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Intraspecific Genetic Variations, Fitness Cost and Benefit of *RPW8*, A Disease Resistance Locus in *Arabidopsis thaliana*

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ABSTRACT

The *RPW8* locus of *Arabidopsis thaliana* confers broad-spectrum resistance to powdery mildew pathogens. In many *A. thaliana* accessions, this locus contains two homologous genes, *RPW8.1* and *RPW8.2*. In some susceptible accessions, however, these two genes are replaced by *HR4*, a homolog of *RPW8.1*. Here, we show that *RPW8.2* from *A. lyrata* conferred powdery mildew resistance in *A. thaliana*, suggesting that *RPW8.2* might have gained the resistance function before the speciation of *A. thaliana* and *A. lyrata*. To investigate how *RPW8* has been maintained in *A. thaliana*, we examined the nucleotide sequence polymorphisms in *RPW8* from 51 *A. thaliana* accessions, related disease reaction phenotypes to the evolutionary history of *RPW8.1* and *RPW8.2*, and identified mutations that confer phenotypic variations. The average nucleotide diversities were high at *RPW8.1* and *RPW8.2*, showing no sign of selective sweep. Moreover, we found that expression of *RPW8* incurs fitness benefits and costs on *A. thaliana* in the presence and absence of the pathogens, respectively. Our results suggest that polymorphisms at the *RPW8* locus in *A. thaliana* may have been maintained by complex selective forces, including those from the fitness benefits and costs both associated with *RPW8*.

DURING the long-time conflict between plants and potential pathogens, plants have evolved disease resistance (*R*) genes to detect the invasion of infectious pathogens and trigger effective defenses (Chisholm *et al.* 2006). In the past 15 years, >60 plant *R* genes have been isolated (Xiao 2006), of which the majority encode nucleotide-binding site (NBS) and leucine-rich-repeat (LRR) domains. The *NBS-LRR* genes constitute the largest *R* gene class and are abundant and ubiquitously expressed in all higher plants (Dangl and Jones 2001; McHale *et al.* 2006). A less frequent class of *R* genes comprises members of extracellular (e) LRR-containing receptor-like proteins (eLRR-RLPs) (Jones *et al.* 1994; Hammond-Kosack and Jones 1997) and receptor-like kinases (eLRR-RLKs) (Song *et al.* 1995; Sun *et al.* 2004). These two classes of LRR-containing R proteins are thought to be intracellular or cell-surface receptors that detect pathogen-derived virulence proteins (referred to as Avr effectors if recognized by R proteins) through direct or indirect interaction (Dangl and Jones 2001). The remaining characterized *R* genes encode proteins that either resemble the overall structure or a domain of the above two classes with some degree of structural variations, or have a novel protein structure that does not show significant homology to any other R proteins (Xiao 2006). Therefore, in terms of protein structures, they are atypical *R* genes in contrast to the typical LRR-encoding *R* genes.

The evolution and maintenance of plant *R* genes has become a research focus in recent years. Different mechanisms for sequence evolution have been documented for *R* genes (Michelmore and Meyers 1998; Meyers *et al.* 2005). However, the type and strength of selection acting on specific *R* genes is not well characterized. In conjunction with the recent advances in understanding of the molecular mechanisms of R-Avr interaction, several recent evolutionary analyses suggest that the mode of R-Avr recognition may profoundly influence the patterns of *R-Avr* coevolution (Dangl and McDowell 2006; Dodds *et al.* 2006).

While the simplest "arms-race" model used for describing the coevolution between plants and pathogens predicts directional selection or selective sweeps, a recent genomewide survey of *R* gene polymorphisms in Arabidopsis did not detect convincing evidence for a recent selective sweep for any of the *R* genes analyzed (Bakker *et al.* 2006). For some *R* genes in Arabidopsis, balancing selection appears to play a central role in molecular evolution (Stahl *et al.* 1999; Tian *et al.* 2002;

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Shen *et al.* 2006). For example, Arabidopsis *RPM1* and *RPS5*, whose protein products detect their Avr proteins indirectly by association with the host target proteins of the Avr effectors (Mackey *et al.* 2002; Shao *et al.* 2003), are subject to balancing selection on resistance and susceptible alleles. At these loci, there are simple presence/absence polymorphisms (for the entire R-protein coding regions) that respectively correspond to the resistant and susceptible phenotypes. The *R-Avr* recognition in both cases appears to be of ancient origin and has been maintained for millions of years by balancing selection, presumably in a frequency-dependent fashion (Stahl *et al.* 1999; Tian *et al.* 2002). Relatively low genetic diversity with simple resistance/susceptibility allelism has been found at the Arabidopsis *RPS2* locus, which was also interpreted as being consistent with balancing selection (Caicedo *et al.* 1999; Mauricio *et al.* 2003). Similar to RPM1 and RPS5, RPS2 recognizes its cognate Avr (avrRpt2) through indirect interaction (Axtell and Staskawicz 2003; Mackey *et al.* 2003).

On the other hand, some R proteins may recognize cognate Avr proteins by direct physical interaction (Jia *et al.* 2000; Deslandes *et al.* 2003; Dodds *et al.* 2006). These *R* genes seem to have been under diversifying selection for amino acid differentiation to generate new R proteins, which could recognize modified Avr effectors. This *R-Avr* coevolution would result in high genetic diversity at the *R* and the corresponding *Avr* loci (Dangl and McDowell 2006; Dodds *et al.* 2006). Compelling evidence for diversifying selection comes from recent studies by Ellis *et al.* (1999) on the flax *L* locus and the cognate *AvrL567* locus in the flax rust pathogen, *Melampsora lini*. The *L* locus encodes at least 11 *R* alleles (including *L5*, *L6*, and *L7*) capable of recognizing distinct *Avr* genes belonging to different loci, including *AvrL567*, in the pathogen (Ellis *et al.* 1999). The *AvrL567* locus also contains multiple *Avr* genes that are recognized by the *R* alleles at the *L* locus (Dodds *et al.* 2004). More significantly, they demonstrated that the R proteins L5, L6, and L7 physically interact with the corresponding Avr proteins in the yeast two-hybrid system in a specific manner that matches the specificity of the genetic interaction observed (Flor 1956; Dodds *et al.* 2006). These results strongly suggest diversifying selection at the *R* and the corresponding *Avr* loci for high levels of amino acid sequence polymorphism. Such high amino acid sequence diversity has also been observed at the Arabidopsis *R* genes *RPP13* and *RPP1* (Botella *et al.* 1998; Rose *et al.* 2004) and at the corresponding *Avr* genes *ATR13* and *ATR1* in *Hyaloperonospora parasitica* (Allen *et al.* 2004; Rehmany *et al.* 2005), implying that these two R-Avr pairs may be engaged in direct interaction.

The *Arabidopsis thaliana RPW8* locus from accession Ms-0 confers broad-spectrum resistance to powdery mildew (Xiao *et al.* 2001). This locus contains two homologous genes, *RPW8.1* and *RPW8.2*, both of which contribute to resistance. All tested Arabidopsis accessions contain three homologs of *RPW8*, *i.e.*, *HR1*, *HR2*, and *HR3*, that are closely linked to the *RPW8* locus (Xiao *et al.* 2001, 2004). Based on the presence/absence of *RPW8.1* and *RPW8.2*, there are two basic Arabidopsis haplotypes at the *RPW8* locus: one contains both *RPW8.1* and *RPW8.2* and the other contains *HR4* in replacing *RPW8.1* and *RPW8.2* (Xiao *et al.* 2001, 2004). *HR4* shares the most recent common ancestor with *RPW8.1*, and they might be orthologous (Xiao *et al.* 2004). *RPW8.1* and *RPW8.2* (hereafter referred to as *RPW8* unless otherwise indicated) are unique because they confer broad-spectrum resistance to polyphagous Erysiphe pathogens that cause powdery mildew disease on many different plant species and they encode novel proteins showing no significant homology to other proteins (Xiao *et al.* 2001). How powdery mildew pathogens cause disease and how *RPW8* detects the pathogens and induces resistance in *A. thaliana* are not clear. However, *RPW8* appears to activate defense through a conserved signaling pathway that is also utilized by a subset of *NBS-LRR R* genes (Xiao *et al.* 2003, 2005). Our previous evolutionary analysis indicated that the origin of the *RPW8* locus is relatively young, probably after the separation of Arabidopsis from the Brassica lineages and that *RPW8.1* and *RPW8.2* evolved from an *HR3*-like progenitor gene by duplication and functional diversification (Xiao *et al.* 2004). However, it is not known how divergent *RPW8* alleles have evolved and been maintained in the *A. thaliana* populations. In the present study, we analyze the intraspecific sequence polymorphism at *RPW8.1* and *RPW8.2* to examine the evolutionary mechanism of *RPW8* in *A. thaliana*. We relate the disease reaction phenotypes to the evolutionary history of the *RPW8.1* and *RPW8.2* alleles and identify allelic mutations that likely contribute to phenotypic variations. More significantly, we provide evidence that gene expression of *RPW8* is associated with both fitness benefits and costs and that activation of defense-related cell death in the absence of the pathogen may account for the fitness cost of *RPW8* expression.

MATERIALS AND METHODS

Plant materials: Fifty-one accessions of *A. thaliana* from different geographical locations (Figure 1) were selected for sequence determination for *RPW8.1* and *RPW8.2*, or *HR4*, if present, and *HR3* (for 26 accessions). Nucleotide sequences of *RPW8.1* and *RPW8.2* or *HR4* in 32 of the 51 accessions were previously determined in Xiao *et al.* (2004), but have not been analyzed at the nucleotide level. Seeds of *A. thaliana* accessions were obtained from the Arabidopsis Biological Resource Center or the Nottingham Arabidopsis Stock Centre. The six *A. lyrata* accessions were provided by Charles Langley, University of California, Davis.

Assessment of disease phenotypes: Most of these accessions were analyzed for their disease reaction phenotypes in response to powdery mildew isolates *Erysiphe cruciferarum* UEA1 and *E. cichoracearum* UCSC1 (Adam *et al.* 1999; Xiao *et al.*

2004). The newly obtained accessions were tested with *E. cichoracearum* UCSC1 three times using the method previously described (Xiao *et al.* 2003, 2005). Among the 51 accessions surveyed, there was a range of disease reaction (DR) phenotypes from very resistant to very susceptible. We used three categories to simplify the data analysis: resistant (R) (no visible fungus, HR, DR score 0–1), intermediate (I) (some fungus with <30% leaf coverage, with or without a slower HR, DR score 1 or 1–2), and susceptible (S) (profuse fungus with >30% leaf coverage, no HR, DR score 2 or 2–3 or higher).

