Broad-spectrum resistance of Arabidopsis C24 to downy mildew is mediated by different combinations of isolate-specific loci

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Summary

- Most natural Arabidopsis thaliana accessions are susceptible to one or more isolates of the downy mildew pathogen Hyaloperonospora arabidopsidis (Hpa). However, Arabidopsis C24 has proved resistant to all Hpa isolates tested so far. Here we describe the complex genetic basis of broad-spectrum resistance in C24.
- The genetics of C24 resistance to three Hpa isolates was analyzed by segregation analysis and quantitative trait locus (QTL) mapping on recombinant inbred and introgression lines.
- Resistance of C24 to downy mildew was found to be a multigenic trait with complex inheritance. Many identified resistance loci were isolate-specific and located on different chromosomes. Among the C24 resistance QTLs, we found dominant, codominant and recessive loci. Interestingly, none of the identified loci significantly contributed to resistance against all three tested Hpa isolates.
- Our study demonstrates that broad-spectrum resistance of Arabidopsis C24 to Hpa is based on different combinations of multiple isolate-specific loci. The identified quantitative resistance loci are particularly promising as they provide an important basis for the cloning of susceptibility- and immunity-related genes.

Introduction

The life of plants is associated with diverse microorganisms, several of which can cause plant diseases. Plants resist the infection attempts of most microorganisms, and disease can therefore be regarded as exception. The reason for this is that plants are nonhosts for most microbes either because of basal immune responses or because the plant does not provide an appropriate environment to support pathogen growth and development. This nonhost resistance or basic incompatibility is considered the major form of resistance effective against the vast majority of potentially pathogenic microbes (Nurnberger & Lipka, 2005). Nevertheless, each plant species can be successfully infected by a limited number of adapted pathogens, a phenomenon known as basic compatibility. Within plant species there is variation in susceptibility to pathogens that, in many cases, is the result of gene-for-gene interaction in which dominant resistance genes (R-genes) of the plant confer resistance to specific pathotypes expressing cognate avirulence genes. Hence, this is isolate-specific resistance. Several mechanisms, supported by experimental data, have been proposed to explain R-gene-mediated resistance, including direct recognition of pathogen proteins by plant receptors, but also indirect recognition as described in the guard and decoy model (Van der Hoorn & Kamoun, 2008). At the same time, certain genotypes within a single plant species may evolve resistance to multiple isolates of the same, otherwise successful, pathogen or even several unrelated adapted pathogenic species. This form of resistance is called broad-spectrum resistance (BSR; Kou & Wang, 2010).

There is no general mechanism underlying BSR in plants, and cloned BSR loci represent different classes of genes. Genetically, mechanisms of BSR can be classified as monogenic or polygenic with complex inheritance and interactions between loci.

In cases of monogenic recessive BSR, plants might lack susceptibility factors that are important for successful pathogen development, thereby leading to a partial or complete incompatibility. For example, rice plants with a mutated promoter of the sugar transporter gene Xa13 are completely or partially resistant to a wide range of races of bacterial blight disease (Yang et al., 2006; Chen et al., 2010). The translation initiation factor eIF4E is ultimately required for the infection of potyviruses in many plants, and mutations in the gene are responsible for loss of susceptibility (Robaglia & Caranta, 2006). The gene Tsn1, encoding for a protein with a nucleotide-binding site and leucine-rich repeats (NBS-LRR), is responsible for the sensitivity of bread wheat to the tan spot and Stagonospora nodorum blotch toxin TsnA. tsn1 mutants are nonsensitive to the toxin and therefore resistant to the fungal disease (Faris et al., 2010).

Also, mutants of negative regulators of immune responses may exhibit recessive nonsololate-specific resistance. For example, recessive mutations in Arabidopsis homologs of the barley gene Mildew...
Resistant Locus O (MLO) confer effective resistance against a powdery mildew fungus as a result of hyperaccumulation of indolic secondary metabolites that have antimicrobial activity (Consonni et al., 2010). Broad-spectrum resistance can also exhibit dominant expression that is simply inherited. For instance, Pi9 residing in a cluster of NBS-LRR genes is responsible for the resistance of rice to more than 20 Magnaporthe oryzae isolates (Qu et al., 2006). Another NBS-LRR gene from Arabidopsis WRKY4 confers resistance to several races of white rust on crucifers (Borhan et al., 2008). The Arabidopsis genes RPW8.1 and RPW8.2 encoded R proteins with transmembrane and coiled-coiled domains provide BSR against powdery mildew (Xiao et al., 2001). Additionally, the dominant R-gene Mi from tomato confers resistance against several pests such as root-knot nematodes, whiteflies and potato aphids (Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003).

There are examples when the genetics of BSR is not governed by single loci. In many of those cases, plants have multiple resistance loci with different race specificities and therefore the plant gains BSR. Interestingly, in this situation, race-specific resistance loci are shown to have quantitative effects on immunity (Rygulla et al., 2006, 2008). The tested lines are designated as in Liseč et al. (2008, 2009). Col-0 plants expressing the R CY1 protein tagged with hemagglutinin (HA) are described in Sekine et al. (2008), and the rey1 mutants in the C24 background are described in Sekine et al. (2006). Plants were grown at 21°C under long-day conditions (16:8 h, light: dark; light intensity 100 μmol m⁻² s⁻¹).

