

# Evolutionary trade-offs at the *Arabidopsis* *WRR4A* resistance locus underpin alternate *Albugo candida* race recognition specificities

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Received 31 March 2021; revised 18 June 2021; accepted 21 June 2021.

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## SUMMARY

The oomycete *Albugo candida* causes white rust of *Brassicaceae*, including vegetable and oilseed crops, and wild relatives such as *Arabidopsis thaliana*. Novel *White Rust Resistance* (*WRR*) genes from *Arabidopsis* enable new insights into plant/parasite co-evolution. *WRR4A* from *Arabidopsis* accession Columbia (Col-0) provides resistance to many but not all white rust races, and encodes a nucleotide-binding, leucine-rich repeat immune receptor. Col-0 *WRR4A* resistance is broken by AcEx1, an isolate of *A. candida*. We identified an allele of *WRR4A* in *Arabidopsis* accession Øystese-0 (Oy-0) and other accessions that confers full resistance to AcEx1. *WRR4A*<sup>Oy-0</sup> carries a C-terminal extension required for recognition of AcEx1, but reduces recognition of several effectors recognized by the *WRR4A*<sup>Col-0</sup> allele. *WRR4A*<sup>Oy-0</sup> confers full resistance to AcEx1 when expressed in the oilseed crop *Camelina sativa*.

**Keywords:** immunity, resistance gene, NLR, natural variation, evolution, effector recognition, crop protection, *Arabidopsis thaliana*, camelina.

## INTRODUCTION

Plants have evolved powerful defence mechanisms that can arrest attempted colonization by microbial pathogens. Timely defence activation requires perception of pathogen-derived molecules by cell-surface pattern-recognition receptors and intracellular nucleotide-binding (NB), leucine-rich repeat (LRR) (NLR), immune receptors (Jones and Dangl, 2006). Extensive NLR genetic diversity within plant populations is associated with robustness of NLR-mediated immunity (Baggs et al., 2017), and plant NLR sequences reveal diversifying selection on NLR genes compared with other genes (Kuang et al., 2004; Meyers et al., 1998; Monteiro and Nishimura, 2018). To investigate NLR diversity, next-generation sequencing technologies were combined with sequence capture to develop *Resistance* (*R*)-gene enrichment sequencing (RenSeq) (Jupe et al., 2013). This method has shed new light on NLR repertoires in several plant genomes, including tomato, potato and wheat

(Andolfo et al., 2014; Steuernagel et al., 2016; Witek et al., 2016). A comparison of 64 *Arabidopsis thaliana* (*Arabidopsis*) accessions using RenSeq documented NLR sequence diversity within a single species, revealing the *Arabidopsis* “pan-NLRome” (Van de Weyer et al., 2019). Each *Arabidopsis* accession contains 150–200 NLR-encoding genes. About 60% are found in clusters (within 200 kb from each other) that show copy number variation (Lee and Chae, 2020). From all the NLRs of the 64 accessions, 10% are singletons and the rest are distributed among 464 orthogroups. Each accession contains a unique subset comprising, on average, 25% of the orthogroups.

NLRs vary in their intramolecular architecture. Plant NLR proteins usually display either ‘Toll, Interleukin-1, *R*-gene’ (TIR), or ‘Coiled-Coil’ (CC), or ‘Resistance to Powdery mildew 8’ (RPW8) N-terminal domain, a central NB domain and a C-terminal LRR domain. Some NLRs also comprise additional C-terminal domains. For example, RRS1 is an

*Arabidopsis* TIR-NLR with a WRKY domain required to detect the effectors AvrRps4 (from the bacterium *Pseudomonas syringae*) and PopP2 (from the bacterium *Ralstonia solanacearum*). The integrated WRKY is called a decoy as it mimics the authentic AvrRps4 and PopP2 effector targets (Le Roux et al., 2015; Sarris et al., 2015). Several other integrated decoy domains have been described (Cesari, 2017). The analysis of NLR-integrated domains can potentially reveal novel effector targets (Kroj et al., 2016).

RPP1 and Roq1, two TIR-NLRs from *Arabidopsis* and *Nicotiana benthamiana* respectively, form tetrameric resistosomes upon activation (Ma et al., 2020; Martin et al., 2020). In this structure, a C-terminal jelly-roll/Ig-like domain (C-JID) physically binds the cognate effector, along with the LRR domain. The C-JID corresponds to previously described motifs found after the LRR of many TIR-NLRs, called post-LRR motifs (Saucet et al., 2021; Van Ghelder and Esmenjaud, 2016). We will refer to this domain as C-JID in the rest of the text.

*Albugo candida* causes white blister rust in *Brassicaceae* and serious annual yield losses in brassica crops such as oilseed mustard (*Brassica juncea*) in India (Gupta et al., 2018). It comprises several host-specific groups, which include race 2 from *B. juncea*, race 7 from *Brassica rapa*, race 9 from *Brassica oleracea* and race 4 from wild relatives (e.g. *Capsella bursa-pastoris*, *Arabidopsis* spp. and *Camelina sativa*) (Table S1) (Jouet et al., 2018; Pound and Williams, 1963). They have been proposed to evolve by rare recombination events that occurred between the races, followed by clonal propagation on susceptible hosts (McMullan et al., 2015). The *Arabidopsis* Columbia (Col-0) allele of *WRR4A* can confer resistance to isolates of all four races (Borhan et al., 2008, 2010). The allele encodes a canonical TIR-NLR and belongs to an orthogroup of three genes in Col-0 at the same locus. The accession Wassilewskija-2 (Ws-2; susceptible to *A. candida* race 4) lacks *WRR4A* but contains the two other paralogs, illustrating intraspecies copy number variation within clusters. Interestingly, one of these paralogs, *WRR4B*, also confers resistance to the Ac2V isolate of race 2 (Cevik et al., 2019). In addition, the CC-NLR-encoding *BjuWRR1*, which confers resistance to several *A. candida* isolates collected on *B. juncea*, was mapped and cloned from the European accession of *B. juncea* Donskaja-IV (Arora et al., 2019).

Several Col-0-virulent isolates of *A. candida* race 4 have been collected from naturally infected *Arabidopsis* plants. They were used to identify an alternative source of broad-spectrum white rust resistance. One of these isolates, AcEx1, was used to reveal a source of resistance in Øystese-0 (Oy-0) that mapped to the *WRR4* locus (Castel, 2019; Fairhead, 2016). We set out to clone the gene conferring AcEx1 resistance in Oy-0, and characterize the corresponding pathogen effector(s).