DNA sequence determination and analysis: Gene-specific primers (sequences available upon request) were used for PCR amplification of the target genes. PCR products were purified and sequenced from both strands. DNA sequences were aligned using AlignX function of Vector NTI Suite (Invitrogen) and corrected manually. Amino acid sequences were deduced from the nucleotide sequences by Vector NTI and aligned by AlignX. DnaSP version 4.0 was used for calculation of nucleotide polymorphism and divergence (Jukes-Cantor corrected) (Rozas and Rozas 1999). The molecular evolutionary genetic analysis program version 3.1 (Kumar *et al.* 2004) was used to generate phylogenetic trees based on nucleotide sequences. Trees generated by neighbor-joining (using Jukes-Cantor distance or *p* distance), maximum parsimony, or minimum evolution (using Jukes-Cantor distance or *p* distance) were very similar, and the trees constructed by neighbor-joining were presented.

Estimation of divergence time: To estimate the divergence time for the resistant and divergent/susceptible *RPW8.1* and *RPW8.2* alleles, we inferred the synonymous mutation rate for *RPW8.2* to be 2.12×10^{-8} per synonymous substitution per site per year, based on the divergence time (T) of 5.3 million years ago (MYA) for the separation of *A. lyrata* and *A. thaliana* (Koch and Kiefer 2005) and the synonymous substitution (ds) of 0.2247 between *AlRPW8.2* and *AtRPW8.2*/Ms-0 (Xiao *et al.* 2004). We then applied this mutation rate to estimate the divergence time for both *RPW8.1* and *RPW8.2* using the formula ds/2T = synonymous mutation rate.

Neutrality tests: Neutrality tests were performed using DnaSP version 4.0 (Rozas and Rozas 1999). *P*-values to obtain the observed Tajima's *D* and Fu and Li's *D* and *F* (Tajima 1989; Fu and Li 1993) were calculated based on 10,000 replicates of coalescent simulations assuming no recombination. Observed test statistics were further tested using empirical distribution in *A. thaliana* populations (Nordborg *et al.* 2005). In McDonald and Kreitman's test (McDonald and Kreitman 1991), Col-0 *HR4*, *AlRPW8.2*, and *AlHR3* were used as outgroups for *RPW8.1*, *RPW8.2*, and *HR3*, respectively. HKA test (Hudson *et al.* 1987) was performed on 23 accessions from which both *RPW8.2* and *HR3* were sequenced, using sequences from *A. lyrata* as an outgroup. We were unable to do the HKA test for *RPW8.1* because it is absent from *A. lyrata*.

Transgene analysis: The genomic DNA fragments containing the coding sequence of *AtRPW8.1* plus 1000 bp upstream of the ATG start codon from Ms-0, Sy-0, Ler, or Ws-0, and *AtRPW8.2* plus 1000 bp upstream of the ATG start codon from Ms-0, Can-0, Ler, or Ws-0, and *AlRPW8.2* (99m9) were amplified with Pfu-turbo with gene-specific primers and cloned into the binary vector pSMB (Mylne and Botella 1998) under control of the *35S* promoter. A genomic fragment containing the *AlRPW8.2* coding sequence plus 753 bp upstream of the ATG start codon (which is the whole intergenic region between *AlHR3* and *AlRPW8.2*) from Al99m9 was amplified and cloned into binary vector pBIN19-plus. All these constructs were introduced to Col-gl (Col-0 harboring the glabrous mutation 1). Homozygous transgenic lines were generated and tested for their DR phenotypes in response to *E. cichoracearum* UCSC1.

Tests of fitness costs: More than 20 Col-gl lines transgenic for a 6.2-kb genomic fragment from Ms-0 containing both *AtRPW8.1* and *AtRPW8.2* under control of their native promoters were generated, of which 7 contained a single copy of the transgene. Six of the 7 lines showed no defects in growth and development and no sign of spontaneous HR-like cell death (at least not visible to the naked eye) under normal growth conditions, but had powdery mildew-induced HR and resistance similar to that in Ms-0. The relative mRNA levels of *AtRPW8.1* from 3 homozygous lines (*i.e.*, S5, T5, and T7) were measured in comparison with Ms-0 by real-time quantitative RT-PCR using the procedures previously described (Xiao *et al.* 2003). The locations of the T-DNA transgenes in these 3 lines were determined by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (Sessions *et al.* 2002). The experiments were carried out in two different environments: a growth room and a greenhouse. For the test in a growth room in 2003, only line S5 was used to compare with Col-gl for vegetative growth (dry mass of the rosette leaves of the entire plant) under three conditions: no infection, early (heavy) infection, and late (light) infection by powdery mildew *E. cichoracearum* UCSC1, and for measuring seed yield in the absence of the pathogen. Plants were cultivated in an autoclaved soil mixture consisting of 2 vol of John Innes compost 3 (Gem Gardening, Lancashire, UK), 2 vol horticultural grit (Gem Gardening), 2 vol peat (Shamrock, Newbridge, Ireland), and 1 vol vermiculate (Vermiperl, Lincolin, UK). The growth conditions were 22°, 65–75% relative humidity (RH), and ~125 μmol · m^{-2} · sec^{-1} light (fluorescent lamps) intensity. Unless otherwise indicated, 2-week-old, short-day grown seedlings were transplanted into round pots (5 cm in diameter) and first kept in short day (8 hr light, 16 hr dark) for 2 weeks and then shifted to long day (16 hr light, 8 hr dark) until sample collection or seed maturation. Plants were irrigated regularly and supplied with fertilizer (1/2 teaspoon of Miracle-Gro in 1 liter of water for 32 pots) twice (at 4 and 6 weeks old) during the entire growth period. These experiments were repeated twice with similar results.

For the experiments in a greenhouse in 2005, three transgenic lines, S5, T5, T7, and Col-gl were used for measuring seed yield in the absence of any powdery mildew pathogens. To assess the effect of the same *AtRPW8* transgene in a different genetic background, the *AtRPW8* transgene from S5 was introduced to Ler background by backcrossing for five generations. This line (denoted as S5/Ler) was then used for comparison with Ler wild type for seed yield. Seeds were sown in Sunshine Mix 1 soil (Maryland Plant & Suppliers, Baltimore) and cold treated (4° for 2 days) before moving out to 22°, 75% RH, short day (8 hr light at ~125 μmol · m^{-2} · sec^{-1}, 16 hr dark). Two weeks later, seedlings were transplanted into 1/2-in. square pots filled with Sunshine Mix 1 soil and kept in the greenhouse under ~22°, 65–75% RH and natural light conditions. Individual plants from different genotypes were placed in the same trays as randomly as possible. Plants were irrigated once with GNATROL (Greenfire, Sacramento, CA) to control fungus gnats, and were supplied with Miracle-Gro once at the same concentration as used in the growth room. Seeds from individual plants were collected and weighed after maturation.

Other analyses: Trypan blue staining for cell death (Xiao *et al.* 2003), mRNA extraction, and RT-PCR (Xiao *et al.* 2004) were performed as previously described. TAIL-PCR (Sessions *et al.* 2002) was used to determine the location of the *AtRPW8* transgenes in S5, T5-3, and T7-10 lines.

RESULTS

***A. lyrata RPW8.2* confers powdery mildew resistance in *A. thaliana*:** The syntenic *RPW8* locus of *A. lyrata* contains

an orthologous gene (*AlRPW8.2*) of the *A. thaliana RPW8.2* (*AtRPW8.2*) (Xiao *et al.* 2004). *AlRPW8.2* and *AtRPW8.2* share 81.2% and 61% sequence identity at the nucleotide and the amino acid level, respectively. We asked whether *AlRPW8.2* is a functional powdery mildew *R* gene in *A. lyrata*. To address this question, we first challenged six *A. lyrata* accessions (99m6, 99m7, 99m8, 99m9, 99m11, and 99m23) with four powdery mildew isolates reported in Xiao *et al.* (2004). All of the six accessions were moderately to highly resistant to these pathogens (see supplemental Table 1 at http://www.genetics.org/supplemental/). We then sequenced the *AlRPW8.2* alleles from the six accessions and found they were nearly identical, with only eight silent substitutions (four in the exons and four in the singular intron). Because *A. thaliana* accession Col-0 lacks *AtRPW8.1* and *AtRPW8.2*, it is ideal for testing if *AlRPW8.2* is a functional *R* gene. We expressed *AlRPW8.2* in Col-0 by its native promoter. More than 60% (14 of 22) transgenic lines obtained were resistant to *E. cichoracearum* UCSC1 (Figure 2A), indicating that *AlRPW8.2* is indeed a functional *R* gene. We also overexpressed *AlRPW8.2* by the strong viral *35S* promoter in Col-0 and found that 3 of 20 transgenic lines developed severe spontaneous HR-like cell death (SHL) and plants of these 3 lines were tiny in size (Figure 2B). This is reminiscent of the results from overexpression of *AtRPW8.1* and *AtRPW8.2* by their native promoters (Xiao *et al.* 2003). Thus, *AlRPW8.2* is capable of inducing cell death and powdery mildew resistance in *A. thaliana*. These results suggest that *AlRPW8.2* can contribute to powdery mildew resistance in *A. lyrata* and that the resistance function of *RPW8.2* might have evolved before the speciation of *A. lyrata* and *A. thaliana*.

Intraspecific genetic variation at *RPW8*: Previously we determined the sequences of the *RPW8.1* and *RPW8.2* alleles from 32 *A. thaliana* accessions mostly collected from Europe and found that there were two basic haplotypes: Ms-0-like and Col-0-like based on the presence and absence of *RPW8.1* and *RPW8.2* (Xiao *et al.* 2004). To systematically investigate the levels of genetic variation at this locus, we further conducted nucleotide sequence analyses on *RPW8.1* and *RPW8.2* from 51 worldwide samples (the 32 accessions described in Xiao *et al.* 2004 plus 19 accessions from wider geographic areas, including the USA and Japan; Figure 1). Among the 51 *A. thaliana* accessions analyzed, 43 contain *RPW8.1* and *RPW8.2*, and the remaining 8 accessions lack *RPW8.1* and *RPW8.2* but contain *HR4*.

Genetic variation at RPW8.1: The 43 *RPW8.1* alleles have the same overall gene structure (two exons split by a single intron) and they all encode full-length proteins. The length of the complete alignment of the 43 alleles was 718 nucleotides, including 281 bp for the first exon, 208 bp for the intron, and 229 bp for the second exon. As shown in Table 1 and Figure 3, there were 31 nucleotide segregating sites, of which 9 were singletons. Among the segregating sites, the number of sites causing nonsynonymous substitutions was 15. The average nucleotide diversity ($\pi = 0.012$) and the number of segregating sites per base pair ($S = 0.047$) of *RPW8.1* were close to the average values derived from a set of 27 *NBS-LRR R* genes recently surveyed (Bakker *et al.* 2006), which were both significantly higher than the empirical distribution of polymorphism in 876 randomly distributed genomic regions (Nordborg *et al.* 2005). The 43 *RPW8.1* alleles distinguished 17 haplotypes and encoded 12 distinct proteins (Figure 3).

