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The effect of environmental heterogeneity on *RPW8*-mediated resistance to powdery mildews in *Arabidopsis thaliana*

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• *Background and Aims* The biotic and abiotic environment of interacting hosts and parasites may vary considerably over small spatial and temporal scales. It is essential to understand how different environments affect host disease resistance because this determines frequency of disease and, importantly, heterogeneous environments can retard direct selection and potentially maintain genetic variation for resistance in natural populations.
• *Methods* The effect of different temperatures and soil nutrient conditions on the outcome of infection by a pathogen was quantified in *Arabidopsis thaliana*. Expression levels of a gene conferring resistance to powdery mildews, *RPW8*, were compared with levels of disease to test a possible mechanism behind variation in resistance.
• *Key Results* Most host genotypes changed from susceptible to resistant across environments with the ranking of genotypes differing between treatments. Transcription levels of *RPW8* increased after infection and varied between environments, but there was no tight association between transcription and resistance levels.
• *Conclusions* There is a strong potential for a heterogeneous environment to change the resistance capacity of *A. thaliana* genotypes and hence the direction and magnitude of selection in the presence of the pathogen. Possible causative links between resistance gene expression and disease resistance are discussed in light of the present results on *RPW8*.

Key words: Genotype × environment interaction, *RPW8*, *Arabidopsis thaliana*, *Golovinomyces orontii*, powdery mildew, qPCR, temperature, plant × pathogen interaction, disease resistance.

INTRODUCTION

Natural plant and animal populations often harbour genetic variation for disease resistance and susceptible and resistant individuals co-occur despite a fitness disadvantage of susceptible genotypes in the presence of the parasite (e.g. Jarosz and Burdon, 1992; Jeffery and Bangham, 2000). So why has natural selection not eliminated the susceptible genotypes? The answer is important because the processes acting to maintain variation in disease resistance in uncontrolled environments will determine disease dynamics and can potentially undermine any of our attempts to control disease (Gandon *et al.*, 2001).

Evolutionary theory provides a number of testable hypotheses as to what maintains variation in disease resistance in natural populations, in the form of adaptive (Dawkins and Krebs, 1979; Simms and Rausher, 1987; Jeffery and Bangham, 2000) and non-adaptive processes (Sasaki, 2000; Thrall and Burdon, 2002; Tellier, 2009). However, relatively little attention has been given to the role of environmental heterogeneity in maintaining such variation (Thompson, 2005; Laine and Tellier, 2008; Lazzaro and Little, 2009; Wolinska and King, 2009). A spatially or temporally varying environment may affect the function of genes differentially and hence cause variation in the expression of host and parasite traits (Lynch and Walsh, 1998). Any variation in traits related to infection and disease can lead to variation in the favoured host or parasite genotype and result in heterogeneous selection for host tolerance and resistance and parasite infectivity and virulence (Levene, 1953; Gillespie and Turelli, 1989). The potential importance of heterogeneous selection in host–parasite co-evolution and in maintaining variation in disease resistance is apparent when the performance of host or parasite genotypes varies under different abiotic conditions (e.g. Price *et al.*, 2004; Laine, 2007; Vale *et al.*, 2008), or when distinct host genotypes perform differently with different parasite genotypes (e.g. Burdon and Jarosz, 1991; Salvaudon *et al.*, 2005; Dubuffet *et al.*, 2007).

In plants, the interactive effect of host genotype, parasite genotype and environmental factors on disease outcome has long been recognized (Burdon, 1987). Empirical evidence is plentiful but largely limited to studies at the whole-plant and parasite level and therefore provides little understanding of the underlying genetic mechanisms behind differential disease responses to the environment. Plants rely on their innate immune system to recognize pathogen-associated molecules and trigger an immune response (Jones and Dangl, 2006). Central to this process of recognition and defence response are the disease resistance (*R*) genes. The activation of *R*-genes and their encoded proteins by pathogen effectors sets off strong defence responses which often lead to the death of infected cells and hence the arrest of the infection (Jones and Dangl, 2006). A few *R*-genes and other pathogenesis-related genes are reported to vary in expression across different temperatures, light or humidity levels (e.g. Wang *et al.*, 2001; Yang and Hua, 2004; Noutoshi *et al.*,

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2005; Zhu *et al.*, 2011). In wheat the gene *Yr36* is up-regulated at high temperatures, leading to full or partial resistance against stripe rust (*Puccinia striiformis*) at high, but not at low, temperatures (Fu *et al.*, 2009). Clearly, there is potential for abiotic factors to influence the regulation of *R*-genes and thereby affect the outcome of parasite attack. At present, the general importance of this mechanism in *R*-gene function and plant pathogen defence is unknown. In particular, there is little empirical data showing interacting effects of biotic and abiotic environments on plant disease resistance loci that incorporate genetic variation and hence are applicable to natural conditions.

Here I address these issues by assessing the potential of two abiotic factors to affect the outcome of infection by a powdery mildew pathogen in different genotypes of *Arabidopsis thaliana*. Specifically, I investigate whether varying temperature and soil nutrient conditions result in genotype-specific differences in resistance to powdery mildew pathogens in this species. To explore the mechanisms behind any variation in disease resistance, I investigate the effect of temperature and soil conditions on the expression of *RPW8*, a gene conferring resistance to powdery mildew pathogens in *A. thaliana* (Xiao *et al.*, 2001), and investigate whether variation in resistance under different environmental conditions can be directly associated with variation in the expression of *RPW8*.