AcEx1 was collected from *Arabidopsis halleri* in Exeter, UK. It is also virulent in *C. sativa*, an emerging oilseed crop that has been engineered to provide an alternative source of long chain omega-3 polyunsaturated fatty acids (Petrie et al., 2014; Ruiz-Lopez et al., 2014). Transgenic camelina oil is equivalent to fish oil for salmon feeding and for human health benefits (Betancor et al., 2018; Napier et al., 2015; West et al., 2019). Despite challenges to distribute a product derived from a genetically modified crop (Napier et al., 2019), an increase in camelina cultivation can be expected in the near future. Fields of *C. sativa* will inevitably be exposed to *A. candida* and early identification of *R*-genes will enable crop protection. Furthermore, AcEx1 can suppress *Arabidopsis* non-host resistance to the potato late blight pathogen *Phytophthora infestans* (Belhaj et al., 2017; Prince et al., 2017), and to downy mildews (Cooper et al., 2008) emphasizing the importance of protecting camelina fields from white rust.

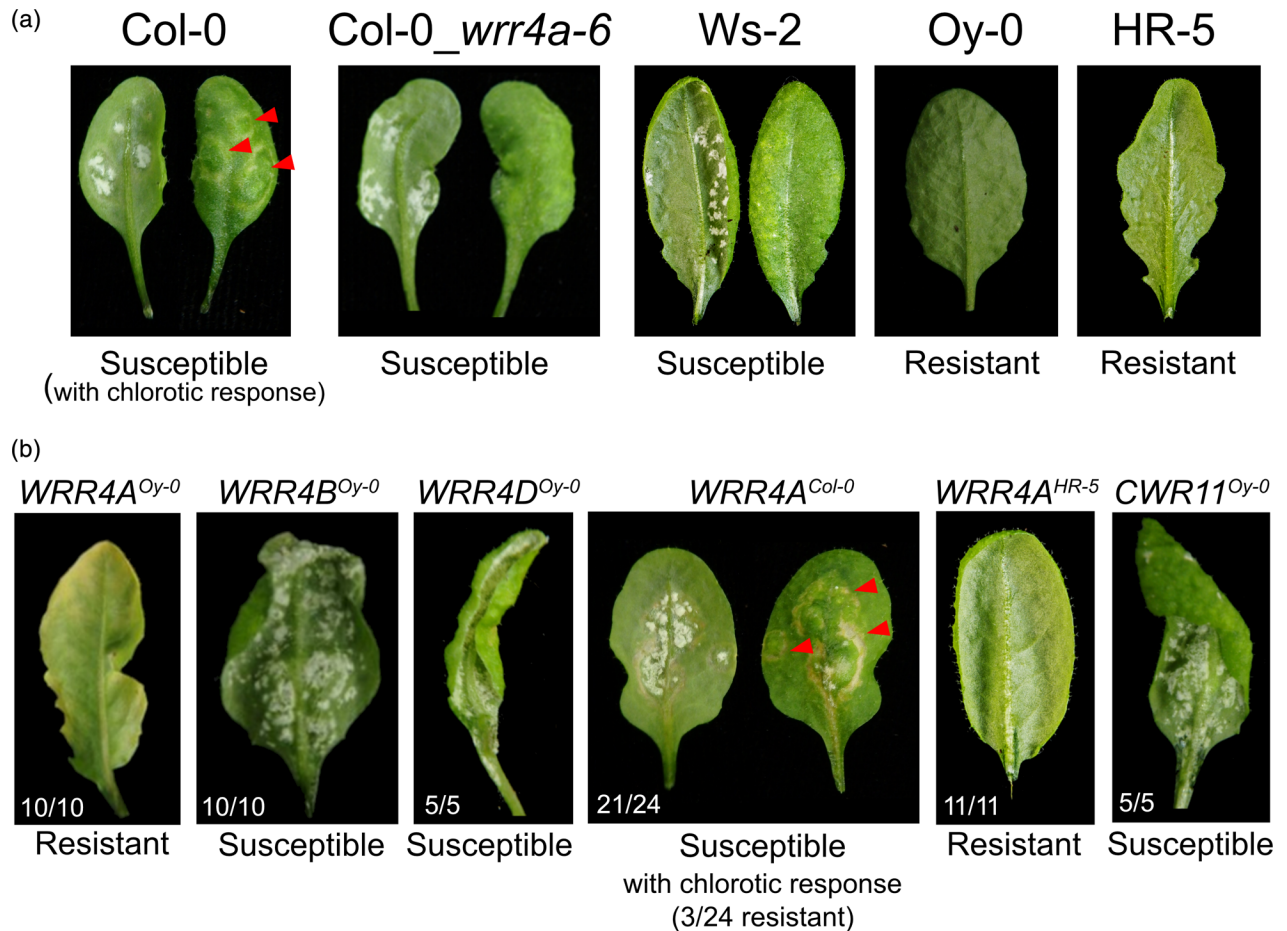
In this study we identified two alleles of *WRR4A* conferring full resistance to AcEx1 from *Arabidopsis* accessions Oy-0 and HR-5. They both encode proteins with a C-terminal extension compared with the Col-0 *WRR4A* allele. This extension enables recognition of at least one effector from AcEx1. We propose that *WRR4A*<sup>Oy-0</sup> is the ancestral state, and that in the absence of AcEx1 selective pressure, an early stop codon in *WRR4A* generated the Col-0-like allele, enabling more robust recognition of other *A. candida* races while losing recognition of AcEx1. Finally, we successfully transferred *WRR4A*<sup>Oy-0</sup>-mediated resistance to AcEx1 from Oy-0 into *C. sativa*.

## RESULTS

### Resistance to AcEx1 is explained by *WRR4A* alleles of HR-5 and Oy-0

AcEx1 growth on Col-0 results in chlorosis that is not seen in the fully susceptible accession Ws-2 (Figure 1a). As *WRR4A* confers resistance to all other *A. candida* races tested and Ws-2 lacks *WRR4A*, we tested if the chlorotic response could be explained by *WRR4A*, by testing a Col-0\_*wrr4a-6* mutant, and found that it shows green susceptibility to AcEx1. We also tested Ws-2 transgenic lines carrying *WRR4A* from Col-0 and observed chlorotic susceptibility (Figure 1b). Thus, *WRR4A* from Col-0 weakly recognizes AcEx1 and provides partial resistance. However, AcEx1 is still able to complete its life cycle on Col-0, which is therefore considered susceptible.