As shown in the phylogenetic tree based on the nucleotide polymorphism in the entire genes (Figure 4A), these alleles can be divided into two major clades. Clade I comprises 30 alleles that can be further divided into three groups (a–c). Group a contains 12 resistant alleles that encode proteins identical to RPW8.1 of Ms-0 from which the *RPW8* locus was isolated (Xiao *et al.* 2001). Group b contains 9 alleles that are similar to the Ms-0-(like) alleles, with one to three amino acid replacements. Among the nine accessions from which the 9 alleles are derived, one was resistant to powdery mildew, three had an intermediate phenotype, and five were susceptible (Table 2, Figure 4A). Group c contains 8 alleles carrying five unique nonsynonymous substitutions in the first exon, and all the accessions except Ob-0 were fully susceptible, suggesting that these are nonfunctional alleles and that resistance of Ob-0 may be conferred by *RPW8.2*/Ob-0 or other *R* loci. Clade II contains 13 alleles all characterized by a 63-bp intragenic duplication at the nucleotide position 546, which resulted in an insertion of 21 amino acids in the C-terminal tails of the proteins (Figure 3). Seven of the accessions were susceptible, and resistance of Uk-1 and Ts-7 can be attributed to the presence of the functional *RPW8.2* alleles (see later text). The associations between these clades and their DR phenotypes were highly significant ($\chi^2 = 26.6$, $P < 0.0001$), confirming the functional significance of *RPW8.1* to the powdery mildew resistance in *A. thaliana*. The minimum number of recombination events (Hudson and Kaplan 1985) among the 43 alleles detected was eight. The Knox-1 allele might have resulted from a recombination between an Ms-0-like allele (such as Na-1) in Clade Ib, and any allele in Clade II (Figure 3).

Genetic variation at RPW8.2: The level of genetic variation at *RPW8.2* was similar to that at *RPW8.1*. All the 43 *RPW8.2* alleles had a similar overall gene structure. The length of the complete alignment of the 43 alleles was 653 nucleotides, including 296 bp for the first exon, 128 bp for the intron and 229 bp for the second exon. As shown in Table 1 and Figure 5, there were 33 nucleotide polymorphic sites (excluding four indels), of which 9 were singletons. Among the 25 segregating sites in the coding region, 18 were nonsynonymous substitutions, whereas only 7 were synonymous substitutions. The average nucleotide diversity ($\pi = 0.010$) and the number of segregating sites per base pair

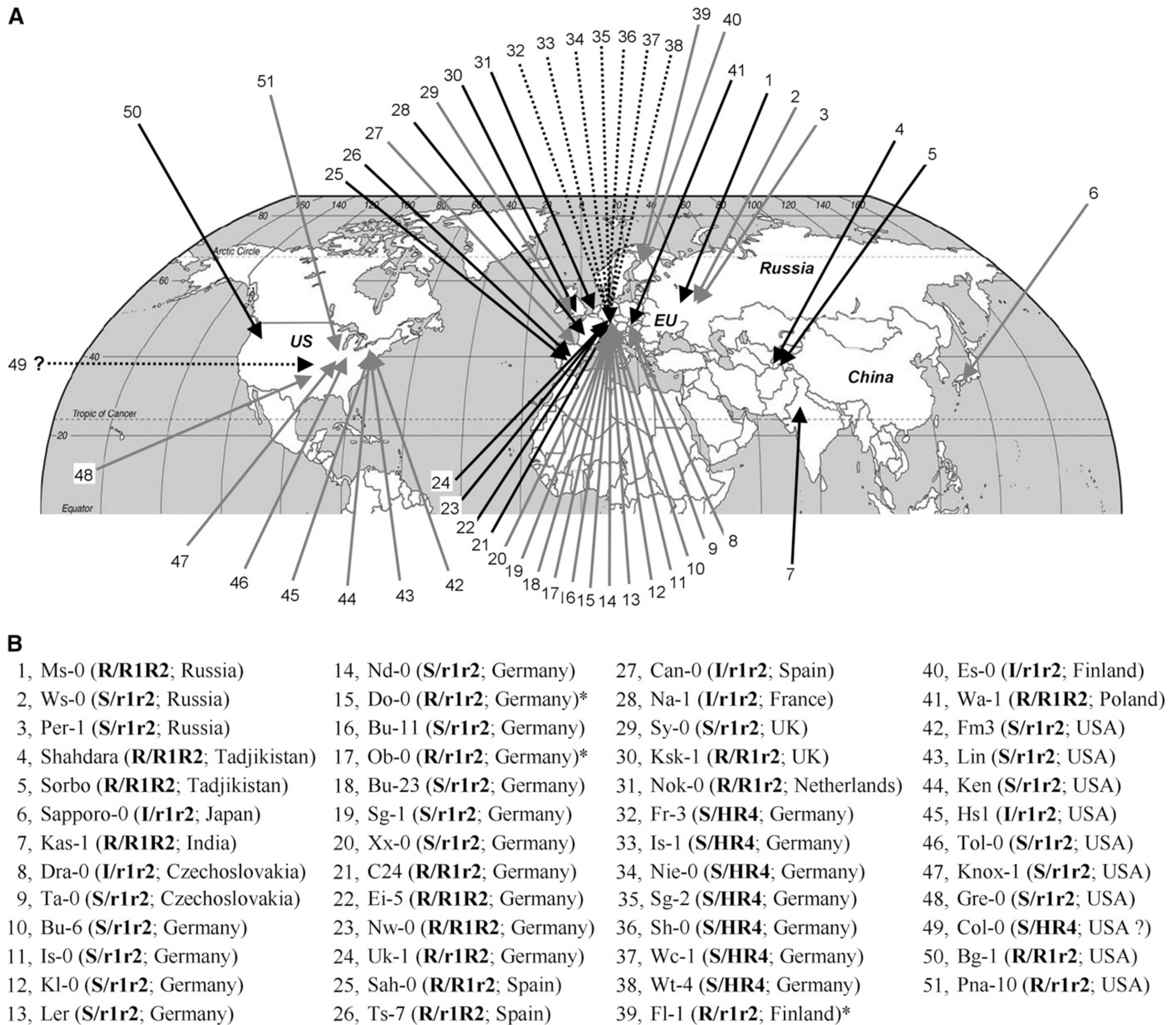


FIGURE 1.—Geographic distribution of the surveyed *A. thaliana* accessions. (A) The accessions containing either *RPW8.1* and/or *RPW8.2* encoding identical protein sequences as those of Ms-0 alleles are indicated by solid arrows; accessions containing *RPW8.1* and *RPW8.2* alleles encoding proteins different from those of Ms-0 are indicated by shaded arrows; accessions lacking *RPW8.1* and *RPW8.2* but having *HR4* are indicated by dashed arrows. Numbers in A match numbers in B. (B) Names, disease reaction phenotypes (R, resistant; I, intermediate; S, susceptible), genotypes (R1, *RPW8.1* identical to that of Ms-0 at the protein level; r1, *RPW8.1* divergent from that of Ms-0; R2, *RPW8.2* identical to that of Ms-0 at the protein level; r2, *RPW8.2* divergent from that of Ms-0), and locations of 51 accessions. Accessions with an asterisk may contain powdery mildew *R* genes different from *RPW8.1* and *RPW8.2*.

($S = 0.048$) of *RPW8.2* is also close to that of *RPW8.1*. The 43 *RPW8.2* alleles distinguish 26 haplotypes, which encode 21 different proteins. Except for the *RPW8.2*/Kl-0 allele, the remaining 42 alleles can be divided into two clades in the phylogenetic tree (Figure 4B), even though the division of the two clades is not as reliable as in the *RPW8.1* tree. Clade I comprises 9 alleles identical to *RPW8.2*/Ms-0, and all of the nine accessions carrying the Clade I allele were resistant. Clade II comprises 33 alleles that differ from *RPW8.2*/Ms-0 by 2 (*e.g.*, *RPW8.2*/Can-0) to 14 (*e.g.*, *RPW8.2*/Is-0) nonsynonymous substitutions (Figure 5). The majority (20 out of 34) of the accessions that carry the Clade II *RPW8.2* alleles were susceptible, indicating that these alleles are less functional or nonfunctional (Table 2). The associations between the clades in *RPW8.2* and the DR phenotypes were highly significant ($\chi^2 = 17.4$, $P = 0.0002$). The *RPW8.2*/Kl-0 allele differs from all other alleles by two small indels and 4 singleton nonsynonymous substitutions in the second exon. Twelve alleles (shaded in Figure 4B) including *RPW8.2*/Kl-0 contain a single base pair indel (at the nucleotide position 542 or 556) that

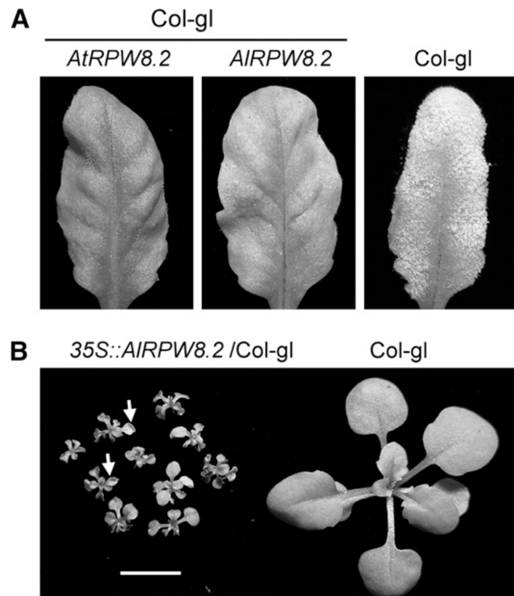


Figure 2.—*AlRPW8.2* confers powdery mildew resistance in Col-0. (A) A 2.3-kb genomic fragment containing the *AlRPW8.2* gene and its native promoter was introduced to Col-gl background and the transgenic plants were inoculated with *E. cichoracearum* USCS1. Disease phenotypes were scored and typical infected leaves were photographed at 10 days post-inoculation (dpi). (B) The *AlRPW8.2* gene was expressed under control of the 35S promoter in Col-gl background. Plants of ~10% (3 of 29) transgenic lines showed SHL and 4-week-old T3 plants of one line with the most severe SHL were shown together with the wild type. Arrows indicate dead or dying leaves. Bar, 1 cm.

resulted in frameshift and a truncation of 28–34 amino acids at the C-termini of the deduced proteins. The minimum number of recombination events (Hudson and Kaplan 1985) among the 43 alleles was eight.