MATERIALS AND METHODS

The study system

The gene *RPW8* confers broad-spectrum resistance to powdery mildew pathogens in *Arabidopsis thaliana* and contains a total of six paralogues that differ in their presence and copy number among individuals (Xiao *et al.*, 2001; Fig. 1). Paralogues *RPW8·1* and *RPW8·2* have been shown to be directly responsible for resistance to powdery mildews in the *A. thaliana* genotype Ms-0 (Xiao *et al.*, 2001) and a prerequisite for, but not exclusively associated with, resistance in other genotypes of the species (Jorgensen and Emerson, 2008). The same combination of *RPW8·1* and *RPW8·2* alleles can be associated with resistance in some individuals and susceptibility in others, suggesting that genetic factors other than allelic variation at *RPW8* are involved in the resistance reaction (Jorgensen and Emerson, 2008). Members of the protein family 14-3-3 have, for example, been suggested as possible regulators of *RPW8·2* (Yang *et al.*, 2009). Long-established natural populations of *A. thaliana* have been shown to have considerable allelic variation at the *RPW8* locus (Jorgensen and Emerson, 2008).

RPW8 acts as a signalling component in the basal resistance against powdery mildew pathogens by inducing the expression of common pathogenesis-related genes and the production of salicylic acid (Xiao *et al.*, 2005; Wang *et al.*, 2007). Resistance conferred by *RPW8* results in a hypersensitive reaction (HR) at the site of infection with rapid, localized cell death that limits the spread of the invading pathogen (Xiao *et al.*, 2001). One locus, *RPW8·2*, may have a dual role in pathogen defence by also acting directly at the plant–pathogen interface to limit the development of the pathogen haustorium (WM Wang *et al.*, 2009). As with many other *R*-genes, transcripts of *RPW8·1* and *RPW8·2* are present at low levels in unchallenged plants but increase continuously for more than 7 d after pathogen infection in resistant genotypes (Xiao *et al.*, 2005). Successful infection by powdery mildew pathogens will decrease leaf biomass and hence cause negative effects on plant fitness (Orgil *et al.*, 2007).

Plant material

Seeds of *A. thaliana* were collected from locations in East Anglia, UK, and had been used previously in a study of natural variation at *RPW8* in this species (Jorgensen and Emerson, 2008). All wild collections were grown and self-seeded in the greenhouse for one generation. Eleven accessions were chosen so that a total of three *RPW8* genotypes were

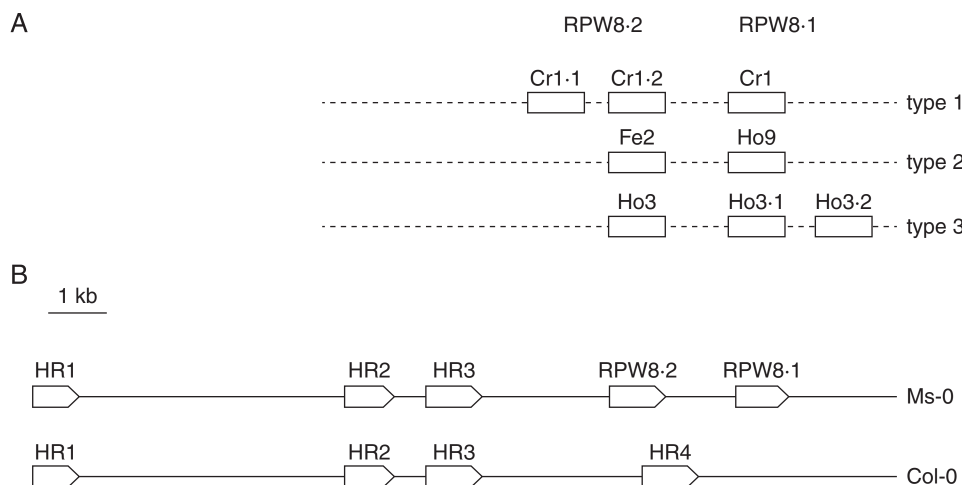

FIG. 1. Composition and organization of the six paralogues at *RPW8*. (A) Copy number of the two paralogues directly involved in disease resistance, *RPW8·1* and *RPW.2*, in the three *RPW8* types used in the study (not to scale). Allele names refer to those of Jorgensen and Emerson (2008). (B) The organization of the six paralogues at the *RPW8* locus on chromosome 3 in *Arabidopsis thaliana* resistant ecotype Ms-0 (GenBank accession no. AF273059) and susceptible Col-0 (TAIR database, http://www.arabidopsis.org/index.jsp) (after Xiao *et al.*, 2001).

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represented by three or four unrelated accessions each (Fig. 1). Accessions with the same *RPW8* genotype had identical alleles at *RPW8·1* and *RPW8·2* but differed at a minimum of four of 14 unlinked microsatellite loci distributed across the rest of the genome (Jorgensen and Emerson, 2008).