Materials and Methods

Plant material and growth

Arabidopsis thaliana (L.) Heynh (referred to as Arabidopsis) RILs derived from the cross Col-0 × C24 (RILs), C24 ILs in the Col-0 background, the genetic map, and the genotype information are described in Torjek et al. (2006, 2008). The tested lines are designated as in Liseč et al. (2008, 2009). Col-0 plants expressing the R CY1 protein tagged with hemagglutinin (HA) are described in Sekine et al. (2008), and the rey1 mutants in the C24 background are described in Sekine et al. (2006). Plants were grown at 21°C under long-day conditions (16:8 h, light: dark; light intensity 100 μmol m⁻² s⁻¹).

Downy mildew infection assays and quantification of the pathogen growth

The downy mildew Hyaloperonospora arabidopsidis (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw. (Hpa) isolates Waco9, Emco5, Noco2 and Maks9 were maintained as previously described (Van Damme et al., 2005). All infection assays were performed on 11-d-old seedlings with a standard inoculum density of 50 conidia per ml. For QTL mapping with the RILs, relative Hpa biomass was quantified based on the content of Hpa DNA relative to Arabidopsis DNA in the infected plants at 5 d post inoculation (dpi). The quantification was performed using TaqMan® quantitative PCR (qPCR, Life Technologies, Carlsbad, CA, USA). Sequences of primers for the amplification of Hpa ACT2, Arabidopsis ACT2, the corresponding TaqMan® probes and a protocol for the assay are presented in the Supporting Information (Tables S1, S2 and Notes S1). For the qPCR, MgCl₂ was added, at a final concentration of 9.5 mM, to TaqMan Universal PCR Master Mix without AmpErase® UNG (Life Technologies, Carlsbad, CA, USA). The efficiency of the primers and probe sets was estimated with the dilution series of the total genomic DNA from the noninfected Col-0 plants and spores of Hpa Waco9. DNA was isolated from the infected seedlings with the CTAB method (Weigel & Glazebrook, 2002a). Five microliters of the isolated DNA (1–5 ng μl⁻¹) were added to TaqMan qPCR (final volume 25 μl). The relative Hpa biomass was expressed as the C₅₀ value obtained for Hpa DNA minus the C₅₀ value obtained for Arabidopsis DNA.

The degree of resistance of ILs to downy mildew was evaluated with spore counting at 6–8 dpi depending on the isolate. Spore counting was performed on five randomly chosen seedlings per replicate per genotype. Each experiment was repeated at least twice with three replicates in every experiment. The segregation analysis of resistance in populations F2 C24 × Col-0 flx3 and BC1 F1 × C24 was performed at 9 dpi to allow full pathogen sporulation. Furthermore, to study the early stages of Hpa infection, we performed trypan blue

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staining (Weigel & Glazebrook, 2002b) and microscopic analysis of infected seedlings at 2 dpi.

QTL mapping

Quantitative trait locus mapping was performed on a subset of 75 RILs Col-0 × C24 (Notes S2). For the mapping, ΔĈ values from the TaqMan qPCR assays (Table S1, Notes S1) were used as phenotype scores without transformation. The composite interval and multiple QTL mapping procedures implemented in R/qtl were applied to locate resistance loci; for details see Notes S2 (Broman et al., 2003). Epistatic interactions were examined with both QTLNetwork (Yang et al., 2008) and R/qtl. For the QTL mapping with ILs in the Col-0 background, we used the results of spore counting. The statistical analysis was performed with ANOVA followed by the least significant difference (LSD) test (P < 0.05, n = 3). The experiment was performed twice; IL QTLs were considered significant only if they were significant in each of the independent experiments. The identified QTLs were named according to their order on the chromosomes; for example, qtl1.2 refers to the second identified QTL on chromosome 1.

Analysis of the inheritance mode of the resistance QTL

To determine whether C24 alleles of identified resistance QTLs are dominant, codominant or recessive, we crossed Col-0 ILs containing resistance QTLs from C24 with the Col-0 parent. Hybrids were checked with PCR primers specific for a given QTL (Table S1). Then, spore counting expressed in spores per seedling was performed for the ILs, Col-0 and F1 hybrids. Groups of genotypes with similar spore counts were determined on the basis of the Tukey–HSD test (α = 0.05, three replicates with five seedlings per replicate). If F1 hybrids were assigned to the same group as Col-0, the corresponding QTL was considered recessive; if the F1 hybrid did not appear in the same groups as the IL and Col-0, the QTL was considered codominant; and if F1 hybrids were in the same group as the ILs, the QTL was denoted as dominant. Each experiment was performed twice with three replicates in every experiment.

