H_2O_2 accumulation in response to fungal penetration and they were resistant to the pathogens. Thus, we envisage that *RPW8.1/2* functions through a SA-dependent signaling pathway involving *EDS1, PAD4, EDS5* and *NPR1*, which is also used by the TIR-NB- LRR *R* genes *RPP5* and *RPS4* (Aarts et al. 1998). The partial resistance when *RPW8.1/2* is in the *npr1-1* background implies that *RPW8* may also recruit an *NPR1*-independent but SA-dependent pathway for cell death and resistance.

Regulation of *RPW8* Expression

Northern analysis showed that *RPW8.2* is expressed at a higher level in leaves inoculated with *E. cichoracearum* than in controls (Xiao et al. 2001). We analysed the transcriptional regulation of *RPW8* expression by fusing the *RPW8.1* and *RPW8.2* promoters to the *E. coli uidA* (*GUS*) gene. Interestingly, wounding induced GUS expression in transgenic Col-0 plants containing either *RPW8.1p::GUS* or *RPW8.2p::GUS*. GUS expression was

also induced when the transgenic seedling were treated with exogenous SA (100 μM for 2 days), but not JA (50 μM for 2 days). These results reveal that *RPW8.1/2* are transcriptionally regulated by wounding and add weight to the growing evidence for inter-connection or convergence of the wound-responsive and pathogen-responsive signal pathways (Romeis et al. 1999). Transcriptional regulation of *RPW8.1/2* is presumably downstream of pathogen perception and wounding. SA-induction of *RPW8*p::*GUS* expression indicates not only that *RPW8* signals through SA accumulation (Xiao et al. 2001), but also that the signal is amplified by SA, possibly through a feedback loop.

Overexpression of *RPW8*

SPONTANEOUS CELL DEATH (SCD)

Approximately one third of Col-0 lines (32 of 97 independent *T1* lines tested) containing a 14 kb genomic fragment with both *RPW8*.1 and *RPW8*.2 controlled by their native promoters had necrotic lesions and reduced stature when germinated on soil. Southern analysis showed a correlation between the number of insertions of the *RPW8* transgene and SCD. *T5* progenies of two lines, S5 carrying a single insertion, and S24 harbouring multiple copies of the insert, were used for further characterisation. S5 plants grown in soil normally did not develop necrotic lesions unless inoculated with the *Erysiphe* isolates, whereas S24 uninoculated plants growing in soil developed extensive SCD in the leaves and failed to survive to maturity. Northern analysis showed *RPW8.1*and *RPW8.2* transcripts were respectively ~12- and ~8- fold greater in S24 than in S5. A detailed comparison of these two lines, cultivated under different growth conditions showed that *RPW8*-mediated SCD was spontaneous, and was attenuated or enhanced by external conditions. The results are summarized in Table 2. The conditions under which S24 plants develop SCD are close to the natural environment of Arabidopsis, whereas the conditions under which the SCD is suppressed or attenuated, are atypical for normal growth of *Arabidopsis*. The mechanisms by which the SCD of S24 is permitted or suppressed are not known.

ENHANCED RESISTANCE TO PERONOSPORA AND CAMV

To determine if S24 has enhanced resistance to pathogens other than powdery mildews, we inoculated S5, S24 and Col-0 plants with *Peronospora parasitica* NOCO2, cauliflower mosaic virus (CaMV) and *Pseudomonas syringae* pv *maculicola* ES4326, all of which are virulent on Col-0. S24 plants grown under SCD-non-permissive conditions were

Table 2. The effect of environmental conditions on spontaneous cell death of S24 line.

Growth Conditions [1]		SCD of S24 plants
medium	MS agar	suppressed
	soil or perlite	permitted
light	normal intensity or higher	permitted or promoted
	dark or low intensity [2]	suppressed or delayed
temperature	22 °C	permitted
	30 °C	suppressed
humidity	85% or lower	permitted
	96% or higher	attenuated

[1] S24 seeds were sown on MS agar plates to germinate where seedlings were grown for 2 weeks before transfer to fresh MS agar plates and grow for another 2 weeks. The seedlings were then transferred to different conditions. The basic conditions were short day (8 hour day and 16 hour dark), 22 °C, with a light intensity of ~85 $\mu MOLm^{-2}s^{-1}$ in autoclaved perlite wetted with MS liquid media without sucrose. Plants were grown in Magenta Vessels (Sigma) under aseptic condition all times.
[2] Low light intensity: 1/6 of normal light intensity (i.e. ~14 $\mu Mm^{-2}s^{-1}$).

susceptible to all pathogens. However, S24 plants grown under SCD-permissive conditions developed necrotic lesions at the site of infection and had enhanced resistance to the *Peronospora.* Under SCD permissive conditions, S24 also exhibited enhanced resistance to CaMV. We did not, however, observe enhanced resistance of S24 to *P. syringae* ES4326 (under SCD permissive conditions, in three independent experiments in which S24, S5 and Col-0 leaves were infiltrated with a bacterial suspension of OD_{600}=0.0002, S24 exhibited the same typical disease symptoms as S5 and Col-0).

Homologues in *Brassica*

We identified three tandemly clustered *RPW8* homologues from both *Brassica oleracea*, and *B. rapa* by screening two *Brassica* BAC libraries using *RPW8* genomic DNA as probe. These *Brassica* homologues (named *BrHR1/BoHR1, BrHR2/BoHR2*, and *BrHR3/BoHR3*) are highly homologous to each other (>88% identity at nucleotide level when the predicted whole genes are compared) and show considerable homology (56-72% identity) to *RPW8.1, RPW8.2, HR1-3 and Col-HR4*. Phylogenetic analysis indicates that the *Arabidopsis RPW8* homologues are more divergent than the *Brassica* homologues. Initial mapping with *BrHR*

homologues placed this *RPW8*-like locus in a 2 cM interval flanked by marker pW207E3N and pW104E2, on chromosome 8 of *B. rapa*. We are testing whether these *Brassica* homologues also function as *R* genes.

Transfer of *RPW8* to Tobacco

We transferred a 7.5 kb genomic fragment containing *RPW8.1* and *RPW8.2* under control of their native promoters into *Nicotiana tabacum* through stable transformation. The transgenic *T1* lines were tested with *Erysiphe orontii* MGH. Wild type *N. tabacum* plants were fully susceptible to this isolate (disease rating 2-3 or 3). In contrast, all the 4 *T1* line tested were resistant (no visible fungus; disease rating 0). Microscopic examination after $DiOC_6$ staining and DAB /trypan blue staining showed that the *RPW8* transgene triggered a hypersensitive response manifested by rapid H_2O_2 accumulation at fungal penetration sites, and localised cell death. A test of the *T2* progenies derived from the 4 *T1* lines further confirmed that *RPW8* confers resistance in in tobacco. Transient expression of *RPW8.1* and *RPW8.2* in *N. benthamiana* by agroinfiltration caused cell death. We believe this represents the first example of an *R* gene that confers specific resistance in a host from a different plant family. Evidently, the *RPW8* signalling pathway(s) for disease resistance is conserved in *Arabidopsis* and tobacco.

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