Experimental conditions

Multiple seeds from one maternal plant per accession were sown in Levington F2 compost containing insecticide (Intercept, Scotts, http://www.scottsprofessional.com/) and 12 % grit, and germinated in a growth chamber under short day conditions (8 h light, approx. 100 μmol m^{-2} s^{-1}, 22 °C and high humidity, >90 %). Seedlings were transplanted to individual 90-mL pots when 2–3 leaves had emerged. Pots contained either Levington F2 compost with Intercept (N/P/K = 150 : 200 : 200 mg L^{-1}) for a high nutrient treatment or a mixture of F2 compost (1/3) and sterile horticultural sand (2/3) with intercept for a low nutrient treatment. Seven days after transplanting, plants were transferred to two identical growth cabinets (Fitotron, Weiss-Gallenkamp) with short day conditions (8 h light, approx. 150 μmol m^{-2} s^{-1}, approx. 85 % humidity) and regular watering. Two temperature regimes were applied. One cabinet had a constant temperature of 20 °C, whereas the other was kept at 20 °C for 16 h followed by an 8-h cold period at 10 °C inserted 4 h into the dark period. Ramping between temperatures was performed over 30 min within the 8-h cold period. Eight plants represented each accession, two in each of the four treatments: (A) nutrient-rich soil/constant temperature, (B) nutrient-rich soil/cold nights, (C) nutrient-poor soil/constant temperature and (D) nutrient-poor soil/cold nights. Treatment A reflects standard growth conditions for experimental studies of the *A. thaliana* – powdery mildew interaction (Xiao *et al.*, 2003; Jorgensen and Emerson, 2008), while the use of sandy soils and the introduction of a cold night period represent more realistic aspects of natural growth conditions for *A. thaliana* in East Anglia. Soil nutrient status was manipulated by the addition of sand because the focus was on the general nutrient condition of the plant and not any specific nutrient component. Plants were randomized within each cabinet every 3 – 4 d.

Inoculations and harvesting

Inoculations with *Golovinomyces orontii* strain MGH (Plotnikova *et al.*, 1998) were performed after 23 d of growth when all plants had formed a minimum of eight proper leaves. This obligate parasite is one of four powdery mildew isolates (*G. cichoracearum*, *G. cruciferarum* and *Oidium lycopersici*) that have been shown to cause near-identical disease phenotypes in a number of different *A. thaliana* accessions (Xiao *et al.*, 2004). Reproduction and dispersal in powdery mildew species are primarily by asexual spores (conidia) formed in conidiophores from upright hyphae (Plotnikova *et al.*, 1998). Prior to the inoculations, one leaf per plant (20–30 mg each) was harvested, snap-frozen in liquid nitrogen and stored at –80 °C for subsequent RNA extraction. All plants were transferred to long day conditions and 22 °C (16 h light, approx. 100 μmol m^{-2} s^{-1}) for immediate inoculation. Only the host plants therefore experienced different temperatures while the pathogen experienced constant temperatures (22 °C) across treatment groups. Conidia from heavily infected plants of the susceptible genotype Col-gl1 (Col-0 harbouring the glabrous mutation 1) were brushed evenly on each individual leaf rosette. Col-0 was used as positive control in the inoculations. Plants were kept at high humidity (>90 %) for 48 h to allow favourable conditions for spore germination after which relative humidity was decreased to 65–75 %. One leaf per plant was harvested 4 d (95–96 h) after inoculation and stored in 70 % ethanol for later staining and measurement of fungal growth. At this stage, fungal colonies had formed asexual spores but were still at a size where single colonies could be easily distinguished. Leaves were harvested for RNA extraction 2 d later (143–145 h after inoculation), snap-frozen in liquid nitrogen and stored at –80 °C for later RNA extraction. The later harvesting for RNA extraction was chosen to increase the chance of detecting a difference in *RPW8* expression after infection because *RPW8·1* and *RPW8·2* transcription is known to increase continuously at least 7 d after infection in resistant genotypes (Xiao *et al.*, 2005). In summary, two leaves from two independent plants per accession per treatment were available for analyses of fungal growth and another two leaves per accession per treatment for analyses of *RPW8* expression.

Staining and measurements

Leaves stored in ethanol were stained with Trypan Blue (Koch and Slusarenko, 1990), mounted in 50 % glycerol and investigated under a microscope. The first ten germinated spores (hereafter termed colonies) that were encountered on a leaf were used for measurements of fungal growth. The longest hyphae in a colony was measured (curved length) using the Auto-Montage microscopy image analysis software (Syncroscopy). The number of conidiophores was counted for each colony.

RNA extraction and qPCR

Total RNA was extracted from two combined leaves (one from each replicate plant) using the RNeasy Plant mini kit (Qiagen) and including a DNase digestion step according to the manufacturer's protocol. A spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA) was used to assess RNA purity and concentration. Reverse transcription was performed by incubating 1 μg of RNA with 1·5 μg random primers (Invitrogen) in a total volume of 11 μL at 70 °C for 10 min followed by the addition of 200 U Superscript II Reverse Transcriptase with buffer and DDT (Invitrogen), 1 μL 10 mM dNTP (Roche) and 40 U RNase inhibitor (Promega) in a final volume of 20 μL and incubation at 42 °C for 1 h. The cDNA was stored at –20 °C. Primers for quantitative real-time PCR were designed by PrimerDesign Ltd to amplify a 105-bp product of *RPW8·1* (GenBank accession number AF273059) (forward: 5′GTGGAAGGTTCACCATTAAGG, reverse: 5′TACTTGTCTGCGTCTGAGTT), a 124-bp product of *RPW8·2* (GenBank accession number AF273059) (forward: 5′CCGTCAAAAGAGCAAAAGATAGAT, reverse: 5′TCTTCCATTTCTTCACTGAACTTATC) and five reference genes (At2g28390, At4g26410, At5g55840, Gapdh and At5g46630) included in