Expression analysis of Pathogenesis Related 1

In order to measure Pathogenesis Related 1 (PR-1) transcript abundance in healthy uninfectected leaves, 11-d-old seedlings were sprayed with demineralized water and placed under conditions used for the Hpa infection. At 3 d after treatment, samples were collected and used for total RNA isolation using the RNeasy Plant mini-kit (Qiagen, Düsseldorf, Germany) followed by DNaseI treatment (Thermo Fisher Scientific Inc., Waltham, MA, USA) and cDNA synthesis with oligo(dT)14, RevertAidTM H Minus reverse transcriptase (Thermo Fisher Scientific Inc.) and RiboLockTM RNase Inhibitor (Thermo Fisher Scientific Inc.) according to the manufacturers’ instructions. For the normalization of PR-1 expression data, the reference gene At5g19840 was selected with the RefGenes tool of Genevestigator (Hruz et al., 2011). Sequences of primers for the quantitative real-time polymerase chain reaction (qRT-PCR) and their efficiency coefficients are presented in Table S1. qRT-PCR was performed with the Power CYBER Green master mix (Life Technologies) according to the manufacturer’s recommendations. The experiment was performed twice with three replicates per genotype in each experiment. Significance of differences in the PR-1 expression was assessed with ANOVA (P < 0.05).

Results

Arabidopsis C24 shows a hypersensitive response upon downy mildew infection

Resistant Arabidopsis plants are known to give different immune responses against downy mildew, for example hypersensitive response (HR) or trailing necrosis, depending on the strength and timing of the cell death response (Coates & Beynon, 2010). We microscopically examined the immune reaction of Arabidopsis C24 to three isolates of Hpa, Waco9, Noco2 and Emco5, that are all virulent on Arabidopsis Col-0. C24 appeared to develop an HR upon infection with each of the tested isolates, visible as cell death in trypan blue-stained leaves (Fig. 1). The susceptible accession Col-0, on the other hand, showed successful hyphal colonization and haustorium formation and only occasionally plant cell death. The cell death response of C24 to Hpa isolates Noco2 and Emco5 was comparable to that of the accession Ws-0 to Noco2, which is determined by the dominant RPP1 locus (Reignault et al., 1996), and to that of the Arabidopsis accession Ler to Emco5, which is mediated by RPP8 (McDowell et al., 1998). By contrast, the RPP5-mediated response in the Ler-Noco2 interaction (Parker et al., 1997) was less severe compared with the reaction of C24 to Hpa Noco2. Interestingly, cell death triggered by Hpa Waco9 in C24 was less pronounced than in the interactions of C24 with Noco2 and Emco5. The microscopically observed HR caused by the downy mildew isolates Noco2 and Emco5 in Arabidopsis C24 resembles HR in known gene-for-gene interactions, suggesting that C24 resistance to these isolates may be based on dominant R genes. The weaker response of C24 to Waco9 might be based on other mechanisms, although it is also associated with cell death.

Resistance of Arabidopsis C24 to Hpa is genetically complex

The genetic basis of downy mildew resistance of C24 was analyzed in segregating progenies of crosses with the susceptible accession Col-0. F1 hybrids from the cross C24 × Col-0 and the reciprocal cross Col-0 × C24 showed an intermediate degree of susceptibility to the isolate Waco9, but full resistance to isolates Emco5 and Noco2 (Fig. 2). As there was no significant difference between F1 plants from the reciprocal crosses, we concluded that resistance of C24 is not differentially affected by cytoplasmic genetic factors but rather depends on nuclear genetic loci, which can be inherited dominantly (to Emco5 and Noco2) or codominantly (to Waco9). Next, the segregation of resistance was analyzed in F2 of C24 × Col-0 flc3 and backcross 1 populations (BC1: F1 × C24). The Col-0 flc3 mutant was used to reduce the extreme segregation in flowering time that occurs in crosses between C24 and Col-0 (Salome et al., 2011), and was not different in resistance to Hpa compared with the wildtype Col-0 (not shown). The segregation
ratios of susceptible and resistant seedlings were determined following infection with the three Hpa isolates (Table 1). Strikingly, segregation ratios were different for each of the tested isolates ($\chi^2$ test, $P < 0.005$ in all pairwise comparisons for F2 results), implying that C24 resistance is at least partially isolate-specific. If resistance is controlled by a single dominant locus from C24, it is expected that the ratio of resistant to susceptible plants in F2 populations is 3 : 1. However, after infection with Emco5 and Waco9, the observed ratio differed significantly from 3 : 1 ($P < 0.001$ for both isolates), implying that the resistance is not monogenic-dominant. At the same time, for Emco5 we did not find susceptible plants in the BC1 population, indicating the presence of at least one completely dominant locus among several resistance loci. The segregation of resistance to Noco2 might be explained by a single completely dominant $R$ gene, although with low confidence ($P = 0.11$), suggesting that several additional small-effect loci may condition resistance. Thus, C24 resistance to Waco9 and Emco5 is governed by more than one locus, suggesting that, in general, C24 resistance to downy mildew is multigenic and genetically complex.