In a search for more robust sources of AcEx1 resistance, we tested 283 *Arabidopsis* accessions (Table S2). We identified 57 (20.1%) fully resistant lines, including Oy-0 and HR-5. We phenotyped 278 recombinant inbred lines (RILs) between Oy-0 (resistant) and Col-0 (susceptible) and conducted a quantitative trait locus (QTL) analysis that revealed one major QTL on chromosome 1 and two minor



**Figure 1.** Oy-0 and HR-5 alleles of *WRR4A* confer full resistance to AcEx1.

5-week-old plants were sprayed inoculated with AcEx1. Plants were phenotyped 14 days after inoculation.

(a) AcEx1 response in nature *Arabidopsis* accessions and mutants. Indicated genotypes always display this phenotype in response to AcEx1.

(b) AcEx1 response in transgenic Ws-2 expressing *WRR4A*<sup>Oy-0</sup>, *WRR4B*<sup>Oy-0</sup>, *WRR4D*<sup>Oy-0</sup>, *WRR4A*<sup>Col-0</sup>, or *CWR11*<sup>Oy-0</sup>. Numbers indicate the number of independent transgenic lines showing similar phenotype out of the number of independent transgenic lines tested. Red arrows indicate a chlorotic response seen in susceptible lines containing *WRR4A*<sup>Col-0</sup> (i.e. Col-0 wild type and Ws-2\_ *WRR4A*<sup>Col-0</sup> transgenic). Adaxial picture of the leaves has been added to illustrate the chlorotic response.

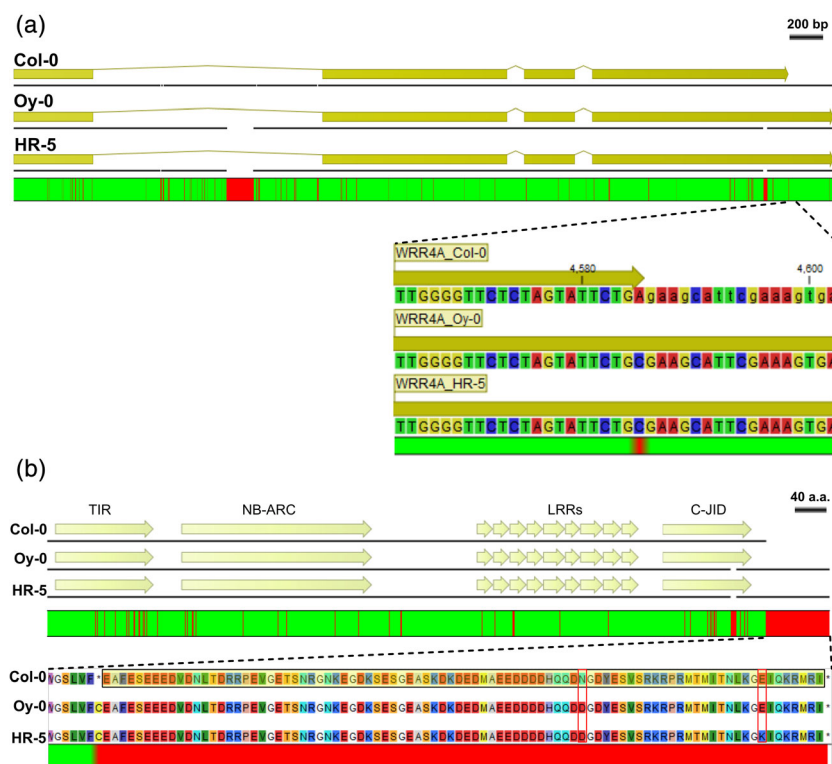
QTLs on chromosomes 3 and 5 (Figure S1a). All loci contribute to resistance, with a predominant contribution of the QTL on chromosome 1 (see figure 3.7 of Fairhead, 2016). We did not investigate the minor QTL on chromosome 5. Fine mapping on chromosome 1 and 3 QTLs refined the QTL boundaries (Figures S2 and S3, see Experimental procedures). Based on sequence identity between the QTL in Col-0 and in an Oy-0 RenSeq dataset (Van de Weyer et al., 2019), we identified four NLRs associated with the QTLs in Oy-0: three TIR-NLR paralogs on chromosome 1 (*WRR4A*, *WRR4B*, and one absent in Col-0 that we called *WRR4D*) and a CC-NLR absent in Col-0 on chromosome 3 (that we called *Candidate to be WRR11* and *CWR11*) (Figure S1b,c).

We expressed these genes, with their own promoters and terminators, in the fully susceptible accession Ws-2. Only *WRR4A*<sup>Oy-0</sup> conferred full resistance (Figure 1b).

*CWR11*, the only NLR from the *WRR11* locus, does not confer AcEx1 resistance. The gene underlying *WRR11* locus resistance remains unknown.

We conducted a bulk segregant analysis using an F2 population between HR-5 (resistant) and Ws-2 (susceptible). RenSeq on bulked F2 susceptible segregants revealed a single locus on chromosome 1, which maps to the same position as the chromosome 1 QTL in Oy-0 (Figure S4a). As *WRR4A*<sup>Oy-0</sup> confers resistance to AcEx1, we expressed its HR-5 ortholog, in genomic context, in the fully susceptible accession Ws-2, and found that *WRR4A*<sup>HR-5</sup> also confers full resistance to AcEx1 (Figure 1b).

In conclusion, *WRR4A* from Col-0 can weakly recognize AcEx1 but does not provide full resistance. We identified two *WRR4A* alleles, in Oy-0 and HR-5, which confer full AcEx1 resistance.



**Figure 2.** Allelic variation between Col-0, HR-5, and Oy-0 alleles of *WRR4A*.

(a) Nucleotide sequence alignment of *WRR4A* alleles. Plain yellow areas represent exons. Yellow lines represent introns. bp, base pair.

(b) Amino acid alignment. a.a., amino acid. The C-terminal extension is framed in yellow for Col-0 to indicate that an early stop codon avoids translation of this sequence. (a,b) Cartoons made with CLC Workbench Main. Green represents identity. Red represents polymorphism. Figures are to scale.