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the GeNorm Arabidopsis Housekeeping Gene Kit (PrimerDesign Ltd). PCR reactions were performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green to monitor double-stranded DNA synthesis. Reactions contained 10 μL 2 × SYBR Green Master Mix (Applied Biosystems), 4·5 ng cDNA (assuming 100 % efficiency in the reverse transcription reaction) and 1 μL of primer-mix (PrimerDesign Ltd) in a final volume of 20-μL reactions. Conditions for the PCR reaction were 2 min at 50 °C, 10 min at 95 °C, and then 50 cycles, each consisting of 15 s at 95 °C and 1 min at 60 °C. Amplicon dissociation curves were recorded after cycle 50 according to the manufacturer's instructions (Applied Biosystems) in order to detect primer dimers and non-specific products in the reaction. Cycle quantification C_q (Bustin *et al.*, 2009) was the cycle number at which amplification entered the exponential phase. Each PCR reaction was performed in triplicate on separate plates. Twofold serial dilutions of one sample covering the range of 18–0·5625 ng RNA were performed and used in separate PCR reactions to calculate efficiencies for each primer pair (Ramakers *et al.*, 2003).

Data analyses

GeNORM v. 3·5 (Vandesompele *et al.*, 2002) was used to analyse the expression stability of the five reference genes in 15 samples and select the most stable reference gene for use in all samples. Quantification of *RPW8·1* and *RPW8·2* expression relative to the chosen reference gene was performed from C_q values using the standard curve method (Pfaffl, 2001).

The effect of temperature and soil nutrient conditions on fungal growth and *RPW8* expression was analysed with factorial analyses of variance in R (http://cran.r-project.org/). *RPW8* genotype (hereafter *RPW8* type 1, 2 and 3, Fig. 1) and accessions nested within *RPW8* type were entered as explanatory variables to separate effects of the *RPW8* locus ('*RPW8* type') from that of other genetic factors ('accessions within *RPW8* type'). Soil nutrient status and temperature were entered as factors to test the effect of different environments on fungal growth and *RPW8* expression. Infection status (before or after inoculation) was included in the analyses of *RPW8* expression to test the effect of pathogen infection on transcription levels. Log-transformed measures of hyphal length and *RPW8* expression were entered as response variables. Effects of inoculation within each treatment were tested with one-way ANOVAs. Residual plots confirmed that data were approximately normally distributed.

RESULTS

There was a strong positive correlation between average hyphae length of *G. orontii* colonies and the proportion of colonies that had formed conidiophores ($r = 0{\cdot}804$, $P \ll 0{\cdot}001$, $n = 52$, data not shown). Hyphae length was therefore a good indicator of the amount of asexual reproduction by *G. orontii* in this experiment. No conidiophores were observed on colonies with hyphae less than 342 μm long ($n = 520$) which was therefore taken as the size below which the host plant is resistant.

Growth of *G. orontii* differed significantly between the three *RPW8* types (Table 1). There were no significant interactions between *RPW8* type and soil nutrient conditions or temperature. The overall effect of the *RPW8* locus on fungal growth was therefore independent of environmental conditions. However, interaction terms between accessions nested within *RPW8* type, soil nutrient conditions and temperature indicate that hyphae length differed significantly across environmental conditions among accessions of each *RPW8* type (Fig. 2, Table 1). Accessions with identical *RPW8·1* and *RPW8·2* alleles did not therefore have similar disease phenotypes across treatments.

Efficiencies of PCRs ranged between 2·04 and 2·29 (R^2 for standard curve > 0·99). All five reference genes had high expression stabilities in GeNorm ($M < 0{\cdot}53$). The most stable, At5g46630 ($M = 0{\cdot}45$), was selected for further use.

Transcript levels of *RPW8·1* and *RPW8·2* increased after infection in all *RPW8* types and independently of environmental conditions (Tables 2 and 3, Fig. 3). However, accessions with the same *RPW8* type differed significantly in their response to infection across soil nutrient conditions and temperature. Transcript levels of *RPW8·1* were mainly affected by soil nutrient conditions (Table 2) while transcript levels of *RPW8·2* were affected by both soil nutrient conditions and temperature (Table 3). Standard growth conditions for controlled experiments with *A. thaliana* (treatment A) in most cases resulted in increased *RPW8·1* and *RPW8·2* expression after infection, which is in line with previous findings for genotype Ms-0 (Xiao *et al.*, 2005).

Transcript levels of *RPW8·1* and *RPW8·2* after infection were not directly associated with the amount of fungal growth across the 11 *A. thaliana* accessions. There was no significant correlation between average *RPW8·1* expression after infection and average hyphae length (Pearson's correlation $r = -0{\cdot}207$, $P = 0{\cdot}178$, $n = 44$) although high levels of expression were never associated with long hyphae (Fig. 4). Likewise, no significant correlation was found between average *RPW8·2* expression after infection and average hyphae length ($r = 0{\cdot}084$, $P = 0{\cdot}589$, $n = 44$; Fig. 4) or between average *RPW8·1* expression and average *RPW8·2*