QTL mapping of resistance to Hpa Waco9

Unraveling complex traits can be effectively performed by QTL mapping. As we are particularly interested in the genetics of...
complex resistance of C24 to downy mildew, we conducted QTL mapping of resistance to the isolate Waco9. To do this, we made use of RILs derived from a cross between the susceptible parent Col-0 and the resistant parent C24 (Torjek et al., 2006). The degree of susceptibility of 75 RILs to Waco9 was quantified based on the relative Hpa DNA content in infected Arabidopsis seedlings with TaqMan qPCR. The advantage of this method is that it provides a ratio of plant and pathogen DNA from a single seedling with TaqMan qPCR. The advantage of this method is that it provides a ratio of plant and pathogen DNA from a single reaction, thereby reducing technical variation. As a measure of pathogen infection, the cycle threshold values for the ACTIN genes of Hpa and Arabidopsis were subtracted, giving a ΔCt value that was used as input for QTL mapping (Notes S1, Fig. S1). Four loci influencing resistance of C24 to Hpa, Waco9 were mapped on chromosomes 1, 3 and 5 (Tables 2, S3, Fig. S2). These loci had mostly additive effects, with a weak interaction between qtl1.2 and qtl3.1 located on chromosomes 1 and 3, respectively (Fig. S3). The C24 alleles of qtl1.1, qtl3.1 and qtl5.1 contribute positively to resistance, with qtl5.1 having the strongest effect. By contrast, the C24 allele of qtl1.2 increased susceptibility of Arabidopsis to Waco9. Together, the four identified loci explain around 50% of the phenotypic variation in the degree of resistance within the subset of RILs. These data confirm that C24 resistance to Waco9 is genetically complex, supporting the results of the segregation analysis in the F2 and BC1 populations.

Next, we used 70 lines with introgressions from C24 covering the entire Arabidopsis genome in the Col-0 genetic background (Col-0 ILs; Torjek et al., 2008) to validate the resistance QTLs identified in the RIL population and to find possible additional genetic factors (Table 3; also see Table S4 for details). Resistance of ILs was evaluated by scoring the amount of sporulation. None of the Col-0 ILs was as resistant to Waco9 as Arabidopsis accession C24. Three RIL QTLs could be confirmed in the ILs.

Table 1 Segregation analysis of resistance to the downy mildew (Hyaloperonospora arabidopsidis, Hpa) in F2 and back-cross progeny of Arabidopsis Col-0 flc3 and C24.

<table>
<thead>
<tr>
<th>Hpa isolate</th>
<th>Population</th>
<th>Susceptible</th>
<th>Resistant</th>
<th>Segregation S : R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 : 1</td>
<td>1 : 3</td>
<td>1 : 1</td>
<td></td>
</tr>
<tr>
<td>Waco9</td>
<td>F2 C24 × Col-0 flc3, N = 1142</td>
<td>916</td>
<td>226</td>
<td>(\chi^2 = 16.5) (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>BC1 (F1 × C24), N = 326</td>
<td>72</td>
<td>254</td>
<td>–</td>
</tr>
<tr>
<td>Emco5</td>
<td>F2 C24 × Col-0 flc3, N = 322</td>
<td>52</td>
<td>270</td>
<td>(\chi^2 = 594.8) (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>BC1 (F1 × C24), N = 99</td>
<td>0</td>
<td>99</td>
<td>–</td>
</tr>
<tr>
<td>Noco2</td>
<td>F2 C24 × Col-0 flc3, N = 317</td>
<td>67</td>
<td>250</td>
<td>(\chi^2 = 490.5) (P &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>BC1 (F1 × C24), N = 99</td>
<td>0</td>
<td>99</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2 Quantitative trait locus (QTL) mapping of C24 resistance to Hyaloperonospora arabidopsidis isolate Waco9 in the population of Arabidopsis Col-0 × C24 recombinant inbred lines (RILs).

<table>
<thead>
<tr>
<th>QTL*</th>
<th>Location of max LOD (cM)</th>
<th>Max LOD</th>
<th>% variance explained</th>
<th>Marginal effect/SE</th>
<th>Interval from mapping with RILs (cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qtl1.1</td>
<td>chr1 9.0</td>
<td>2.4</td>
<td>7.9</td>
<td>3.4**</td>
<td>0–17</td>
</tr>
<tr>
<td>qtl1.2</td>
<td>chr1 20.4</td>
<td>3.9</td>
<td>13.2</td>
<td>–4.4***</td>
<td>17–27</td>
</tr>
<tr>
<td>qtl3.1</td>
<td>chr3 38.2</td>
<td>1.9</td>
<td>6.1</td>
<td>2.9**</td>
<td>0–50</td>
</tr>
<tr>
<td>qtl5.1</td>
<td>chr5 60.1</td>
<td>9.9</td>
<td>41.1</td>
<td>7.8***</td>
<td>55–63</td>
</tr>
</tbody>
</table>

*Name of QTL includes number of chromosome and order of the QTL on chromosome (when several QTLs are present).
†A positive sign indicates that the C24 allele increases resistance, the marginal effects are significant at **, \(P < 0.01\); ***, \(P < 0.001\) (using an F-test).

Table 3 Quantitative trait locus (QTL) mapping of C24 resistance to the Hyaloperonospora arabidopsidis isolate Waco9 in a population of Arabidopsis Col-0 introgression lines (ILs).