### *WRR4A*<sup>Col-0</sup> carries an early stop codon compared with *WRR4A*<sup>Oy-0</sup>

To understand why the Oy-0 and HR-5 alleles of *WRR4A* confer full resistance to AcEx1, while the Col-0 allele does not, we compared the gene and protein sequences (Figure 2). First, we defined the cDNA sequence of *WRR4A*<sup>Oy-0</sup>. The splicing sites are identical between the two alleles. There are 46 polymorphic amino acids among Col-0, HR-5, and Oy-0. Col-0 shares 96.03% amino acid sequence identity with Oy-0 and 96.23% with HR-5, while Oy-0 and HR-5 share 97.15% amino acid sequence identity. *WRR4A*<sup>Col-0</sup> carries a 156-nucleotide insertion in the first intron compared with Oy-0 and HR-5. A more striking polymorphism is a TGC→TGA mutation in *WRR4A*<sup>Col-0</sup>, resulting in an

early stop codon compared with *WRR4A*<sup>Oy-0</sup> and *WRR4A*<sup>HR-5</sup> (Figure 2), located 178 amino acids after the C-JID, resulting in an 89 amino acid extension in *WRR4A*<sup>Oy-0</sup> and *WRR4A*<sup>HR-5</sup>. The nucleotide sequence for this extension is almost identical between HR-5, Oy-0, and Col-0 (two polymorphic sites). Thus, by mutating TGA to TGC in Col-0, we could engineer an allele with the extension that we called *WRR4A*<sup>Col-0\_LONG</sup> (Figure 3a). By mutating TGC to TGA in Oy-0, we could engineer an Oy-0 allele without the extension that we called *WRR4A*<sup>Oy-0\_SHORT</sup>. We expressed these alleles, as well as the wild-type Col-0 and Oy-0 alleles, with their genomic context, in the AcEx1-compatible accession Ws-2. For unknown reasons, none of the *WRR4A*<sup>Col-0\_LONG</sup> and *WRR4A*<sup>Oy-0\_SHORT</sup> transgenic seeds

**Figure 3.** Recognition of CCG effectors by wild-type (WT) and stop codon mutant alleles of *WRR4A*.

CCG effector candidates were transiently expressed in 4-week-old *Nicotiana tabacum* leaves, under the control of the 35S promoter and Ocs terminator, alone or with WT or mutant alleles of *WRR4A*. Leaves were infiltrated with *Agrobacterium tumefaciens* strain GV3101 in infiltration buffer at OD<sub>600</sub> = 0.4. Pictures were taken at 4 dpi.

(a) cartoon of the *WRR4A* alleles: (a) Col-0 WT, (b) Oy-0 WT, (c) Col-0 with TGA-TGC mutation, causing an Oy-0 like C-terminal extension, (d) Oy-0 with a TGC-TGA mutation causing a truncation of the C-terminal extension.

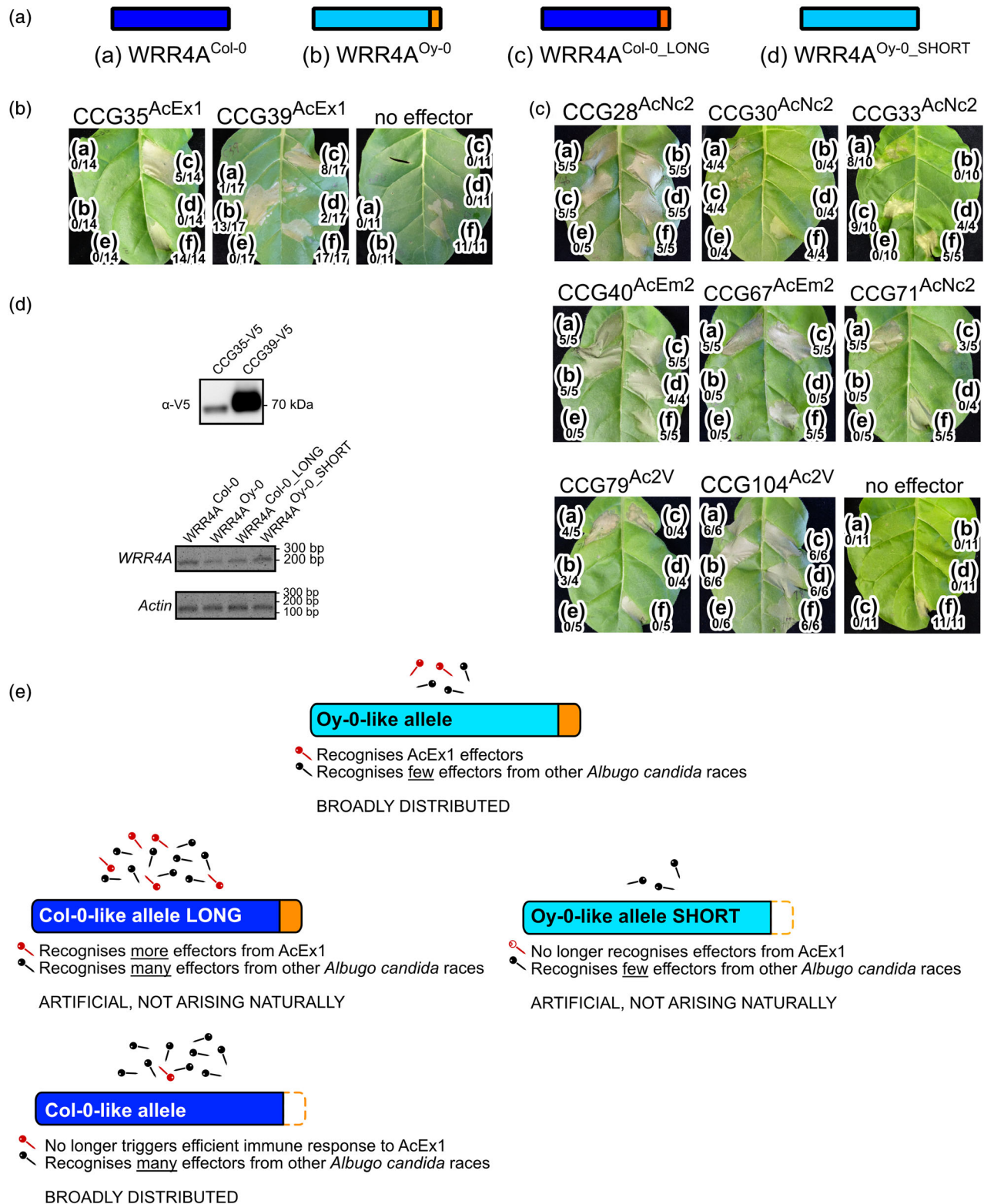
(b) AcEx1 CCG effector candidates alone (e) or with one of the four *WRR4A* alleles as shown in a [a–d]. MLA7 CC domain was used as an HR-positive control (f). Numbers indicate the number of positive HR observed out of the number of infiltrations conducted.