Genetic variation at HR4 (At3g50480): Eight of the 51 *A. thaliana* accessions (15.7%, accessions Fr-3, Is-1, Nie-0, Sg-2, Sh-0, Wc-1, Wt-4, and Col-0) contain *HR4* in the place of *RPW8.1* and *RPW8.2*. They were generally more susceptible to the powdery mildew isolates (Xiao *et al.* 2004). Sequence analysis revealed that there were only two alleles among the 8 accessions, and the average genetic diversity was low ($\pi = 0.002$). The low level of genetic variation in *HR4* was expected *a priori*, because of the small fraction of allelic class in the populations (Innan and Tajima 1997). The expected nucleotide diversity at the *HR4* ($0.013 = 0.002 \times 51/8$), based on the allele frequency (Innan and Tajima 1997), was close to that at *RPW8.1* (0.015) and *RPW8.2* (0.012, Table 1), suggesting that there is no recent selective sweep among these loci. Nie-0 is identical to Col-0, while the remaining six alleles are identical to each other, differing from the Col-0 allele by two nonsynonymous substitutions ($G_{Col\text{-}0}$ 145A and T161A, resulting in E49K and V54E, respectively), one silent (C567T) substitution, and a deletion of 42 bp (GATACAAGTCGACCAATGGACC GATATCAAAGAAATGAAGGC), which is exactly the third copy of the five tandem duplicated segments in the 3′ end of *HR4*/Col-0 (Xiao *et al.* 2004). This suggests that an unequal intragenic recombination occurred between the Col-0-like alleles. Interestingly, unlike *RPW8.1*- and *RPW8.2*-containing accessions that are distributed throughout the surveyed geographical areas, all the *HR4*-containing accessions are from Germany except for Col-0 (annotated as from USA; however, see discussion) (Figure 1).

Genetic variation at HR3 (At3g50470): Based on PCR amplification, all of the 51 accessions surveyed contain *HR3* (data not shown). For comparisons, we sequenced the *HR3* alleles from 26 out of 51 accessions. The *HR3* allelic sequences showed a very low level of nucleotide diversity in the coding region ($\pi = 0.002$). Twenty two of the 26 alleles encode identical proteins, and the remaining 4 alleles encode proteins that differ from the others by only one or two amino acid replacements (Figure 4C and supplemental Figure 1 at http://www.genetics.org/supplemental/), indicating that *HR3* is highly conserved. This is consistent with purifying selection inferred from a low ratio (0.17) of nonsynonymous substitution rate (K_a) and synonymous substitution rate (K_s) between *AlHR3* and *AtHR3* (Xiao *et al.* 2004). There are two types of introns among the 26 alleles (type I and type II, see supplemental Figure 1). These introns shared only ~67% homology between each other and ~70% to the intron of *AlHR3*. The highly divergent nucleotide sequences between two types of intron suggest relaxed selective constraint in this region and an old common ancestor between alleles carrying different types of intron in *HR3*. There was no significant association between these haplotypes and their DR phenotypes ($\chi^2 = 4.8$, $P = 0.09$).

Correlation between sequences and phenotypes: Alleles of *RPW8.1* and *RPW8.2* in the resistant accessions are not scattered throughout the genealogies, but rather are grouped together (see above), suggesting a correlation between functionality of the two *RPW8* genes and powdery mildew resistance phenotype. However, assessing the functionality of the individual *RPW8* alleles is difficult because both *RPW8.1* and *RPW8.2* could function independently and probably additively to confer non-race-specific resistance to powdery mildew (Xiao *et al.* 2001; S. Xiao, unpublished data). Based on our classification (see materials and methods), 17 of 51 accessions surveyed were resistant to *E. cichoracearum* (*Ec*)-UCSC1, 6 were intermediate, and 28 were susceptible. As shown in Figures 3 and 5, 7 of the 17 resistant accessions (Ms-0, Shahdara, Sorbo, Kas-1, Ei-5, Nw-0, and Wa-1) contained nearly identical *RPW8.1* (only one silent substitution A450T in the intron in Ei-5, Nw-0, and Wa-1) and identical *RPW8.2* alleles. Thus, they are identical to Ms-0 in terms of the RPW8.1 and RPW8.2 protein sequences. Five accessions (*i.e.*, C24, Sah-0, Ksk-1, Nok-0, and Bg-1) contained *RPW8.1* alleles that differ from the Ms-0 alleles in four to six silent substitutions

TABLE 1

Nucleotide polymorphism and divergence at the *RPW8* locus of *Arabidopsis thaliana*

Gene (no. of alleles)	Parameter	Entire gene	Coding region	Synonymous	Nonsynonymous
RPW8.1 (43)	No. of sites[a]	644	444	103.29	340.71
	Segregating sites	31	24	9	15
	π	0.0124	0.0127	0.0141	0.0124
	E[π][b]	0.0147	0.0151	0.0167	0.0147
	Tajima's *D*	0.37	0.04		
RPW8.2 (43)	No. of sites[a]	652	519	114.64	404.36
	Segregating sites	33	32	7	25
	π	0.0099	0.0118	0.0125	0.0116
	E[π][b]	0.0118	0.0140	0.0148	0.0138
	Tajima's *D*	−0.63	−0.28		
HR4 (8)	No. of sites	848	555	116.42	438.58
	Segregating sites	4	3	0	2
	π	0.0020	0.0023	0.0000	0.0030
	E[π][b]	0.0129	0.0147	0.0000	0.0188
	Tajima's *D*	0.48	0.46		
HR3 (26)	No. of sites[a]	1004	639	136.13	502.87
	Segregating sites	92	9	4	5
	π	0.01 88	0.0021	0.0060	0.0010
	Tajima's *D*	−0.94	−1.41		

[a] Excluding all gaps; π, the average nucleotide diversity.
[b] Expected average nucleotide diversity was calculated on the basis of allele frequencies (Innan and Tajima 1997).

in the exon and the intron but encode identical proteins, while their *RPW8.2* alleles were divergent from the Ms-0 allele at both the nucleotide and amino acid levels. Two resistant accessions (Uk-1 and Ts-7) contained *RPW8.2* alleles identical to those of Ms-0, but their *RPW8.1* alleles were divergent. Therefore, the DR phenotypes of these 14 accessions could be at least in part attributable to the presence of the functional *RPW8.1* and/or *RPW8.2* alleles. The remaining 3 resistant accessions (Do-0, Ob-0, and Fl-1) contain divergent *RPW8.1* and *RPW8.2* alleles, suggesting that either these alleles may also be functional or resistance in these 3 accessions is controlled by genes that differ from the *RPW8* genes.

Also as shown in Figures 3 and 5, among the 6 accessions with an intermediate DR phenotype, 3 (Dra-0, Na-1, and Sapporo-0) contained identical *RPW8.1* and *RPW8.2* alleles that differ from those of Ms-0 alleles by only two amino acids, $F_{\text{Ms-0}}$77L (nucleotide/amino acid of Ms-0 is placed first here in all polymorphic sites) and S108R, for RPW8.1 and three amino acids, T64S, D116G, and T161K, for RPW8.2 at the protein level. Accession Can-0 contained an *RPW8.2* allele that differs from the Ms-0 allele by only two amino acids (T64S and D116G) and a more divergent *RPW8.1* allele. The sequence and phenotypic data together suggest that these slightly different *RPW8* alleles may be (partially) functional. Accessions Hs-1 and Es-0 contained divergent *RPW8.1* and *RPW8.2* alleles carrying four and five nonsynonymous substitutions that were also present in 7 and 10 susceptible accessions, respectively, implying that the *RPW8* locus may not be responsible for the intermediate phenotypes of the 2 accessions.

Among the 28 susceptible accessions, 5 (Bu-11, Bu-23, Sg-1, Sy-0, and Tol-0) contain *RPW8.1* alleles with only one (F77L) or two amino acid replacements (F45L and F77L), implying that either these two residues are critical to the resistance function, or possibly these (partially) functional alleles are not sufficiently expressed. Fifteen accessions either contained more divergent *RPW8.1* and *RPW8.2* alleles with three or more nonsynonymous substitutions and/or indels that resulted in an insertion of 21 amino acids in RPW8.1 or a truncation of ∼30 amino acids at the C-terminus of RPW8.2. The extreme susceptibility of the remaining 8 accessions was associated with the absence of *RPW8.1* and *RPW8.2* and the presence of *HR4* (Figure 1) (Xiao *et al.* 2004). Since *HR4* showed the highest homology to *RPW8.1* (72.5%; the homology between *HR4*-*RPW8.2* is only 50%) and shared two unique indels with *RPW8.1* that are not present in other members of the *RPW8* gene family (Figure 2 in Xiao *et al.* 2004), it is reasonable to consider *HR4* and *RPW8.1* to be orthologous, albeit not typical. Thus, there are two basic haplotypes at the *RPW8* locus, *RPW8.1*- (and *RPW8.2*-) containing and *HR4*-containing haplotypes, and the sequence diversity at the *RPW8* locus can account for a major (∼80%) proportion but not all of the phenotypic variations among the 51 *A. thaliana* accessions (Table 2).

Neutrality tests: Based on the nucleotide sequence polymorphisms at the *RPW8* locus, we applied several statistical tests to examine the selective neutrality (see