TABLE 1. *Analyses of variance in hyphae length of* Golovinomyces orontii *grown on 11* Arabidopsis thaliana *accessions representing three different* RPW8 *types*

	d.f.	Mean square	$F_{(d.f.)}$	P
RPW8 type (R)	2	4·887	$8{\cdot}88_{(2,8)}$	0·009
Soil (S)	1	0·018	$0{\cdot}03_{(1,8)}$	0·860
Temperature (T)	1	0·264	$0{\cdot}48_{(1,8)}$	0·509
R × S	2	1·097	$1{\cdot}99_{(2,8)}$	0·199
R × T	2	0·151	$0{\cdot}27_{(2,8)}$	0·767
S × T	1	2·818	$5{\cdot}12_{(1,8)}$	0·054
R × S × T	2	1·542	$2{\cdot}80_{(2,8)}$	0·120
Accession within *RPW8* type (R[A])	8	4·404	$5{\cdot}08_{(8,396)}$	<0·001
R[A] × S	8	3·341	$3{\cdot}86_{(8,396)}$	<0·001
R[A] × T	8	1·806	$2{\cdot}09_{(8,396)}$	0·036
R[A] × S × T	8	6·373	$7{\cdot}36_{(8,396)}$	<0·001
Error	396	42·876	$5{\cdot}08_{(8,396)}$	<0·001

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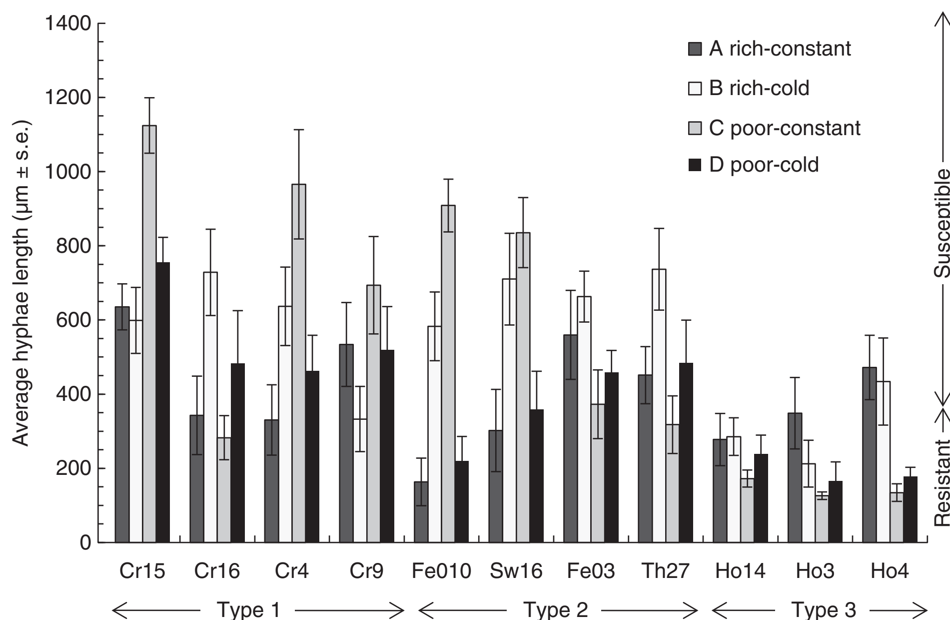

FIG. 2. Growth of *G. orontii* on accessions of *A. thaliana* raised in four different environments prior to infection. A, nutrient-rich soil/constant temperature; B, nutrient-rich soil/cold nights; C, nutrient-poor soil/constant temperature; D, nutrient-poor soil/cold nights. Temperatures were held constant after infection. Accessions represented three different *RPW8* types (see Fig. 1). Hyphae lengths below 342 µm had no conidiophore production and were hence associated with resistance.

TABLE 2. *Analysis of variance in* RPW8·1 *transcript levels in 11* Arabidopsis thaliana *accessions before and after infection with* Golovinomyces orontii

	d.f.	Mean square	$F_{(\mathrm{df})}$	P
Infection (I)	1	21·626	$11{\cdot}83_{(1,8)}$	0·009
I × *RPW8* type (R)	2	0·147	$0{\cdot}08_{(2,8)}$	0·924
I × Soil (S)	1	8·064	$4{\cdot}41_{(1,8)}$	0·069
I × Temperature (T)	1	0·316	$0{\cdot}17_{(1,8)}$	0·688
I × R × S	2	0·112	$0{\cdot}06_{(2,8)}$	0·941
I × R × T	2	0·058	$0{\cdot}03_{(2,8)}$	0·969
I × S × T	1	0·202	$0{\cdot}11_{(1,8)}$	0·748
I × R × S × T	2	0·170	$0{\cdot}09_{(2,8)}$	0·912
Accession within *RPW8* type (R[A])	8	1·827	$21{\cdot}90_{(8,173)}$	<0·001
I × R[A]	8	0·328	$3{\cdot}94_{(8,173)}$	<0·001
I × R[A] × S	8	0·231	$2{\cdot}77_{(8,173)}$	0·007
I × R[A] × T	8	0·115	$1{\cdot}38_{(8,173)}$	0·207
I × R[A] × S × T	7	0·175	$2{\cdot}10_{(7,173)}$	0·046
Error	173	0·083		

A. thaliana accessions represent three different *RPW8* types. Factors and interactions not including 'Infection' are not reported. Four missing data points lowered total degrees of freedom from 263 to 259.