(c) Eight CCGs from other races of *Albugo candida* known to be recognized by Col-0 allele of *WRR4A* were tested with the three others *WRR4A* alleles. CCG effector candidates alone (e) or with one of the four *WRR4A* alleles as shown in a [a–d]. For CCG28, CCG30, CCG33 and the control leaf, *WRR4A*<sup>Oy-0</sup> (b) is infiltrated on the top right of the leaf, instead of *WRR4A*<sup>Col-0\_LONG</sup>. Numbers indicate the number of positive HR observed out of the number of infiltrations conducted.

(d) Expression of CCG35 (68 kDa) and CCG39 (72 kDa) showed by Western blot with anti-V5 antibody. Expression of *WRR4* alleles (not tagged) by reverse transcription–polymerase chain reaction (197 bp), using *NbActin* as a control (143 bp).

(e) Summary of the CCG recognition by *WRR4A* alleles. Red nails: AcEx1 effectors, black nails: CCGs from other *A. candida* races.





germinated. We tried to generate Arabidopsis Col-0 lines with *WRR4A*<sup>Col-0-STOP</sup> using CRISPR adenine base editor (see Experimental procedures). Of 24 transformed plants,

none displayed editing activity at all. Thus, we did not generate stable *WRR4A* stop codon mutants in Arabidopsis. We therefore cloned these alleles under the control of the

35S promoter and the *Ocs* terminator for transient overexpression in *Nicotiana tabacum* (Figure 3).

As many TIR-NLRs carry a C-JID, we conducted a Hidden Markov Model search and found one in *WRR4A* [www.ebi.ac.uk/Tools/hmmer/search/hmmsearch on *A. thaliana* using the Hidden Markov Model previously reported (Ma et al., 2020), e-value =  $5.7 \times 10^{-14}$ ]. This C-JID is present in Oy-0, HR-5, and Col-0 alleles (Figure 2b). The C-terminal extension in *WRR4A*<sup>Oy-0</sup> relative to *WRR4A*<sup>Col-0</sup> does not show homology with known protein domains.

### Extension in *WRR4A* confers specific recognition of AcEx1 candidate effectors

To identify AcEx1 effectors specifically recognized by *WRR4A*<sup>Oy-0</sup>, we tested for a hypersensitive response (HR), a typical phenotype upon NLR activation, after transient expression of *WRR4A*<sup>Oy-0</sup> along with AcEx1 candidate effectors in *N. tabacum* leaves. Secreted CxxCxxxxG (CCG) proteins are expanded in the genomes of *Albugo* species and are effector candidates (Furzer et al., 2021; Kemen et al., 2011). We identified 55 CCGs in the AcEx1 genome (Jouet et al., 2018; Redkar et al., 2021), and polymerase chain reaction (PCR)-amplified and cloned 21 of them, prioritizing those that showed allelic variation with other races. From them, CCG39 induces a *WRR4A*<sup>Oy-0</sup>-dependent HR (Figure 3) and explains AcEx1 resistance in Oy-0. *WRR4A*<sup>Col-0\_LONG</sup> can also recognize CCG39, but *WRR4A*<sup>Oy-0\_SHORT</sup> cannot. Hence, the C-terminal extension fully explains the acquisition of recognition of CCG39. In addition, *WRR4A*<sup>Col-0\_LONG</sup> recognizes CCG35 (Figure 3b). Recognition of CCG35 is not explained solely by the C-terminal extension (as *WRR4A*<sup>Oy-0</sup> does not recognize it) or by the core region of the Col-0 allele (as *WRR4A*<sup>Col-0</sup> does not recognize it).

*WRR4A*<sup>Col-0</sup> can recognize eight CCG effectors from other races of *A. candida* (Redkar et al., 2021). We found that *WRR4A*<sup>Oy-0</sup> is able to recognize CCG28, CCG40, and CCG104, but not CCG30, CCG33, CCG67, CCG71, and CCG79 (Figure 3c). *WRR4A*<sup>Col-0\_LONG</sup> recognizes all the CCGs indistinguishably from *WRR4A*<sup>Col-0</sup>, indicating no influence of the C-terminal extension on their recognition.

In conclusion, we identified one AcEx1 effector specifically recognized by *WRR4A*<sup>Oy-0</sup>. The C-terminal extension is required and sufficient for its recognition. We also found that *WRR4A*<sup>Oy-0</sup> does not recognize several of the Col-0-recognized CCG from other races.

### *WRR4A* alleles carrying a C-terminal extension are associated with AcEx1 resistance

The NLR repertoire of 64 Arabidopsis accessions has been determined using resistance gene enrichment sequencing (RenSeq) (Van de Weyer et al., 2019). We found 20 susceptible and five resistant genotypes that belong to the 64 accessions (Table S2). We retrieved *WRR4A* from these 25

accessions (<http://ann-nblrrrome.tuebingen.mpg.de/apollo/jbrowse/>). The read coverage was insufficient to resolve *WRR4A* sequence in Bur-0 (susceptible) and Mt-0 (resistant). *WRR4A* is absent from the *WRR4* cluster in Ws-2, Edi-0, and No-0. Consistently, these accessions are fully susceptible to AcEx1. From the DNA sequence of the 20 other accessions, we predicted the protein sequence, assuming that the splicing sites correspond to those in Col-0 and Oy-0 (Figure 4 and Dataset S1). There are two well-defined groups of *WRR4A* alleles. One includes *WRR4A*<sup>Col-0</sup>; the other includes *WRR4A*<sup>Oy-0</sup>. The Col-0-like and Oy-0-like groups are also discriminated in a phylogeny constructed based on predicted protein sequences (Figure S5 and Dataset S2). All alleles from the Col-0 group carry TGA (apart from ULL2-5, TGC, but *WRR4A* is pseudogenized in this accession), while all alleles from the Oy-0 group carry TGC, at the Col-0 stop codon position. Several alleles from both groups, including Bay-0, ULL2-5, Wil-2, Ler-0, Ws-0, and Yo-0, carry an early stop codon (i.e. upstream of the Col-0 stop codon position), so the resulting proteins are likely not functional. Consistently, all the accessions from the Col-0 group and all the accessions carrying an early stop codon are susceptible to AcEx1. The only exception is Kn-0, which carries an Oy-0-like allele of *WRR4A* but is susceptible to AcEx1. Otherwise, the presence of an Oy-0-like C-terminal extension associates with resistance.