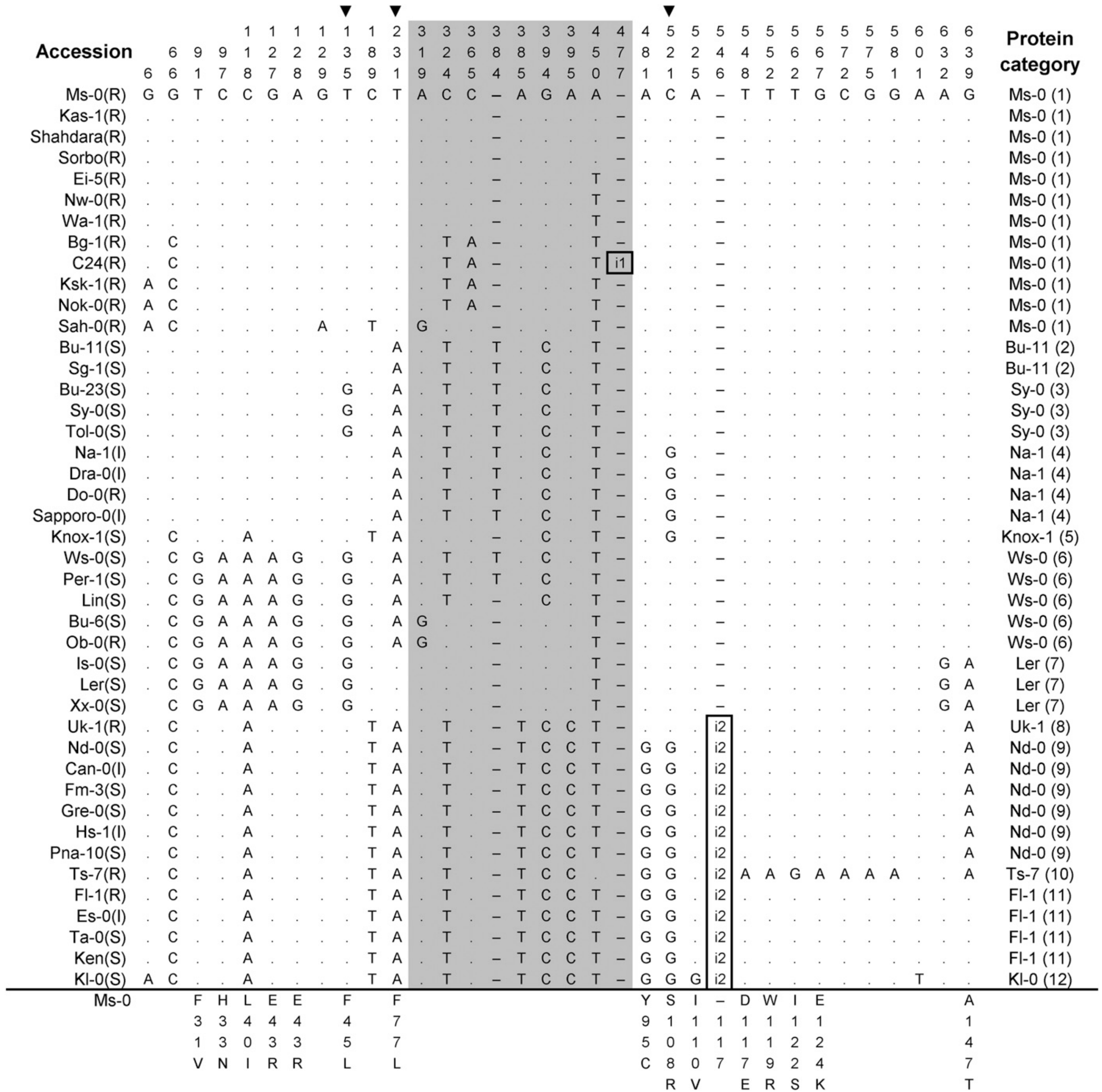


Figure 3.—Polymorphic sites of *RPW8.1* aligned against the Ms-0 *RPW8.1* allele (GenBank accession no. AF273059). A dot indicates an identical nucleotide and a dash indicates a gap. Shaded are the substitutions in the intron. The numbers at the top indicate the nucleotide position relative to the start codon of the Ms-0 alleles. Amino acid replacements caused by nucleotide substitutions are indicated at the bottom. The arrowhead at the top indicates the nonsynonymous substitution that may affect functionality of the proteins (site that showed statistically significant association with the DR phenotype at the 5% level after Bonferroni correction). The disease phenotypes (R, resistant; I, intermediate; S, susceptible) are indicated in the parentheses after the names of the accessions. Identical deduced proteins are represented by one allele only and the numbers in the parentheses indicate the group numbers. Boxed are two insertions: i1, an insertion of 10 bp (GTTTATCTTT); and i2, an insertion of 63 bp (GATCAATGGGACGATATCAAAGAAATCAAGGCCAAGATATCTGAAACGGACACTAAACTTGCT), which was an intragenic duplication (from nucleotide 546–608) and resulted in an insertion of 21 amino acids (DQWDDIKEIKAKISETDTKLA).

materials and methods for details). They include tests based on allele frequencies (Tajima 1989; Fu and Li 1993), polymorphism level differences between loci [HKA test; (Hudson *et al.* 1987)], the distribution of synonymous and nonsynonymous polymorphism, and divergence (McDonald and Kreitman 1991). Although none of the tests detected statistical significance for natural selection at the 5% level in *RPW8.1*, *RPW8.2*,

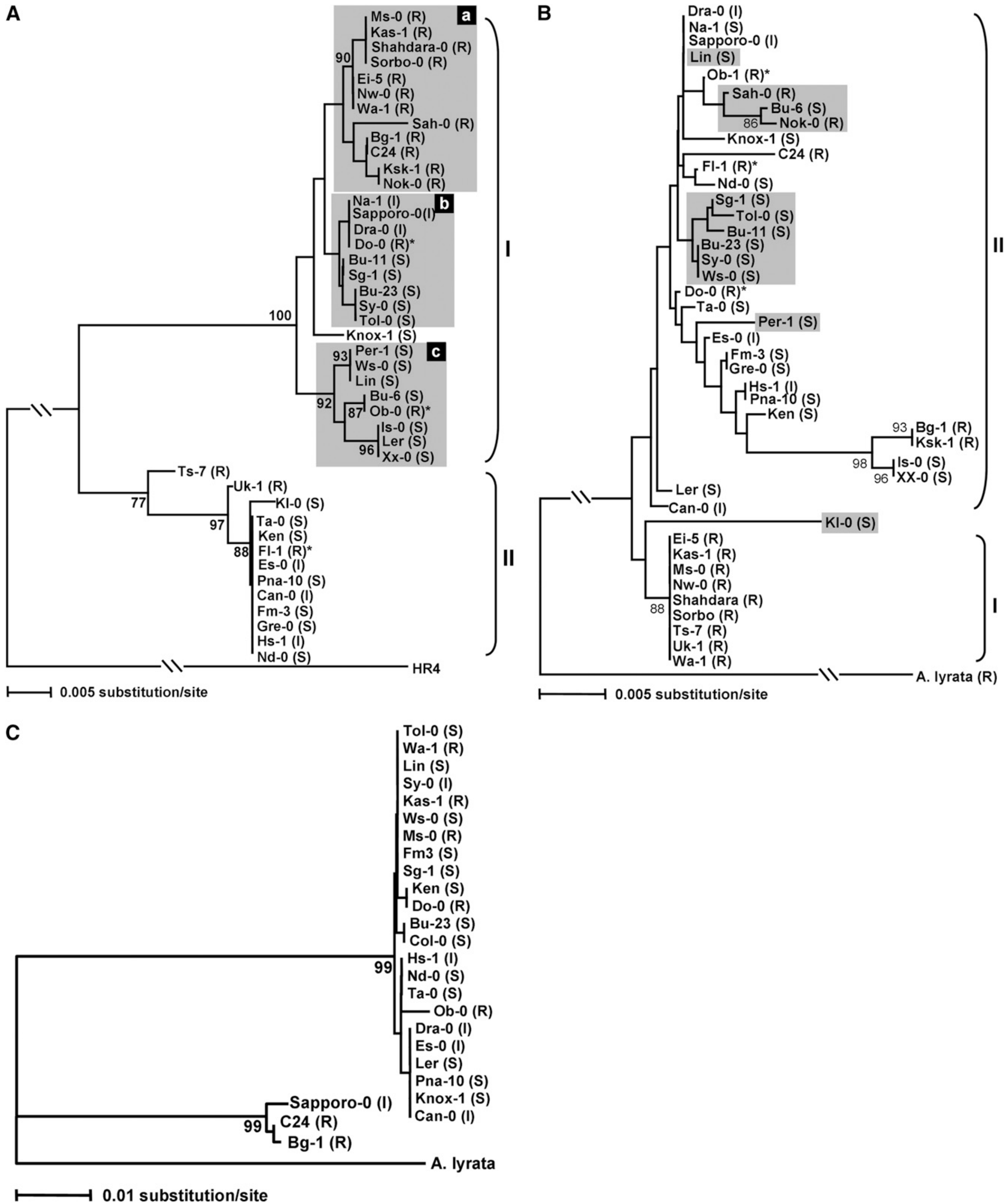


FIGURE 4.—Neighbor-joining tree of 43 *RPW8.1* alleles (A), *RPW8.2* alleles (B), and 26 *HR3* alleles (C) constructed on the basis of nucleotide sequences of the entire genes by MEGA3.1 using Jukes-Cantor or *p* distance. Bootstrap proportions of 500 bootstrap replicates >70 are indicated under the branches. Alleles with an asterisk may not be responsible for the resistance (R) phenotype. (A) The *RPW8.1* tree was rooted using *HR4*, which shows the highest similarity with *RPW8.1*. Shaded areas highlight the subgroups in Clade I. (B) The *RPW8.2* tree was rooted using *AlRPW8.2*. Shaded alleles contain insertions or deletions that resulted in frameshifts. (C) The *HR3* tree was rooted using *AlHR3*.

TABLE 2

Genotype-phenotype relationship concerning the *RPW8* locus and powdery mildew resistance

Phenotypic class	Accession no.	Genotype at *RPW8.1*				Genotype at *RPW8.2*	
		Clade-Ia	Clade-Ib	Clade-Ic[a]	Clade-II	Clade-I	Clade-II
Resistant	17	12	1	1	3	9	8
Intermediate	6	0	3	0	3	0	6
Susceptible	20	0	5	8	7	0	20

[a] *RPW8.1*/Knox-1 is not subgrouped (Figure 4A) but is added to Clade-Ic for simplicity.

and *HR3* (data not shown), Tajima's *D* value (Tajima 1989) was negative and the lowest in the coding sequences of *HR3* ($D = -1.41$, Table 1), agreeing with a previous study that suggested purifying selection at *HR3* (Xiao *et al.* 2004). In contrast, coding sequences of *RPW8.1* and *RPW8.2* showed no sign of purifying selection or selective sweep ($D \sim -0.28$–0.04, Table 1).

To further test the selective neutrality on the maintenance of *RPW8*, we applied the Tajima's and Fu and Li's tests to the nucleotide sequence alignment including both the 43 *RPW8.1* alleles and 8 *HR4* alleles, assuming that these two genes are orthologous (Xiao *et al.* 2004). The average genetic divergence [Jukes-Cantor corrected D_{XY}, (Nei 1987)] between *RPW8.1* and *HR4* alleles was 0.29. Although Tajima's *D* did not detect statistical significance ($D = 0.85$), Fu & Li's D^* and F^* were both positive ($D^* = 2.08$, $F^* = 1.93$) and showed statistical significance by both the coalescent simulation and the empirical distribution (D^*: $P < 0.001$; F^*: $P < 0.05$, see materials and methods). These results suggest that if *HR4* and *RPW8.1* have been maintained as orthologous alleles in *A. thaliana* populations, their genetic differences cannot be explained by the simplest neutral mutation model, and that balancing selection may be acting on these two haplotypes.

***RPW8.1*/Sy-0 and *RPW8.2*/Can-0 alleles confer enhanced resistance when overexpressed:** To obtain genetic evidence to support the idea that some alleles slightly divergent from the Ms-0 alleles at the protein level may be (partially) functional, we cloned three Clade I *RPW8.1* alleles [Sy-0 (Clade Ib), Ler (Clade Ic), and Ws-0 (Clade Ic)] and three Clade II *RPW8.2* alleles (Can-0, Ler, Ws-0 = Sy-0) by placing the genomic fragments containing the genomic sequence of the genes and 1000-bp native promoter (Np) sequence upstream of their start codons downstream of the *35S* promoter to enhance the expression of the transgenes. We found that ~25% (14/57) of the transgenic lines containing *35S::Np-RPW8.1*/Sy-0 and ~16% (5/32) of the transgenic lines containing *35S::Np-RPW8.2*/Can-0 exhibited enhanced resistance to *Ec*-UCSC1 with a DR score of 0–1 to 1–2 and a slower HR (Figure 6, B and C), slightly less resistant compared to plants expressing the corresponding Ms-0 alleles. These results indicate that *RPW8.1*/Sy-0 and *RPW8.2*/Can-0 are at least partially functional. However, none of transgenic lines (>30 for each construct) containing *35S::Np-RPW8.1*/Ler, *35S::Np-RPW8.1*/Ws-0, *35::Np-RPW8.2*/Ler, or *35S::Np-RPW8.2*/Ws-0 examined showed obviously enhanced resistance (Figure 6, B and C), suggesting that these alleles are not functional. It is worth noting that *RPW8.1*/Sy-0 differed from *RPW8.1*/Ms-0 by two amino acids (F45L and F77L), both of which showed statistically significant associations with the DR phenotype (Figure 3). *RPW8.2*/Can-0 differed from *RPW8.2*/Ms-0 by two amino acids (T64S and D116G), and these sites were also significantly associated with the DR phenotype (Figure 5). These results suggest that these four sites may be important for full function of RPW8.1 and RPW8.2 but additional, functionally relevant amino acid replacements are required for nullification of the functions of these two proteins.