<table>
<thead>
<tr>
<th>Col-0 IL*</th>
<th>Spore count (× 10^7 spores per seedling)</th>
<th>QTL</th>
<th>Interval from mapping with ILs (cM)</th>
<th>Interval from mapping with ILs (bp) (TAIR8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>4.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N29/11/1</td>
<td>0.7 (P &lt; 0.001)</td>
<td>qtl1.1, qtl1.3</td>
<td>(0–5) + (62–83)</td>
<td>chr1:0…1189392 (chr1:18980358…24843636)</td>
</tr>
<tr>
<td>N87/12/10</td>
<td>5.9 (P &lt; 0.01)</td>
<td>qtl1.2</td>
<td>20–27</td>
<td>chr1:5855350…8168405</td>
</tr>
<tr>
<td>N52/2</td>
<td>1.7 (P &lt; 0.02)</td>
<td>qtl3.1</td>
<td>21–33</td>
<td>chr2:6535963…8980078</td>
</tr>
<tr>
<td>N68/5</td>
<td>1.7 (P &lt; 0.02)</td>
<td>qtl4.1</td>
<td>38–56</td>
<td>chr4:6810745…10992260</td>
</tr>
<tr>
<td>N2/13/4</td>
<td>0.3 (P &lt; 0.001)</td>
<td>qtl5.1</td>
<td>41–63</td>
<td>chr5:11108011…18282539</td>
</tr>
</tbody>
</table>

*Only one line is shown for each QTL; for more details see Supporting Information Table S4.
†The effect of qtl1.1 was significant only in the line N 29/11/1, which contained an additional introgression on chromosome 1, which we named qtl1.3.
reinforcing these loci as being significant. Col-0 IL N 2/13/4, which contains the major QTL qtl5.1 on chromosome 5, was the most resistant to the isolate Waco9, in both cotyledons and first true leaves. ILs with qtl1.2 were more susceptible to Hpa than their parental line Col-0 \((P < 0.05)\), while the ILs containing qtl3.1 were more resistant \((P < 0.05)\). Unexpectedly, we could not confirm the effect of qtl1.1, as Col-0 ILs with qtl1.1 from C24 did not show altered resistance phenotypes compared with the parent Col-0. However, an additional line N 29/11/1 was found to be more resistant than the parental accession Col-0 \((P < 0.05)\). Line N 29/11/1 contains two introgressions: one introgression corresponds to qtl1.1 and the second, further down on chromosome 1, to the interval 62–83 cM, which we now refer to as qtl1.3. Previously, this line was noted to have a severely altered metabolic profile compared with Col-0 and C24 parents (Lisec et al., 2009). Other tested Col-0 ILs with the C24 allele of qtl1.3 did not show any resistance to Waco9 in comparison with Col-0, suggesting that resistance of line N 29/11/1 is not determined by effects of the individual QTLs qtl1.1 and qtl1.3. Interestingly, with the ILs, we identified qtl4.1 on chromosome 4 \((37–56\ cM, \ P < 0.02)\), which appeared to be as effective as qtl3.1 on chromosome 3, since the resistance of ILs with qtl3.1 and qtl4.1 did not differ significantly \((P > 0.7)\). However, in QTL mapping on the RILs, qtl4.1 was not detected as significant. Thus, ILs allowed us to identify more resistance QTLs than RILs. We concluded that Arabidopsis C24 contains four major loci, qtl1.2, qtl3.1, qtl4.1 and qtl5.1, on chromosomes 1, 3, 4 and 5, respectively, that contribute to resistance to the downy mildew isolate Waco9.

**Individual QTLs are not responsible for BSR of C24 to Hpa**

The differences in genetics of C24 resistance to three isolates of Hpa, observed in the analysis of F1, F2 and BC1 populations, have already indicated that Arabidopsis C24 has isolate-specific immune responses. To analyze this further, Col-0 ILs were screened with Hpa isolates Noco2 and Emco5 (Tables 4, S4). Resistance of the ILs to Emco5 revealed three QTLs on chromosomes 3 \((qtl3.1 \text{ and } qtl3.2)\) and 5 \((qtl5.1)\). Only two QTLs for resistance to Noco2 were uncovered on chromosomes 1 \((qtl1.4)\) and 3 \((qtl3.2)\). In contrast to infection with Waco9, we did not find alleles of C24 that increase susceptibility to the isolates Emco5 and Noco2, indicating that the small-effect qtl1.2 is isolate-specific. In total, we identified six loci that affect resistance to downy mildew. Among all identified loci, we could not find a single locus in C24 that provides resistance to all three tested isolates; qtl3.2 gave full resistance only to the isolates Emco5 and Noco2, but not to Waco9, and qtl5.1 provided resistance to Waco9 and Emco5, but it was not effective against Noco2. Thus, there is no single locus in C24 that is responsible for BSR to Hpa.

**C24 resistance to Hpa involves recessive, codominant and dominant loci**

Despite the fact that C24 resistance is overall a dominant or codominant trait based on the analysis of F1 hybrids, some of the individual QTLs can be inherited differently. We generated F1 hybrids between Col-0 and Col-0 ILs with partial or complete resistance to different Hpa isolates to determine inheritance of individual resistance loci. The degree of susceptibility of these hybrids was quantified and compared with Col-0 and the corresponding parental ILs (Table 5, Fig. 3). C24 alleles of resistance QTLs for Emco5 and Noco2 were either dominant \((qtl3.2)\) or codominant \((qtl1.4)\) and \((qtl3.1)\). Intriguingly, the dominant qtl3.2 locus conferred full resistance to Hpa isolates Emco5 and Noco2 and could contain an R-gene that recognizes effector proteins from these downy mildew isolates. In the case of Waco9, only the susceptibility qtl1.2 was found to be dominant, as F1 hybrids N 87/12/12 x Col-0 were more susceptible than Col-0 but not different from IL N 87/12/12: one could also state that qtl1.2 in Col-0 is a recessive resistance locus. Interestingly, qtl3.1, conferring resistance to both Emco5 and Waco9, inherited differently depending on the isolate. It provided dominant and complete immunity against Emco5, but was codominant and contributed to resistance quantitatively in the case of the isolate Waco9. Two Waco9-specific resistance QTLs, qtl3.1 and qtl4.1, were recessive. In summary, although the overall resistance of C24 to Hpa is either dominant or codominant, the effects of individual resistance loci can be different: dominant \((qtl3.2, \ qtl5.1)\), codominant \((qtl1.4, \ qtl3.1, \ qtl5.1)\) or even recessive \((qtl3.1, \ qtl4.1)\).