TABLE 3. *Analysis of variance in* RPW8·2 *transcript levels in 11* Arabidopsis thaliana *accessions before and after infection with* Golovinomyces orontii

	d.f.	Mean square	$F_{(\mathrm{df})}$	P
Infection (I)	1	13·678	$7{\cdot}58_{(1,8)}$	0·025
I × *RPW8* type (R)	2	1·276	$0{\cdot}71_{(2,8)}$	0·522
I × Soil (S)	1	1·351	$0{\cdot}75_{(1,8)}$	0·412
I × Temperature (T)	1	0·186	$0{\cdot}10_{(1,8)}$	0·757
I × R × S	2	1·064	$0{\cdot}59_{(2,8)}$	0·577
I × R × T	2	0·104	$0{\cdot}06_{(2,8)}$	0·944
I × S × T	1	0·630	$0{\cdot}35_{(1,8)}$	0·571
I × R × S × T	2	0·292	$0{\cdot}16_{(2,8)}$	0·854
Accession within *RPW8* type (R[A])	8	1·805	$41{\cdot}32_{(8,174)}$	<0·001
I × R[A]	8	0·513	$11{\cdot}75_{(8,174)}$	<0·001
I × R[A] × S	8	0·168	$3{\cdot}86_{(8,174)}$	<0·001
I × R[A] × T	8	0·330	$7{\cdot}55_{(8,174)}$	<0·001
I × R[A] × S × T	7	0·158	$3{\cdot}61_{(7,174)}$	0·001
Error	174	0·044		

A. thaliana accessions represent three different *RPW8* types. Only factors and interactions including 'Infection' are reported. Three missing data points lowered total degrees of freedom from 263 to 260.

expression after infection ($r = 0{\cdot}023$, $P = 0{\cdot}885$, $n = 44$, data not shown).

DISCUSSION

The abiotic environment can have significant effects on the interaction between *A. thaliana* and the powdery mildew pathogen *G. orontii*. The majority of host genotypes in the experiment presented here change from susceptible to resistant across temperatures and soil nutrient conditions with the ranking of genotypes changing between treatments. There is therefore strong potential for a heterogeneous environment to change the sign and magnitude of selection on host genotypes in the presence of the pathogen and hence to influence the maintenance of variation in *Arabidopsis* powdery mildew resistance in spatially structured populations.

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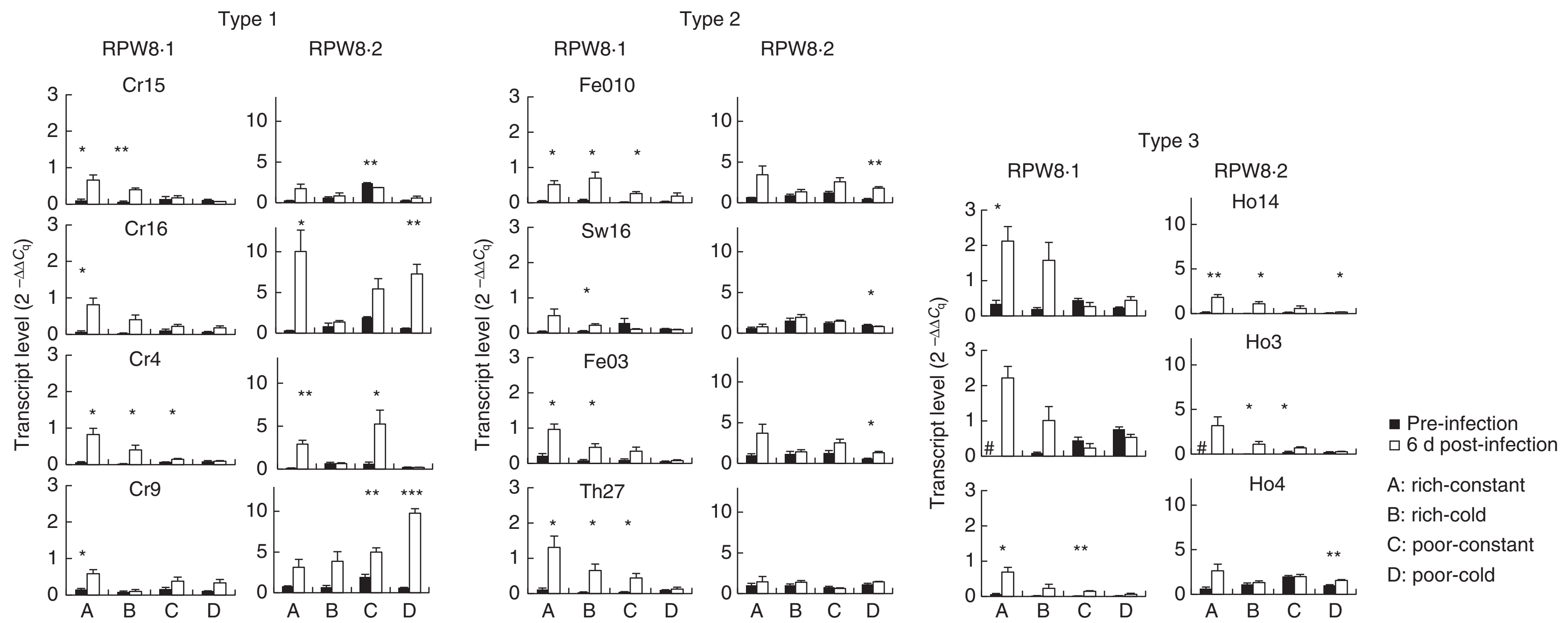

FIG. 3. The effect of temperature, nutrients and infection on transcript levels of *RPW8·1* and *RPW8·2* in *A. thaliana*. Names of accessions in each bar chart refer to those of Jorgensen and Emerson (2008). Bars represent averages of three replicates + s.e. for each treatment: A, nutrient-rich soil/constant temperature; B, nutrient-rich soil/cold nights; C, nutrient-poor soil/constant temperature; D, nutrient-poor soil/cold nights. Black bars: before infection; white bars: 6 d after infection with *G. orontii*. Simple effects of infection within a treatment are presented for each accession (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$, all other others: no significant effect; no corrections for multiple tests). #No statistics reported due to missing data (fewer than three data points).