### AcEx1 resistance can be transferred from Arabidopsis to *Camelina*

AcEx1 can grow on *C. sativa* (Figure 5), which like Arabidopsis, can be transformed using the floral dip method (Liu et al., 2012). We generated a *WRR4A*<sup>Oy-0</sup>-transgenic camelina line. We obtained four independent transformants, including two with a single-locus T-DNA insertion (Figure S6). From these lines we obtained five and four lines showing no symptoms upon AcEx1 inoculation, from which we obtained one bag of homozygous seeds (the others giving either no seeds or segregating seeds). We tested the single homozygous resistant line obtained for stable resistance to AcEx1 (Figure 5). Of 12 individuals, eight showed resistance without symptoms, three showed resistance with a chlorotic response (probably *WRR4A*-mediated HR) and one showed susceptibility (white pustule formation caused by sporulation of *A. candida*). All 12 wild-type camelina control plants showed mild to severe white rust symptoms. This indicates that *WRR4A*<sup>Oy-0</sup> can confer resistance to AcEx1 in *C. sativa*.

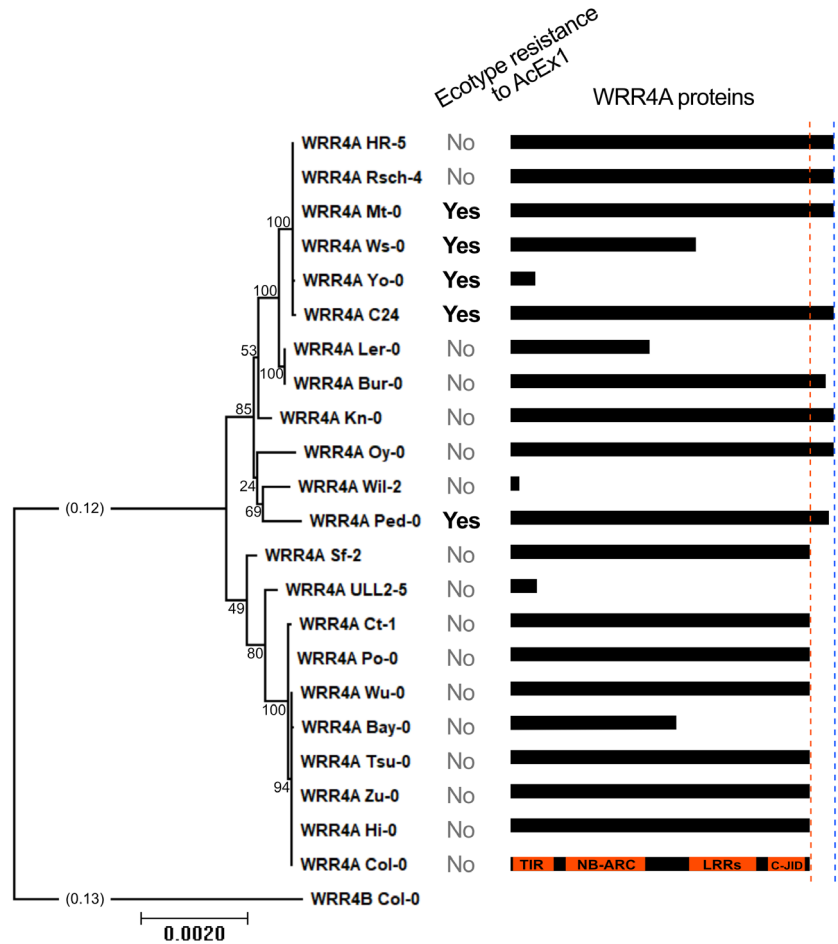
## DISCUSSION

### Col-0 and HR-5 *WRR4A* alleles recognize effectors from AcEx1

A screen for novel sources of resistance to AcEx1 identified accessions HR-5 and Oy-0 as worthy of further

**Figure 4.** An early stop codon in *WRR4A* is associated with AcEx1 susceptibility.

*WRR4A* genomic sequence of 20 *Arabidopsis* accessions were extracted from <http://ann-nbl.rome.tuebingen.mpg.de/apollo/jbrowse/> (Van de Weyer et al., 2019). Nucleotide sequences corresponding from ATG to TAA of the Oy-0 allele (including introns) were aligned using MUSCLE (software: MEGA10, the alignment is available as Dataset S1 on Supporting materials). *WRR4B* from Col-0 was used as out-group. A phylogenetic tree was generated using the maximum likelihood method and a bootstrap (100 replicates) was calculated (software: MEGA10). The tree is drawn to scale (apart the two broken branches, whose length is indicated in parenthesis), with branch lengths measured in the number of substitutions per site. The resistance/susceptibility phenotypes are indicated. Cartoons on the right represent *WRR4A* predicted protein, on scale. TIR, NB-ARC, LRR, and C-JID are indicated in the Col-0 allele. Dashed orange line represents the Col-0 stop codon. Dashed blue line represents the Oy-0 stop codon.



investigation. Positional cloning from Oy-0 and then allele mining in HR-5 showed that this immunity is mediated by alleles of *WRR4A* in HR-5 and Oy-0 with distinct recognition capacities compared with the Col-0 allele. In Oy-0, two additional dominant loci, *WRR11* on chromosome 3 and *WRR15* on chromosome 5 contribute resistance to AcEx1 but the molecular basis of these resistances was not defined. Further investigation on *WRR11* was conducted but did not reveal the causal gene (Castel, 2019, Chapter 3).

*WRR4A<sup>Oy-0</sup>* recognizes at least one AcEx1 effector that is not recognized by *WRR4A<sup>Col-0</sup>* (Figure 3). Conceivably, *WRR4A<sup>Oy-0</sup>* could be combined with *WRR4A<sup>Col-0</sup>* and *WRR4B<sup>Col-0</sup>* to expand the effector recognition spectrum of a stack of *WRR* genes that could be deployed in *B. juncea* or *C. sativa* (Pedersen, 1988).