---

**Table 4 Downy mildew (Hyaloperonospora arabidopsidis) resistance quantitative trait loci (QTLs) in Arabidopsis C24**

<table>
<thead>
<tr>
<th>QTL</th>
<th>Location (Mbp) (TAIR8)</th>
<th>Waco9</th>
<th>Emco5</th>
<th>Noco2</th>
<th>Effect on PR-1 expression</th>
<th>Col-0 IL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>qtl1.1</td>
<td>chr1, 0–1.2†</td>
<td>R†</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>N 29/11/1</td>
</tr>
<tr>
<td>qtl1.2</td>
<td>chr1, 5.9–8.2</td>
<td>S</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>N 38/6/12/12</td>
</tr>
<tr>
<td>qtl1.4</td>
<td>chr1, 9.3–13.8</td>
<td>C</td>
<td>C</td>
<td>R</td>
<td>C</td>
<td>N 2/11/6</td>
</tr>
<tr>
<td>qtl3.1</td>
<td>chr3, 6.5–9.0</td>
<td>R</td>
<td>R</td>
<td>C</td>
<td>C</td>
<td>N 63/14</td>
</tr>
<tr>
<td>qtl3.2</td>
<td>chr3, 15.7–16.5</td>
<td>R</td>
<td>R</td>
<td>C</td>
<td>C</td>
<td>N 21/3/14</td>
</tr>
<tr>
<td>qtl4.1</td>
<td>chr4, 6.8–11.0</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>N 68/5</td>
</tr>
<tr>
<td>qtl5.1</td>
<td>chr5, 11.1–18.2</td>
<td>R</td>
<td>R</td>
<td>C</td>
<td>C</td>
<td>N 2/13/4</td>
</tr>
<tr>
<td>Number of loci</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

S, QTL from C24 increases susceptibility \((\text{LSD} \ P < 0.05)\); R, QTL from C24 increases resistance \((\text{LSD} \ P < 0.05)\); C, the line with the QTL is not significantly different from Col-0 in resistance to Hpa or expression of PR-1 \((\text{least significant difference}, \ P > 0.05)\).

*Only one introgression line (IL) is shown if several ILs contained QTLs.

†The effect of qtl1.1 was significant only in the line N 29/11/1 with an additional introgression qtl1.3.
Table 5 Inheritance of the Arabidopsis C24 resistance quantitative trait loci (QTLs)

<table>
<thead>
<tr>
<th>QTL</th>
<th>Location (Mbp) (TAIR8)</th>
<th>Waco9</th>
<th>Emco5</th>
<th>Noco2</th>
<th>Col-0 IL</th>
</tr>
</thead>
<tbody>
<tr>
<td>qtl1.2</td>
<td>chr1, 5.9–8.2</td>
<td>Dominant</td>
<td>n.a.</td>
<td>n.a.</td>
<td>N 87/12/12</td>
</tr>
<tr>
<td>qtl1.4</td>
<td>chr1, 9.3–13.8</td>
<td>Recessive</td>
<td>n.a.</td>
<td>Codominant</td>
<td>N 2/11/6</td>
</tr>
<tr>
<td>qtl3.1</td>
<td>chr3, 6.5–9.0</td>
<td>Recessive</td>
<td>n.a.</td>
<td>Dominant</td>
<td>N 63/14</td>
</tr>
<tr>
<td>qtl3.2</td>
<td>chr3, 15.7–16.5</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Dominant</td>
<td>N 21/3/14</td>
</tr>
<tr>
<td>qtl4.1</td>
<td>chr4, 6.8–11.0</td>
<td>Recessive</td>
<td>n.a.</td>
<td>n.a.</td>
<td>N 68/5</td>
</tr>
<tr>
<td>qtl5.1</td>
<td>chr5, 11.1–18.2</td>
<td>Codominant</td>
<td>n.a.</td>
<td>n.a.</td>
<td>N 2/13/4</td>
</tr>
</tbody>
</table>

IL, introgression line; n.a., not applicable.