Expression of *RPW8.1* and *RPW8.2* incurs fitness costs: In the above analyses, we detected a general association between the genotypes at *RPW8.1* and/or *RPW8.2* and the DR phenotypes of the accessions surveyed. This was based on the assumption that *RPW8.1* and *RPW8.2* are expressed in all the accessions. To provide evidence for this assumption, we examined the expression of *RPW8.1* and *RPW8.2* in 14 accessions along with Ms-0 and Col-gl transgenic for *RPW8* (line S5; see below) by RT-PCR and found that all had expression of both *RPW8.1* and *RPW8.2* (see supplemental Figure 2 at http://www.genetics.org/supplemental/).

The high levels of genetic and phenotypic variations in RPW8 invite an important question: why have so many divergent alleles been maintained in the *A. thaliana* populations? We hypothesized that expression of the *RPW8* functional genes renders fitness benefits to plants when infected by the pathogens; however, it may incur fitness costs. Hence, in the absence of the pathogens, natural selection may favor accumulation of deleterious mutations in or complete elimination of the two genes. We took a transgene approach to test this idea of fitness costs associated with *RPW8* expression. We generated >30 Col-gl lines transgenic for a 6.2-kb genomic fragment containing both *RPW8.1* and *RPW8.2* and their promoters from Ms-0 and selected three lines (S5, T7-10,

Accession	18	43	49	54	57	94	102	134	141	148	150	154	168	175	▼ 190	202	208	225	236	266	359	458	459	▼ 475	542	543	549	555	556	559	560	562	564	565	567	585	610	613	Protein category
A. lyrata (R)	T	C	G	T	G	A	C	G	T	C	G	C	C	G	C	G	G	C	A	T	A	A	T	A	–	C	C	A	A	T	C	–	C	–	C	G	C	A	A. lyrata
Ms-0(R)	C	C	G	C	C	A	C	G	A	G	G	C	C	G	A	G	A	C	A	T	A	A	T	A	–	.	C	A	A	T	A	–	C	–	C	C	C	A	Ms-0 (1)
Ei-5 (R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Kas-1(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Nw-0(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Shahdara(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Sorbo(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Ts-7(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Uk-1(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Wa-1(R)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ms-0 (1)
Can-0(I)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Can-0 (2)
Dra-0(I)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
Na-1(I)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
Sapporo-0(I)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
Ob-0(R)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	T	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
Fl-1(R)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
Nd-0(S)	G	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Dra-0 (3)
C24(R)	G	.	.	T	A	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	G	A	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	C24 (4)
Ler(S)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	.	.	Ler (5)
Do-0(R)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Do-0 (6)
Ta-0(S)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Do-0 (6)
Es-0(I)	.	.	.	.	A	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Es-0 (7)
Fm-3(S)	.	.	.	T	A	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Es-0 (7)
Gre-0(S)	.	.	.	T	A	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Es-0 (7)
Hs-1(I)	.	.	.	T	A	.	.	.	T	.	.	.	.	A	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Hs-1 (8)
Pna-10(S)	.	.	.	T	A	.	.	.	T	.	.	.	.	A	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Hs-1 (8)
Ken(S)	.	.	.	T	A	.	.	C	T	.	.	.	.	A	T	.	G	.	.	.	G	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Ken (9)
Knox-1(S)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	T	.	.	.	.	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Knox-1 (10)
Is-0(S)	.	.	.	T	A	.	.	C	T	A	T	A	G	A	T	T	G	.	T	A	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Is-0 (11)
Xx-0(S)	.	.	.	T	A	.	.	C	T	A	T	A	G	A	T	T	G		T	A	.	.	.	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Is-0 (11)
Bg-1(R)	.	.	.	T	A	.	.	C	T	A	T	A	G	A	T	T	G	.	T	.	.	G	A	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Bg-1 (12)
Ksk-1(R)	.	.	.	T	A	.	.	C	T	A	T	A	G	A	T	T	G	.	T	.	.	G	A	G	–	.	.	.	.	.	.	–	.	–	.	.	A	.	Bg-1 (12)
Bu-6(S)	.	.	.	.	.	.	T	.	T	.	.	.	.	.	T	.	.	T	.	.	.	.	.	G	G	.	.	.	.	.	.	–	.	–	.	T	A	C	Bu-6 (13)*
Sah-0(R)	.	.	.	.	.	.	T	.	T	.	.	.	.	.	T	.	.	T	.	.	.	.	.	G	G	.	.	.	.	.	.	–	.	–	.	.	A	.	Bu-6 (13)*
Nok-0(R)	.	.	.	.	.	.	T	.	T	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	–	.	–	.	T	A	C	Nok-0 (14)*
Kl-0(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	G	–	T	T	C	C	C	AC	A	AG	A	.	.	.	Kl-0 (15)*
Lin(S)	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Lin (16)*
Bu-11 (S)	.	.	T	.	A	.	.	.	T	.	.	.	.	.	T	.	G	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Bu-11(17)*
Sg-1(S)	.	.	T	.	A	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Sg-1(18)*
Tol-0(S)	.	T	T	.	A	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Tol-0 (19)*
Bu-23(S)	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Ws-0 (20)*
Sy-0(S)	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Ws-0 (20)*
Ws-0(S)	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Ws-0 (20)*
Per-1(S)	.	.	.	.	A	C	.	.	T	.	.	.	.	A	T	.	G	.	G	.	.	.	.	G	–	.	.	.	–	.	.	–	.	–	.	.	A	.	Per-1(21)*
A. lyrata		L	V		Q	T		S		L	L	Q	V	E	Q	V	E		K	L			L	D	S		L	K	K	I	I	M					Q		
Ms-0		L	V		H	T		S		V	V	Q	F	E	T	V	K		E	L			L	D	S		L	K	K	I	I	M					T		
		1	1		1	3		4		5	5	5	5	5	6	6	7		7	8			1	1	1		1	1	1	1	1	1					1		
		5	7		9	2		5		0	0	2	6	9	4	8	0		7	9			1	1	3		4	4	4	4	4	4					6		
		F	F		Q	P		T		M	I	K	L	K	S	F	E		V/G	Q			1	6	8		1	3	3	4	4	5					1		
																							I	G	R		F	S	S	T	T	N					K		

FIGURE 5.—Polymorphic sites of *RPW8.2* aligned against the Ms-0 *RPW8.2* allele (GenBank accession no. AF273059). This figure was made in the same way as Figure 3 except that the *AlRPW8.2* sequence was used as an ancestral reference. Proteins with * have 29–34 aa deletion at the C-termini due to early stop codons introduced by the single nucleotide insertion (boxed G) or deletion (boxed —).

and T5-3) that were homozygous for a single copy transgene and did not develop SHL in soil. We then determined the locations of the T-DNA by TAIL-PCR (SESSIONS *et al.* 2002). We found that the transgenes in S5 and in T7-10 were inserted in intergenic regions and the transgene in T5-3 was inserted in the intron of At1g43970, an expressed gene with unknown function (see supplemental Figure 3 at http://www.genetics.org/supplemental/). Compared with Col-gl, these three transgenic lines showed no noticeable developmental defects and more importantly 5-week-old plants grown on MS-agar medium in which *RPW8* expression is suppressed (XIAO *et al.* 2003) had no difference in the amount of vegetative growth (data not shown). We then measured their mRNA levels of *RPW8.1* relative to that in Ms-0 by quantitative RT-PCR. The mRNA levels of *RPW8.1* in the three transgenic lines were about the same as in Ms-0 in 3-week-old seedlings, and were ~2.5–4 times of that in Ms-0 in the 6-week-old plants (see supplemental Figure 3B), presumably due to the self-transcriptional amplification nature of the *RPW8* genes in mature plants (XIAO *et al.* 2003).

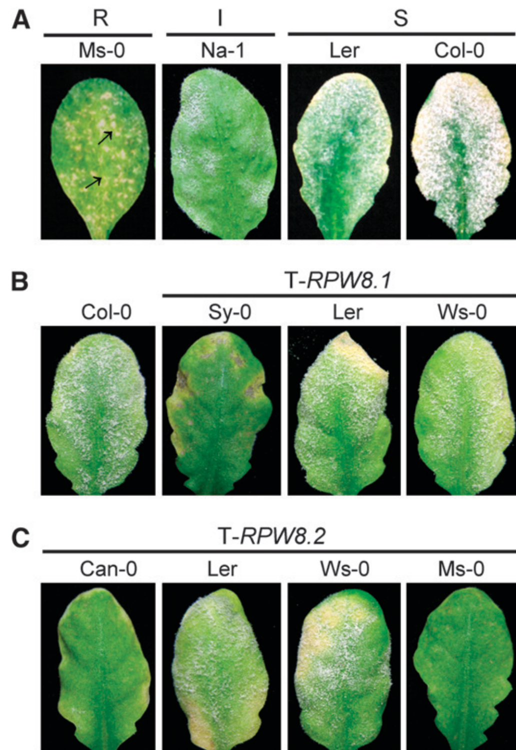


Figure 6.—Some *RPW8* alleles are (partially) functional. Six-week-old plants were inoculated with *E. cichoracearum* USCS1 and a typical infected leaf from each indicated genotype was photographed at 12 dpi and presented. (A) Disease reaction phenotypes of four representative *A. thaliana* accessions. R, resistant; I, intermediate; S, susceptible. Arrows indicate HR lesions. (B and C) Genomic sequences of *RPW8.1* (B) or *RPW8.2* (C) including its native promoter (1 kb upstream of the ATG start codon) from indicated accessions were placed downstream of the *35S* promoter and the constructs were introduced into Col-gl. T3 homozygous lines were tested with the pathogen together with Col-gl wild type.