#### **WRR4A alleles fall into two groups that can or cannot confer AcEx1 resistance**

Analysis of *WRR4A* allele diversity in *Arabidopsis* revealed *WRR4A<sup>Oy-0</sup>*-like and *WRR4A<sup>Col-0</sup>*-like alleles. As *WRR4A<sup>Col-0</sup>*-like alleles show near-identity to *WRR4A<sup>Oy-0</sup>*-like alleles in nucleotide sequence after the premature stop codon, the

latter are likely to be ancestral, and the *WRR4A<sup>Col-0</sup>*-like early stop codon occurred once, in the most recent common ancestor of Sf-2 and Col-0. Other early stop codons, resulting in loss-of-function proteins, occurred randomly in both Oy-0- and Col-0-containing groups. About one-third of the investigated accessions contain another early stop codon resulting in a likely non-functional allele (Figure 4). The full-length Oy-0-like alleles are associated with resistance to AcEx1, while the Col-0-like alleles are associated with susceptibility (Figure 4). The only exception is Kn-0, which displays a full-length Oy-0-like allele but is susceptible to AcEx1. Susceptibility in Kn-0 could be explained by single nucleotide polymorphisms (SNPs), lack of expression or mis-splicing of *WRR4A<sup>Kn-0</sup>*.

#### **Col-0 allele C-terminal truncation correlates with gain of recognition for some CCGs and loss of recognition for others, suggesting an evolutionary trade-off**

*Albugo candida* isolates that are identical or almost identical to AcEx1 are broadly distributed, at least across Europe (Jouet et al., 2018). Similarly, the *WRR4A<sup>Col-0</sup>* allele is not associated with a geographic location, indicating that it is maintained by a non-climatic factor (Figure S7). We

***Albugo candida* race Ex1**  
5-week-old plants, 12 dpi



***Camelina sativa* (cv Celine)**



***Camelina sativa* (cv Celine) + *WRR4A-Oy-0***

**Figure 5.** WRR4A confers resistance to AcEx1 in camelina crop.

Five-week-old camelina (cultivar Celine) plants were sprayed inoculated with AcEx1 race of the white rust oomycete pathogen *Albugo candida*. Pictures were taken 12 days postinoculation (dpi). Top row: 12 wild-type plants all show mild to severe white rust symptoms. Bottom row: 12 lines transformed with *WRR4A<sup>Oy-0</sup>* were tested. One shows mild white rust symptoms, three show local chlorotic response and eight show complete green resistance. White dash line indicates sporulation; red dash line indicates a chlorotic response with no pustule formation.

propose that, in the absence of AcEx1 selection pressure, the Col-0-like early stop codon occurred to provide a new function, along with the loss of AcEx1 effector recognition. This new function enables recognition of additional CCGs from other *A. candida* races.

By combining the C-terminal extension on *WRR4A<sup>Oy-0</sup>* with the core region of *WRR4A* in Col-0 (Figure 3e), recognition of additional AcEx1 CCGs was enabled. Furthermore, Arabidopsis natural accessions carrying the core region of the Col-0-like allele also lack the C-terminal extension (Figure 4; Figure S5, Dataset S1 and S2). This could be an example of intramolecular genetic suppression (Brasseur et al., 2001; Davis et al., 1999; Kondrashov et al., 2002; Schülein et al., 2001). The combination between the core region of the Col-0 allele with the C-terminal extension may form a hyperactive *WRR4A* allele with excessive fitness cost for the plant, which may explain why no

transgenic Arabidopsis could be recovered that carry *WRR4A<sup>Col-0\_LONG</sup>*. The early stop codon may have occurred in Col-0 to compensate for hyperactivation of an ancestral *WRR4A* allele. Hyperactivation of the immune system is deleterious, as shown for example by hybrid incompatibility caused by immune receptors (Wan et al., 2021).

Many TIR-NLRs contain conserved post-LRR motifs (Meyers et al., 2002; Van Ghelder and Esmenjaud, 2016), which cover a functional C-JID motif involved in effector binding (Ma et al., 2020; Martin et al., 2020). We found that *WRR4A* also contains this domain. Both *WRR4A<sup>Col-0</sup>* and *WRR4A<sup>Oy-0</sup>* carry the C-JID, so it does not explain the unique CCG recognition of each allele. Instead, the polymorphism that explains AcEx1 recognition is a short sequence, particularly enriched in negatively charged residues (Glu and Asp, Figure 2), located after the C-JID. Polymorphism within the C-JID between RPP1 and Roq1



contributes to effector recognition specificity (Ma et al., 2020; Martin et al., 2020). In the case of WRR4A, it seems that polymorphism after the C-JID also contributes to specific effector recognition. Biochemical studies of WRR4A should provide more insights into the mechanism of CGG recognition.

#### Arabidopsis WRR4A resistance to AcEx1 can be transferred to the crop camelina

*Camelina sativa* was recently engineered to produce long chain omega-3 polyunsaturated fatty acids, an essential component in the feed used in fish farming (Petrie et al., 2014). Currently, fish farming uses wild fish-derived fish oil. Fish oil-producing camelina offers a solution to reduce the need for wild fish harvesting, potentially reducing pressure on world marine fish stocks (Betancor et al., 2018). There are challenges in delivering products derived from transgenic crops but fish oil-producing crops could reduce the environmental impact of fish farming. White rust causes moderate symptoms on camelina. Moreover, *A. candida* is capable of immunosuppression (Cooper et al., 2008). *Albugo candida*-infected fields constitute a risk for secondary infection of otherwise incompatible pathogens. To safeguard camelina fields against white rust, both chemical and genetic solutions are possible. Genetic resistance offers the advantage of a lower cost for farmers and reduces the need for fungicide release in the environment. Since the first report of white rust on camelina in France in 1945, no genetic resistance has been characterized. All the strains collected on camelina can grow equally irrespective of camelina cultivars (Séguin-Swartz et al., 2009). This absence of phenotypic diversity precludes discovery of resistance loci using classic genetic tools. We found that WRR4A<sup>Oy-0</sup> confers resistance to AcEx1 in camelina (Figure 5). Arabidopsis WRR4A resistance is functional in *B. juncea* and *B. oleracea* (Cevik et al., 2019), suggesting that the mechanism of activation and the downstream signalling of WRR4A is conserved, at least in *Brassicaceae*.