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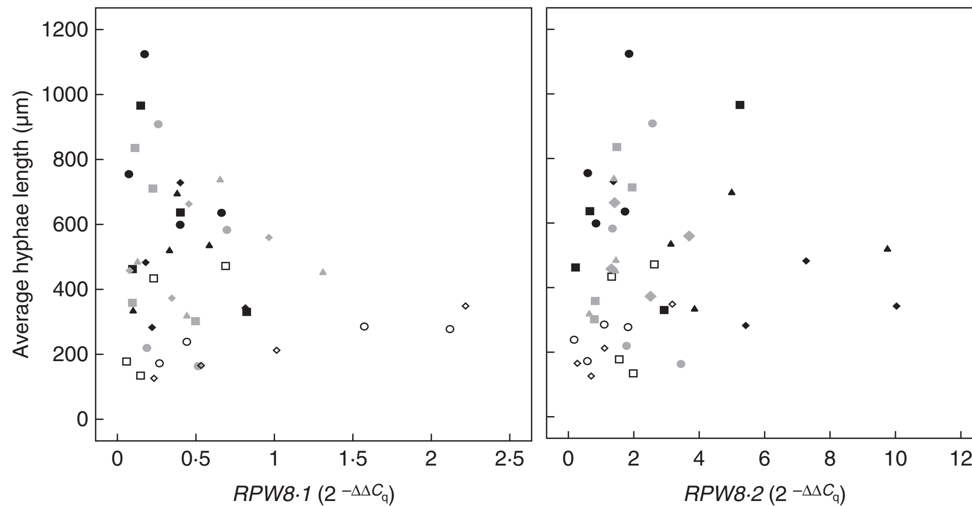

FIG. 4. Associations between *G. orontii* growth and transcript levels of *RPW8·1* and *RPW8·2* 6 d after infection. Each symbol represent different *A. thaliana* accessions in four different treatments (see text). Black symbols are *RPW8* type 1, grey symbols are type 2 and open symbols are type 3 (see Fig. 1).

The impact of temperature on the phenotypes of interacting plants and pathogens has been widely reported (e.g. Chongo and Bernier, 2000; de Jong *et al.*, 2002; Yan and Chen, 2008). A general expectation is that higher temperatures will accelerate the breakdown of resistance, either through higher pathogen pressure or through a negative effect on genes mediating resistance (Webb *et al.*, 2010). However, the empirical support for this prediction is ambiguous. Reports can be found for decreasing (Gijzen *et al.*, 1996; Chongo and Bernier, 2000; Y. Wang *et al.*, 2009) as well as increasing host resistance with increasing temperature (Yan and Chen, 2008; Fu *et al.*, 2009; Webb *et al.*, 2010). Likewise, the effect of host nutritional status on the plant–pathogen interaction appears to vary between host and pathogen species and, importantly, with the form in which the nutrients are available to the host (Agrios, 1997). For example, powdery mildews are known to have increased fitness on host plants grown with high levels of nitrogen but other nutrients may have the opposite effect (Jensen and Munk, 1997; Sander and Heitefuss, 1998). In the *A. thaliana* – *G. orontii* interaction investigated here it is impossible to generalize about the effect that changing temperature and nutrient conditions have on host resistance because of the significant differences among host genotypes. *RPW8* type determines the average level of host resistance independent of environmental conditions but accessions with the same *RPW8* type have different disease phenotypes across different environments. This is in line with our previous work showing that other genetic factors must interact with *RPW8* to mediate resistance (Jorgensen and Emerson, 2008) and suggests that these genetic factors are affected by environmental conditions. The results not only demonstrate the effect that multiple genotypes can have on the level of infection in a population as a whole in fluctuating environments, they also highlight the importance of including multiple host genotypes in the assessments of disease phenotypes across environmental gradients.