Experiments for measuring likely fitness costs of *RPW8*-expression were done under two environmental conditions. We first compared Col-gl and Col-gl transgenic line S5 for plant vegetative growth and seed yield in *Ec*-UCSC1-inoculated and -uninoculated plants in a growth room. For plants inoculated at an early stage (2 weeks old), the transgenic plants were performing much better at the time (6 weeks old) when the rosette leaves were sampled for measuring dry mass (see supplemental Figure 4A at http://www.genetics.org/supplemental/). On average, S5 plants had ~40% more dry mass than Col-gl plants ($P < 0.001$ by Student's *t*-test, Figure 7A), indicating fitness benefits of *RPW8*-conferred resistance. For those inoculated at 4 weeks old, there was no significant difference in plant size and dry mass ($P = 0.238$, Figure 7A; supplemental Figure 4B), implying a balance between benefits and costs conferred by *RPW8* upon pathogen infection at this timing point. However, for uninoculated plants, S5 plants were slightly smaller in size, had 7.5% less dry mass ($P = 0.008$, Figure 7A, supplemental Figure 4C) and 6.1% lower seed yield compared with Col-gl plants ($P < 0.001$, Figure 7B). To consolidate these results, we carried out a larger-scale experiment for measuring seed yield in a greenhouse. Besides using S5, T5-3, and T10-7 transgenic lines, we also introduced the *RPW8* transgene from S5 to Ler background, which probably contains nonfunctional *RPW8.1* and *RPW8.2* alleles (Figures 3, 5, and 6, B and C), by backcrossing for five generations and then the homozygous line S5/Ler was compared to Ler wild-type plants. As shown in Figure 7C, S5 and S5/Ler had slightly lower seed yield compared to Col-gl and Ler wild types, respectively, but the difference was not statistically significant ($P = 0.24$). However, the average seed yield of T5-3 and T7-10 was 92.2% and 85.3% of that of Col-gl, respectively, and these differences were statistically significant ($P < 0.01$, Figure 7C), suggesting that T5-3 and T7-10 plants had reduced fitness that may be attributable to the expression of the *RPW8* genes. The diminished difference in seed yield between S5 and Col-gl in the latter experiment could be due to differences in overall growth conditions (see discussion).

We previously observed that enhanced expression of *RPW8* leads to spontaneous cell death lesions and constitutive activation of defense gene expression (Xiao *et al.* 2003). One probable cause of fitness costs in *RPW8*-expressing plants is the constitutive activation of cell death and defense under certain environmental conditions. To test this, we examined the leaves of >20 6-week-old plants of Ms-0, S5, and Col-gl grown in long-day (16 hr light, 8 hr dark) conditions for sign of cell death by trypan blue staining and for expression of *PR-1*, a reporter gene for defense activation. We detected death of individual as well as clustered mesophyll cells in mature leaves of Ms-0 and S5 plants, but not in leaves of Col-gl plants (Figure 7D). We also found that the mRNA levels of *PR-1* in Ms-0 and S5 were 3.5 and 4.0 times higher, respectively, than that of Col-gl (data not shown). We did not, however, observe cell death and difference in *PR-1* expression in plants of these three genotypes grown in short day (8 hr light and 16 hr dark) or on MS-agar medium during the same time frame (Xiao *et al.* 2003; data not shown). This, together with our earlier observations (Xiao *et al.* 2003, 2005) indicates that fitness costs of *RPW8* expression may be attributable to the energy consumption caused by constitutive activation of a defense-related cell death pathway by *RPW8* in the absence of pathogens under permissive conditions, such as long day in soil.

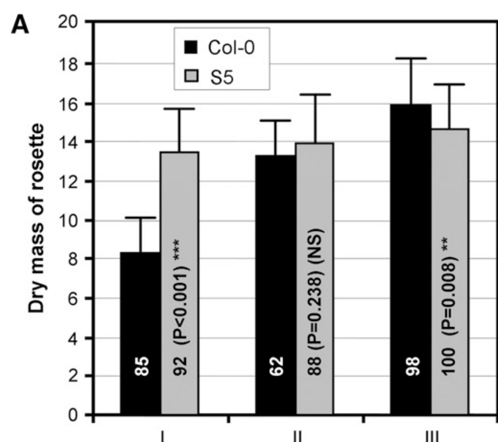
DISCUSSION

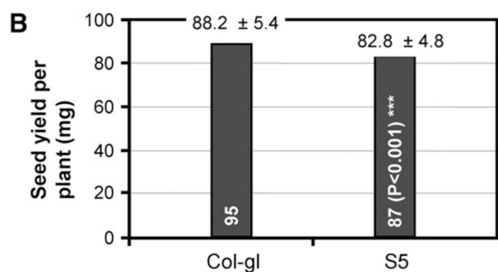
In this study, we made two major observations regarding the maintenance of a unique *R* gene locus in *A. thaliana*. First, we found relatively high levels of genetic variation at the *RPW8* locus, suggesting no recent selective sweep for *RPW8*. Second, we found that gene

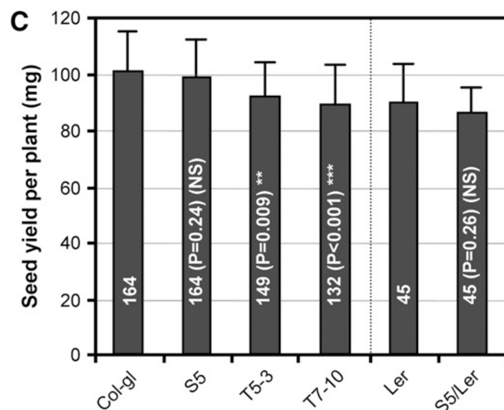
expression of *RPW8* has both benefits and costs on the fitness of individuals, depending on the presence and absence of the pathogens. These results suggest that polymorphisms at the *RPW8* locus in *A. thaliana* may have been shaped and maintained by complex selective forces, including those from the fitness benefits and costs associated with *RPW8*.

Genetic variation at the *RPW8* locus: *RPW8* represents a complex *R* gene locus identified in *A. thaliana*. The relatively high genetic diversity in the *RPW8.1* and *RPW8.2* coding regions ($\pi = \sim 0.01$; Table 1) is in sharp contrast to that in the coding regions of *HR3* ($\pi = 0.002$), the presumable progenitor of the *RPW8* gene family located at the same genomic region (Xiao *et al.* 2004). This result is consistent with our previous study, in which a strong selective constraint on *HR3* was suggested between *A. lyrata* and *A. thaliana* (Xiao *et al.* 2004). Within *A. thaliana*, the *HR3* alleles are highly conserved in amino acid sequence (see supplemental Figure 1 at http://www.genetics.org/supplemental/). For the coding region of *HR3*, the ratio of π_{non} (average nucleotide diversity at nonsynonymous sites) to π_{syn} (average nucleotide diversity at synonymous sites) is only 0.174 (π_{non} = 0.001 and π_{syn} = 0.006, Table 1), confirming the strong selective constraint on the amino acid changes in *HR3*. In *RPW8.1* and *RPW8.2*, on the other hand, π_{syn} is about two times larger than π_{syn} in *HR3*, whereas π_{non} is >10 times larger in both *RPW8.1* and *RPW8.2* than in π_{non} *HR3* (Table 1). The ratios of π_{non}:π_{syn} are 0.880 in *RPW8.1* and 0.929 in *RPW8.2*, suggesting that the amino acid changes in *RPW8* are relatively free from selective constraints in *A. thaliana*. It is unclear why the *HR3* nonfunctional gene with respect to powdery mildew resistance is under the strong selective constraint but the functional *RPW8* genes are not in *A. thaliana*. Our preliminary data indicate that *HR3* might be involved in the basal defense mechanism of *A. thaliana*, which may explain the selective constraint on *HR3* (S. Xiao, unpublished data). At the *RPW8* locus, the functional redundancy due to gene duplication might, in part, be responsible for the high genetic variation in *A. thaliana*.

Evolution of the *RPW8.2* resistance function: Inferring the time when *RPW8.1* gained resistance







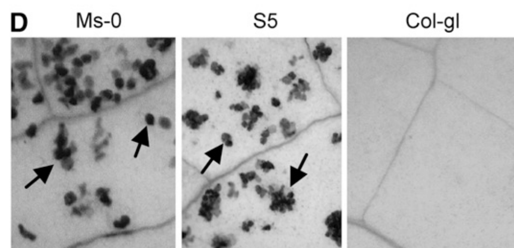


Figure 7.—Fitness cost associated with *RPW8*. The experiments were carried out under two environmental conditions: a growth room (A and B) and a greenhouse (C). The number of plants of an indicated genotype used in these experiments was shown inside the shaded column and the *P*-value from the paired (wild type *vs.* a transgenic line) Student's *t*-test is also shown inside the column. *Significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$; NS, not significant. For detailed experimental conditions, see materials and methods. (A) Arabidopsis seedlings were inoculated with *E. cichoracearum* UCSC1 when they were 2 weeks old (I) or 4 weeks old (II) or remained uninoculated (III). Plants were shifted from short day (8 hr light) to long day (16 hr light) when they were 3 weeks old and all rosette leaves were sampled for measuring dry mass at 6 weeks old. (B) Seedlings of Col-gl and S5 were shifted from short day to long day at 3 weeks old and were maintained in long day until seed maturation in the absence of the pathogen. (C) Seedlings from indicated genotypes were grown in a growth chamber under short day for 2 weeks and then transferred to a greenhouse for further growth until seed maturation. (D) Leaves of 6-week-old, long-day grown plants were stained with trypan blue for cell death. Arrows indicate single and clustered mesophyll cells.

function is difficult, because *RPW8.1* is absent from *A. lyrata*, presumably due to a deletion event (Xiao *et al.* 2004). The presence of an ortholog of *AtRPW8.2* in *A. lyrata* provides an opportunity to address such a question regarding *RPW8.2*. Our demonstration that *AlRPW8.2* confers powdery mildew resistance when expressed by the native promoter (Figure 2) suggests that *AlRPW8.2* may be functionally equivalent to *AtRPW8.2*. There are two likely scenarios for the evolutionary history of the *RPW8.2* function: (i) *RPW8.2* gained resistance function before the divergence of *A. lyrata* and *A. thaliana*, or (ii) *AlRPW8.2* and *AtRPW8.2* evolved resistance function independently after the speciation by convergent evolution. If the first scenario is true, functional *AlRPW8.2* and *AtRPW8.2* alleles would more likely be conserved at those sites that encode amino acids that are critical for resistance. Sequence alignment (Figure 5) showed that *AlRPW8.2* possesses the same nucleotides as the nine functional *AtRPW8.2* alleles at 17 (including 12 nonsynonymous) sites, whereas *AlRPW8.2* possesses the same nucleotide at only 3 sites (including 2 nonsynonymous) with the remaining (likely) susceptible *AtRPW8.2* alleles in Clade II (Figure 4B). At the remaining 5 sites, *AlRPW8.2* differs from both. Thus, *AlRPW8.2* is in general more similar to the resistant *AtRPW8.2* alleles at the sites important for function. Based on this inference, we favor the first scenario, that *RPW8.2* evolved the resistance function before the speciation. The eight amino acid changes (V17F, S45T, Q52K, E59K, V68F, L89Q, L111I, and D116G) between the functional and likely susceptible *AtRPW8.2* alleles but conserved between *AlRPW8.2* and *AtRPW8.2*/Ms-0 may be important for the resistance function of *RPW8.2* (Figure 5). Among those, the D116G mutation is especially interesting, since the change from aspartic acid (D) to glycine (G) happened in all but two divergent alleles that encode truncated, thus most likely nonfunctional, proteins. Indeed, association test suggested statistically significant correlation between the DR phenotypes and alleles that encode different amino acids at this site (Figure 5). Therefore, the aspartic acid residue is probably important for full function of *RPW8.2*. However, since overexpression of *RPW8.2*/Can-0, which harbors D116G and an additional mutation (T64S), resulted in enhanced resistance (Figure 6C), the D116G mutation may not completely abolish RPW8.2 resistance function; rather it may affect RPW8.2 function in an incremental manner. A similar situation was found for the F77L mutation in RPW8.1 (Figure 3). Future site-directed *in vitro* mutagenesis should help assess functional importance of this aspartic acid and other residues encoded by the segregating sites.