Resistance conferred by qtl5.1 is not caused by RCY1

The major QTL on chromosome 5 qtl5.1 was found to be responsible for resistance to the Hpa isolates Waco9, Emco5 (Table 4) and Mak9 (data not shown). This QTL in C24 contains the RCY1 locus, which was previously reported as a dominant R gene underlying resistance of C24 to Cucumber mosaic virus yellow strain (CMV(Y)) (Takahashi et al., 2002). Besides this, the RCY1 gene is allelic to RPP8 (At5g43470), which confers resistance to Hpa Emco5 in the Arabidopsis accession Ler (McDowell et al., 1998). Therefore we checked whether RCY1 underlies the major QTL on chromosome 5. An Arabidopsis Col-0 transgenic line expressing RCY1 and resistant to CMV(Y) (line no.12) (Sekine et al., 2008) was as susceptible to the tested downy mildew isolates Waco9, Emco5 and Noco2 as the Col-0 control (Fig. 4a). The expression of RCY1 in transgenic plants was confirmed by western blot analysis (Fig. S4). In addition, we compared resistance of F1 hybrids among Col-0, C24 and several rcy1 mutants in the C24 background (Sekine et al., 2006). If RCY1 confers resistance to Hpa, F1s C24 × Col-0 should be more resistant than F1 hybrids between Col-0 and rcy1 mutants. However, it appeared that the F1 hybrids are equally susceptible to the isolate Waco9 (Fig. 4b). These results demonstrate that RCY1 is not responsible for the downy mildew resistance of C24.

Elevated expression of PR-1 in C24 is not linked to downy mildew resistance

Previously, it was reported that C24 accumulates increased amounts of salicylic acid (Lisec et al., 2008; Bechtold et al., 2010), the defense hormone required to establish immune responses against biotrophic pathogens (Pieterse et al., 2009). In addition, C24 was shown to have high levels of the SA-responsive marker gene PR-1 (At2g14610; Bechtold et al., 2010). To determine if the elevated expression of PR-1 is linked to downy mildew resistance of C24, we checked if any of the Col-0 ILs with introgressed resistance QTLs showed elevated levels of PR-1 expression in healthy uninfected seedlings. Intriguingly, the expression of PR-1 in the ILs was not significantly different from Col-0 (Table 4, Fig. S5, P > 0.25), whereas C24 had higher PR-1 expression levels compared with all tested lines (t-test P < 0.001). Thus, even though individual QTLs contribute significantly to downy mildew resistance, they do not affect PR-1 expression in Col-0 ILs. Increased expression of PR-1 in Arabidopsis C24 is therefore not directly linked to resistance against Hpa.

Discussion

Arabidopsis C24 is resistant to all tested isolates of the downy mildew Hpa (Holub & Beynon, 1997). This accession was reported to have elevated concentrations of SA, hydrogen peroxide and expression of defense-related genes (Lisec et al., 2008; Bechtold et al., 2010). However, downy mildew resistance is not caused directly by the elevation in SA concentrations and SA-induced gene expression because, in the first place, PR-1 expression, which is a marker of SA-dependent defense pathways, is not linked to Hpa resistance QTLs. Secondly, downy mildew resistance QTLs, except for qtl1.3 detected only in one IL N 29/11/1, did not overlap with QTLs responsible for the accumulation of signal molecules SA and glycerol-3-phosphate in C24 (Lisec et al., 2008), which are important for the establishment of systemic acquired resistance (Spoel & Dong, 2012). Thirdly, there are no Hpa broad-resistance QTLs in C24, which one would expect in the case of unspecific activation of immune responses. Finally, F2 plants C24 × Col-0 flc-3 resistant to downy mildew did not show any growth abnormalities, which in some cases are characteristic of plants with a constitutive activation of defense, for instance in the case of hybrid necrosis (Bomblies & Weigel, 2007). Thus, there is no evidence that BSR of Arabidopsis C24 is caused by constitutively high defense responses. Furthermore, it is known that Arabidopsis accessions may have different transcriptional responsiveness to exogenous application of SA (Van Leeuwen et al., 2007), but yet the responsiveness per se does not determine resistance against downy mildew. For instance, the accession Mt-0 was shown to be hyper-SA-responsive, but it does not confer BSR to downy mildew (Nemri et al., 2010). Similarly, the accession Bur-0 has higher SA-induced PR-1 expression than Col-0 (Ahmad et al., 2011), but it is still susceptible to multiple Hpa isolates (Nemri et al., 2010; Krasileva et al., 2011).

Our study is the first example of genetically characterized multigenic BSR in Arabidopsis, which led to the identification of seven resistance QTLs against the downy mildew Hpa in a single Arabidopsis accession C24. Most of the loci identified in our study appeared to confer resistance against Hpa in a quantitative manner, and only two loci, qtl3.2 (to Emco5 and Noco2) and qtl5.1 (to Emco5), were qualitative and completely dominant. A combination of both quantitative and qualitative isolate-specific resistance mechanisms was found in a study on the Arabidopsis–Hpa interactions (Krasileva et al., 2011) and other pathosystems (Poland et al., 2009). The importance of the combination of qualitative and quantitative resistance was demonstrated in a study
on the resistance-breaking capacity of Potato virus Y (PVY) in pepper, where the strong-effect R gene PVR2\textsuperscript{2} was not overcome by PVY when it was combined with smaller-effect resistance QTLs, but it was overcome in the PVR2\textsuperscript{2}-only situation (Palloix et al., 2009).