At present we have limited knowledge about the genetic factors involved in *RPW8*-mediated resistance and therefore few candidate genes to explain the variation across environments (Xiao *et al.*, 2005; Wang *et al.*, 2007; Yang *et al.*, 2009). Studies on other *R*-genes reveal many factors that affect *R*-gene signalling upstream and downstream of activation (Eitas and Dangl, 2010; Elmore *et al.*, 2011) and some of these are known to respond to variable environments. For example, mitogen-activated protein kinase cascades are functionally linked protein kinases involved in signal transduction downstream of *R*-genes (Pedley and Martin, 2005) and may also respond to abiotic stresses (e.g. Shi *et al.*, 2011). Temperature-sensitive heat shock proteins (HSPs) act to fold and stabilize proteins and one family, HSP90, appears to be essential for the upstream regulation of several *R*-genes (reviewed in Shirasu, 2009). As a first step to understand the importance of *RPW8* regulation, I investigated whether the significant phenotypic differences in host response to *G. orontii* infection across treatments are associated with variation in the expression of *RPW8*. In the present study, transcription levels differed between infected and non-infected individuals independent of environmental conditions when averaged across accessions with the same *RPW8* type. However, in no case did accessions with the same *RPW8·1* and *RPW8·2* alleles show the same change in gene expression across treatments. There are a few reports of other *R*-genes varying in expression between infected and non-infected plants (Yoshimura *et al.*, 1998; Wang *et al.*, 1999; Mohr *et al.*, 2010), across abiotic environments (Yoshimura *et al.*, 1998; Wang *et al.*, 2001; Yang and Hua, 2004; Noutoshi *et al.*, 2005) or between different tissue types or developmental stages (Collins *et al.*, 1999). Yet other *R*-genes are found to be expressed at the same low level across infection regimes and other environmental gradients (Century *et al.*, 1999; Tan *et al.*, 2007; Millett *et al.*, 2009). None of these studies reports quantitative measures of host resistance and we therefore know little about the effect that variation in *R*-gene expression has on the host phenotype. However, a number of scenarios can be envisaged. It is possible that increased *R*-gene expression will increase the general ability (sensitivity)

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of the plant to detect the pathogen and mount a defence response. High transcript levels would then be expected to be associated with resistance and low or no transcription to be associated with susceptibility. A study of transgenic *A. thaliana* lines containing *RPW8·1* and *RPW8·2* suggested such an association (Xiao *et al.*, 2003) but it is clear from the present results that this pattern cannot be generalized to all *A. thaliana* genotypes. Instead, while high levels of *RPW8·1* and, to some extent, *RPW8·2* expression were only associated with resistance, low levels could be associated with both resistance and susceptibility. An alternative scenario is that only low concentrations of R-proteins are necessary for full pathogen recognition and/or defence response and that the degree of host resistance is independent of *R*-gene expression once it is above a certain threshold level. If *R*-genes are costly to express in evolutionary terms we would then expect a tight transcriptional control at constant low levels and not the highly variable expression levels we see at *RPW8* (Brown, 2003). Finally, it is possible that mRNA levels do not accurately reflect R-protein abundance. Post-transcriptional regulation has been reported for *RPM1* in *A. thaliana* (Boyes *et al.*, 1998) and may explain a general lack of correlation between expression and host resistance. The gene *RPW8* is an essential component of resistance to powdery mildews in *A. thaliana*, but other (largely unknown) genetic factors are also involved in this process (Jorgensen and Emerson, 2008; Yang *et al.*, 2009). Based on the present results, I hypothesize that high levels of *RPW8·1* and/or *RPW8·2* expression may result in resistance. However, in some genotypes and/or in some environments *RPW8·1* and *RPW8·2* expression levels are low, and here interacting genetic factors may instead function with *RPW8·1* and/or *RPW8·2* to mediate resistance. The function of these other genetic factors is also likely to be environment-dependent. This hypothesis remains to be tested. However, the suggested scenario provides a mechanism by which environmental heterogeneity can vary the resistance capacity of *A. thaliana* individuals and lead to the maintenance of variation in disease resistance in natural populations.

CONCLUSIONS

The abiotic conditions of hosts and parasites may have important effects on both interacting parties. The present experiment was designed to investigate the isolated effects of host–temperature and host–nutrient interactions on infection outcome in a plant–pathogen interaction. Host plants were grown under different temperature and soil-nutrient conditions before infection, but under constant temperatures after infection to avoid any direct effect of the different temperatures on the pathogen. This is a significant step forward compared with the vast majority of studies reporting host phenotypic responses to temperature gradients because previous experimental designs have rarely allowed the separation of effects on host and pathogen (but see Ramage and Sutherland, 1995). It is possible that in the present experiment the change in growth conditions immediately before infection (a move between growth cabinets) may have affected *RPW8* expression independent of the pathogen infection. There is also a small risk that the effects of infection on *RPW8* expression was confounded by a 6-d age difference in leaves harvested before and after inoculation or by the harvesting per se. However, expression patterns in the standard treatment A (nutrient-rich soil/constant temperature) before and after infection are similar to the patterns of *RPW8* expression reported from studies where no change in growth conditions or harvesting were enforced (Xiao *et al.*, 2005). Therefore, it seems unlikely that any change in *RPW8* expression after infection in the present experiment is due to other factors than the pathogen.

Our understanding of the mechanisms of pathogen infection and host plant defence has been significantly advanced by the discovery of complex signalling pathways and highly variable recognition and effector genes across a wide range of interacting plant and pathogen species (Jones and Dangl, 2006; Oliva *et al.*, 2010). It is clear, however, that the homogeneous laboratory conditions used in most mechanistic research fail to provide a full insight into the molecular and biochemical factors affecting phenotypic traits. The present study exemplifies how a host genotype is not consistently 'resistant' or 'susceptible' and how the expression (and hence potentially the function) of genes involved in resistance can vary considerably across environments. It is even possible that the loci and genetic pathways identified under laboratory conditions are not the same as those important in natural environments, as seen from studies of other traits (Weinig *et al.*, 2002; Carroll *et al.*, 2004). Ignoring the environmental conditions within which traits arose and persist is therefore likely to result in significantly biased interpretations of the mechanism, and the evolution, of infection and defence. It is clear that field studies will be the ultimate approach if we want to obtain a complete understanding of the mechanism and dynamics of disease in natural populations.

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