Functional and genetic divergence of *RPW8* in *A. thaliana*: Classifying a particular allele into resistant (functional) or susceptible (nonfunctional) is difficult for *RPW8.1* and *RPW8.2*, because they could function independently (Xiao *et al.* 2001) and there may exist other powdery mildew *R* genes unlinked to the *RPW8* locus (Adam and Somerville 1996; Xiao *et al.* 1997). While we could infer that alleles encoding identical proteins as the Ms-0 alleles are resistant alleles and alleles from susceptible accession are most likely susceptible, we were not sure whether alleles that are derived from three resistance accessions (Do-0, Fl-1, and Ob-0) and six moderately resistant accessions (Can-0, Dra-0, Es-0, Hs-1, Na-1, and Sapporo) are resistant or susceptible alleles as they are divergent from those of Ms-0 at the protein level. Despite this ambiguity, we noticed several features of the genetic variations at *RPW8*. First, we found that resistant alleles of both *RPW8.1* and *RPW8.2* tend to cluster together in the *RPW8.1*-Clade Ia and the *RPW8.2*-Clade I (Table 2, Figure 4, A and B), respectively, and that the (likely) susceptible alleles are more diversified, implying that (i) there are more sequence constraints on the functional *RPW8* genes, and (ii) resistant and susceptible alleles have been separated for a relatively long time. Similar observation was made for *RPS2* and interpreted as an indication of long-time maintenance of the resistant and susceptible alleles in the populations (Caicedo *et al.* 1999; Mauricio *et al.* 2003). We estimated the average divergence time of the resistant *vs.* susceptible/divergent alleles to be on average 0.5 MYA for *RPW8.1* and 0.4 MYA for *RPW8.2* (see materials and methods), indeed reflecting the relatively long evolutionary time for the maintenance of the resistant/susceptible alleles at the *RPW8* locus.

The relatively long-time maintenance of the *RPW8.1* and *RPW8.2* alleles may also be reflected by the worldwide distribution of the accessions carrying *RPW8* (Figure 1). The *HR4*-containing accessions, on the other hand, are exclusively from German populations except for Col-0 from USA. Among the 22 accessions from Germany (Figure 1), 7 (32%) are the *HR4*-containing, and only 6 (27%) are resistant to powdery mildew pathogens. These proportions are contrasting to 3% (1/29) and 62% (18/29), respectively, among the other populations when intermediate phenotype is considered as resistant phenotype. The deviations of German populations from the rest were statistically significant ($P =$ 0.014 and 0.0058, respectively, by the Chi-square test of independence), indicating that *RPW8* might be selected against especially strongly in the German populations, possibly due to a shift of the fitness cost–benefit balance related to activities of powdery mildew pathogens on this plant.

Furthermore, the only *HR4*-containing accession from outside of Germany, Col-0, might actually have been collected from Germany too. Col-0 is annotated in both the Nottingham Arabidopsis Stock Centre and the Arabidopsis Biological Resource Center to have an origin from Columbia, Missouri. However, George P. Rédei at the University of Missouri, who is among the earliest

scientists using Arabidopsis as the model genetic material, recalled that he had received seeds from F. Laibach (the first Arabidopsis collector) in Germany in 1955 and named one line 5-13 as Col-0 (Rédei 1992; George P. Rédei, personal communication), which agrees with our speculation based on the information from the *HR4* gene.

The second feature regarding the nucleotide polymorphism at *RPW8.1* and *RPW8.2* is that compared with the resistant alleles, all nonsynonymous polymorphisms at *RPW8.1* and *RPW8.2* are exclusively biallelic between resistant and susceptible alleles (Figures 3 and 5). This biallelic polymorphism is in contrast to that at *RPP13*, in which 55% of the polymorphic codons in the LRR domain of *RPP13* (which is considered to be under diversifying selection) encode three or more amino acids and more than one-quarter encode for four or more amino acids (Rose *et al.* 2004). This pattern of polymorphism at *RPW8* might reflect functional attenuation of the *RPW8* alleles, rather than diversification as shown at *RPP13*. This is in agreement with our sequence-phenotypic data, in which *RPW8.1* and *RPW8.2* alleles that are less divergent from the resistant alleles (with one or two amino acid replacements) may be partially functional (Figure 6, B and C), whereas more divergent alleles tend to be nonfunctional. Similar allelic polymorphism was reported for *RPS2*. Apart from resistant and susceptible alleles (Caicedo *et al.* 1999; Mauricio *et al.* 2003), the *Rps2*/Po-1 allele appeared to be partially functional against *averRpt2*-expressing bacterial strain when ectopically expressed in the Col-0 background (Banerjee *et al.* 2001).

Given that resistant alleles of *RPW8.1* or *RPW8.2* are highly similar and clustered together, one would reason that the resistant alleles are relatively young compared with the more diversified susceptible alleles. This is contradictory to the inference that *RPW8.2* may have gained resistance function before the speciation of the two Arabidopsis species (above). A plausible explanation is that resistant alleles have been conserved and maintained in populations exposed to powdery mildew pathogens due to the benefits they confer to plants, and that the formation and maintenance of susceptible alleles reflect natural selection against the resistant alleles in the absence of powdery mildew pathogens due to the fitness costs associated with them. In this scenario, both resistant and susceptible alleles may have been maintained in a spatiotemporal manner, and the derived alleles (*i.e.*, susceptible alleles) can have multiple origins. In addition, susceptible alleles must have been created by loss-of-function mutations, and they might evolve more or less in the neutral manner after loss of function. The maintenance of the susceptible *HR4* alleles in replacing *RPW8.1* (and *RPW8.2*) in eight accessions is also supportive of the evolutionary disadvantage of *RPW8*, at least in some circumstances. Thus, this scenario well explains the high levels of genetic variation and the lack of evidence for natural selection in the sequence variation, although further study is required to test this evolutionary hypothesis.

Fitness costs of *RPW8* expression: It has been assumed that long-time maintenance of resistant and susceptible polymorphisms at several *R* loci in *A. thaliana* populations is determined by two opposing forces, fitness benefits and fitness costs, both of which are associated with expression of the *R* genes (Stahl *et al.* 1999; Tian *et al.* 2002; Mauricio *et al.* 2003). Indeed, fitness costs have been demonstrated for *RPM1* in the absence of the cognate pathogen (Tian *et al.* 2003), suggesting that at least some *NBS-LRR R* genes may incur fitness costs in the absence of pathogens. The features of sequence polymorphisms at the *RPW8* locus prompted us to ask the question: does *RPW8* expression really incur fitness costs in the absence of the pathogens?

Our experimental data (Figure 7) strongly support the conclusion that expression of the functional *RPW8.1* and *RPW8.2* genes indeed incurs fitness costs. Although we cannot formally exclude the possibility that reduced yield in the three *RPW8* transgenic lines may be caused by T-DNA insertion-introduced genomic perturbance, we have strong reasons to believe that the reduced yield is associated with *RPW8* expression, because the reduction of seed yield was not only detectable but it also seemed to be correlating with the levels of *RPW8* expression (Figure 7). In addition, we showed that S5 plants had increased fitness when challenged by the pathogen at an early developmental stage (Figure 7A), further linking the plant performance with expression of the functional *RPW8* genes. Therefore, although there is no statistical evidence of natural selection from the *RPW8.1* and *RPW8.2* sequences, our results suggest that fitness costs associated with resistant *RPW8.1* and *RPW8.2* alleles may explain selective advantage of susceptible *RPW8.1* and *RPW8.2* alleles and the *HR4* gene in the absence of the pathogen that led to formation and maintenance of those alleles in *A. thaliana* populations. However, it should be pointed out that the fitness costs we detected with *RPW8* as a transgene in this analysis do not necessarily reflect the exact levels of fitness costs in natural *A. thaliana* accessions carrying the *RPW8* gene. Also, we have not directly tested if the maintenance of susceptible or partially resistant alleles is indeed due to reduced fitness costs in plants expressing those alleles. Comparative analysis of the performance of plants expressing the resistant or susceptible *RPW8* alleles in the same background is needed to consolidate our conclusion.

Previously, we showed that enhanced expression of the *RPW8* genes led to SHL and greatly reduced plant stature, and suppression of *RPW8*-expression by growing plants on MS-agar medium led to suppression of SHL and restoration of normal plant size (Xiao *et al.* 2003, 2005). These observations provided indirect

evidence for *RPW8*-dosage-dependent fitness costs. We also found that high-light or long-day conditions enhanced *RPW8* expression, whereas high humidity and high temperature attenuated *RPW8* expression (Xiao *et al.* 2003). This environmental regulation of *RPW8* expression may explain why S5 showed clear fitness costs when plants were grown in a growth room (in the earlier experiments) but was not significantly different from Col-gl when plants were grown in a greenhouse (in the later experiment): *RPW8* expression was attenuated under the environmental conditions in the greenhouse, and so were the associated fitness costs. Recognition of this environmental regulation of *RPW8* expression and its associated fitness costs has an important implication. That is: fitness costs and, as a corollary, fitness benefits (in the presence of the pathogens), are not only determined by the strength of the functionality of the *RPW8.1* and *RPW8.2* alleles but also influenced by physical environment conditions besides the pathogens that influence their expression levels.

Source of fitness costs: Our recent work suggested that *RPW8* may activate a conserved defense-related cell death pathway through a salicylic acid (SA)-dependent feedback amplification circuit (Xiao *et al.* 2003, 2005). Detection of hypersensitive response-like cell death (Figure 7D) and *PR1* expression in leaves of 6-week-old plants of S5 and the naturally *RPW8*-expressing accession Ms-0 grown in soil in the absence of any pathogens, along with the observation that enhanced *RPW8* expression leads to spontaneous cell death and reduced plant size (Xiao *et al.* 2003) strongly supports our speculation that reduced fitness in the *RPW8* transgenic lines was due to *RPW8*-expression-triggered constitutive activation of an SA-dependent defense pathway under certain environmental conditions. This is consistent with the recent observations that constitutive activation of the SA-dependent defenses in several *A. thaliana* mutants incurred fitness costs in the absence of pathogens (Heidel *et al.* 2004) and that normal pathogen-inducible SA-dependent defenses may be beneficial to plants under pathogen pressure (Heidel and Dong 2006). Our results in this work, together with similar results from others, suggest that activation of the SA-dependent defenses in plants is costly and thus *R* genes as triggers at the top of the signaling pathway are under stringent selection to balance the fitness benefits and costs associated with the expression of the *R* genes according to the temporally and spatially variable physical and pathogen environments.

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