Our results show that downy mildew resistance loci in C24 can be dominant, codominant and recessive. Genetically controlled resistance in many previously studied interactions between Arabidopsis and downy mildew is mediated by dominant R-genes with NBS-LRR domains (Slusarenko & Schlaich, 2003). We cannot exclude that a classical R-gene confers resistance in a codominant manner, for example when one copy of the R gene leads to pathogen detection and defense activation to a degree that is not sufficient to induce a strong immune response. This could be because of dosage of the R protein, epistasis by modifiers at other genetic loci in the plant genome, or effectors of the pathogen that suppress the immunity. There are also examples of recessive forms of Hpa resistance, for example mutants downy mildew resistant 1 and 6 (Van Damme et al., 2005, 2008, 2009), rar1 suppressors 1 and 2 (Stuttmann et al., 2011). The corresponding wildtype genes (which might be considered susceptibility genes) encode for proteins with predicted metabolic activities. Future cloning of the genes underlying some of the C24 QTLs will reveal the mechanisms of resistance and might explain their mode of inheritance.

In addition to BSR against downy mildew, caused by the oomycete pathogen Hpa, Arabidopsis C24 has complex resistance to the powdery mildew fungus Golovinomyces orontii (Gollner et al., 2008), dominant resistance mediated by the R CY1 gene effective against CMV(Y) (Takahashi et al., 2002), and dominant resistance to Pseudomonas syringae pv. tomato DC3000 (D. Lapin et al., unpublished). Resistance of C24 to a surprisingly broad spectrum of biotrophic and hemibiotrophic pathogens raises questions about evolution of C24 defense mechanisms. The so-called evolved recycling polymorphism model (Holub, 2001) suggests that in plant populations there is extensive proliferation of resistance genes, and, as a result, a pool of constantly appearing ‘unused’ resistance specificities is always present. BSR of C24 could be the result of such proliferated new resistance specificities.

Our study demonstrates that BSR of C24 to downy mildew is multifactorial and depends on combinations of isolate-specific loci; some of the loci contribute to the resistance against only one of three isolates, but others confer resistance to two Hpa isolates. This is not unique to C24, as similar mechanisms were found in other...
plant species, such as rice (fungal blast disease; Shi et al., 2010), Medicago truncatula (oomycete pathogen Aphanomyces euteiches; Hamon et al., 2010), pepper (potyviruses; Caranta et al., 1997), bread wheat (tan spot disease; Faris & Friesen, 2005), barley (powdery mildew; Silvar et al., 2011), and potato (late blight disease; Leonards-Schippers et al., 1994). In these examples, as well as in our study, there was at least one QTL that conferred resistance to several pathogen isolates, but these broad-resistance QTLs were found in combination with other isolate-specific QTLs. Unfortunately, in the studies mentioned, the identified multiple QTLs were not cloned. The Arabidopsis C24-downy mildew pathosystem allows a detailed genetic and genomic analysis of complex BSR in plants.

Ongoing fine-mapping and cloning of the identified Hpa resistance QTLs in Arabidopsis C24 is expected to lead to the identification of susceptibility- and immunity-related genes that will improve our understanding of the molecular mechanisms and evolution of complex resistance in plants.

Acknowledgements

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References


Fig. 4 Sporulation of the downy mildew (Hyaloperonospora arabidopsidis, Hpa) isolates on Arabidopsis thaliana Col-0 plants expressing the RCY1 gene (a); and F1 hybrids of rcy1 mutants and Col-0 (b). qtl/5.1 is a major Waco9 and Emco5 resistance QTL, harboring the RCY1 locus which confers resistance of C24 to cucumber mosaic virus Y and is homologous to the Hpa resistance gene RPP8. (a) Transgenic Arabidopsis Col-0 plants expressing the C24 RCY1 gene were inoculated with three downy mildew isolates: Waco9, white bars; Emco5, light gray bars; Noco2, dark gray bars. The transgenic plants appeared to be as susceptible as the wildtype Col-0 (t-test, P > 0.2 for all Hpa isolates), (b) Three independent ethyl methanesulfonate (EMS) mutants of RCY1 in the C24 background rcy1-3, rcy1-5, and rcy1-7, impaired in resistance to the virus, were crossed with Col-0. The hybrids F1 did not differ in resistance from F1 C24 × Col-0 (ANOVA P = 0.30), which provides evidence that RCY1 does not underlie C24 resistance qtl5.1. Error bars represent ± 1 SD. Statistical analysis was performed with ANOVA (P < 0.05, n = 3) for two independent experiments with three replicates each and five seedlings per replicate.


Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Optimization of the TaqMan® assay for quantification of *Hpa* growth on Arabidopsis.

**Fig. S2** LOD profile of interval mapping of C24 resistance in RILs Col-0 × C24.

**Fig. S3** Interaction plot for qtl1.2 and qtl3.1.

**Fig. S4** HA-tagged RCY1 is expressed in the tested Col-0 transgenic line no. 12.

**Fig. S5** Expression of PR-1 in noninfected but water-sprayed Col-0, C24 and ILs.

Table S1 Primers and probes used in this study

Table S2 Master mix for TaqMan®-based quantification of *Hpa* growth in Arabidopsis

Table S3 Estimates of QTL effects

Table S4 Results of mapping of C24 resistance and susceptibility loci in the population of Col-0 ILs

Notes S1 Optimization and conditions for TaqMan® based *Hpa* quantification in Arabidopsis.

Notes S2 QTL mapping in a population of RILs Col-0 × C24.

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