In conclusion, we found a novel example of post-LRR polymorphism within an NLR family, associated with diversified effector recognition spectra. By investigating the diversity of WRR4A, we identified an allele that confers white rust resistance in the camelina crop.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

Arabidopsis accessions used in this study are Oy-0 (NASC: N1436), HR-5 (NASC: N76514), Ws-2 (NASC: N1601) and Columbia (Col-0, NASC: N1092). Col-0\_ *wrr4a-6* mutant is published (Borhan et al., 2008). Seeds were sown directly on compost and plants were grown at 21°C, with 10 h of light and 14 h of dark, at 75% humidity. For seed collection, 5-week-old plants were transferred under long-day conditions: 21°C, with 16 h of light and 8 h of dark, at 75% humidity. For *N. tabacum* (cultivar Petit Gerard) and

*C. sativa* (cultivar Celine), seeds were sown directly on compost and plants were grown at 21°C, with cycles of 16 h of light and 8 h of dark, at 55% humidity.

### Albugo candida infection assay

For propagation of *A. candida*, zoospores from the infected leaf inoculum were suspended in water (approximately 10<sup>5</sup> spores/ml) and incubated on ice for 30 min. The spore suspension was then sprayed on plants using a Humbrol® spray gun (approximately 700 µl per plant) and plants were incubated at 4°C in the dark overnight to promote spore germination. Infected plants were kept under 10-h light (20°C) and 14-h dark (16°C) cycles. Phenotypes were monitored 14–21 days after inoculation.

### QTL analysis

QTL mapping of the bipartite F8 Oy-0 × Col-0 population (470 RILs, <http://publiclines.versailles.inra.fr/page/27>) (Simon et al., 2008) was performed on a genetic map of 85 markers across the five linkage groups that accompanied the population using R/qtl (Broman et al., 2003). Standard interval mapping using a maximum likelihood estimation under a mixture model (Lander and Botstein, 1989) was applied for interval mapping. Analysis revealed two major QTLs: on chromosome 1 and on chromosome 3.

Chromosome 1 QTL is located between 20 384 and 22 181 Mb (Figure S2). It includes the TIR-NLR cluster *WRR4* and the CC-NLR cluster *RPP7*. Six RILs (three resistant and three susceptible) recombine within the QTL and were used for fine mapping. We designed a SNP (21 195 Mb, forward (F): TCAGATTGTAAC-TGATCTCGAAGG, reverse (R): CCATCAAGCACACTGTATTCC, amplicon contains two SNPs, Oy-0: A and G, Col-0: G and C) and an amplified fragment length polymorphism (21 691 Mb, F: AAGGCAATCAGATTAAGCAGAA, R: GCGGGTTTCCTCAGTTGAAG, Oy-0: 389 bp, Col-0: 399 bp) markers between *WRR4* and *RPP7*. Four lines eliminate *RPP7* from the QTL. The only NLR cluster in chromosome 1 QTL is *WRR4*.

Chromosome 3 QTL is located between 17 283 and 19 628 Mb (Figure S3). It includes the atypical *resistance*-gene cluster *RPW8*, the CC-NLR *ZAR1* and the paired TIR-NLRs *At3g51560-At3g51570*. Six RILs (three resistant and three susceptible) recombine within the QTL and were used for fine mapping. We designed an amplified fragment length polymorphism (18 016 Mb, F: gctacgccactgcatttagc, R: CCAATTCCGCAACAGCTTTA, Oy-0: 950 bp, Col-0: 1677 bp) and a cleaved amplified polymorphic sequence (CAPS, 18 535 Mb, F: TCAAGCCTGTTAAGAAGAAGAAGG, R: GCCCTCCACAAA-GATTCTGAAGTA, enzyme: *DdeI*, Oy-0: uncleaved, Col-0: cleaved) markers between the QTL border and *RPW8*. We designed a CAPS marker (18 850 Mb, F: TCTCGGGGAAAATATGATTAGA, R: GGTTGATTTTTATTGTGGTAGTCGT, enzyme: *SwaI*, Oy-0: cleaved, Col-0: uncleaved) between *RPW8* and *ZAR1*. We designed a SNP (18 937 Mb, F: CCACAAGGTCGGAATCTGTAGC, R: TGCACA-GAAGTAACCCACCAAC, Oy-0: C, Col-0: T) and a CAPS (19 122 Mb, F: ACCACCACCTCGATGCATTTC, R: CCTTCCCTGCGAAAGACACTC, enzyme: *BsrI*, Oy-0: uncleaved, Col-0: cleaved) markers between *ZAR1* and the TIR-NLR pair. Three recombinants eliminate the TIR-NLR pair, two eliminate *ZAR1* and one eliminates *RPW8*. None of the Col-0 NLR clusters orthologs are present in the QTL. The gene underlying chromosome three resistance is located between the border of the QTL and *RPW8*.

### Bulk segregant analysis and RenSeq

We generated an F2 population from a cross between HR-5 (resistant) and Ws-2 (susceptible). We phenol/chloroform extracted

DNA from 200 bulked F2 lines fully susceptible to AcEx1. The bulked DNA sample was prepared as an Illumina library and enriched using the Arabidopsis v1 RenSeq bait library (Arbor Bioscience, MI, USA) (Table S3), as described by (Jupe et al., 2013). The sample was sequenced in a pooled MiSeq run (data available on request). First, reads were aligned with BWA mem (Li and Durbin, 2009) to the Col-0 reference genome and SNPs called with Samtools (Li et al., 2009). The genome was scanned for regions of high linkage with the next generation mapping tools at <http://bar.utoronto.ca/ngm/> (Austin et al., 2011). Secondly, the reads were mapped using BWA to the RenSeq PacBio assembly generated for HR-5 (Van de Weyer et al., 2019). Highly linked regions were confirmed visually with the integrated genome viewer (Robinson et al., 2017).

### Gene cloning

Vectors were cloned with the USER method (NEB, Ipswich, MA, USA) following the manufacturer's recommendations. For expression of resistance gene candidates in Arabidopsis, genes were cloned with their natural 5' and 3' regulatory sequences into LBJJ233-OD (containing a FAST-Red selectable marker, pre-linearized with *PacI* and *Nt. Bbvcl* restriction enzymes). For overexpression in *N. tabacum*, genes were cloned into LBJJ234-OD (containing a FAST-Red selectable marker and a 35S/Ocs expression cassette, pre-linearized with *PacI* and *Nt. Bbvcl* restriction enzymes). Primers, template and vectors are indicated in (Table S4). *WRR4A<sup>Col-0</sup>* is published (Cevik et al., 2019). CCGs recognized by *WRR4A<sup>Col-0</sup>* are published (Redkar et al., 2021).

All the plasmids were prepared using a QIAPREP SPIN MINIPREP KIT on *Escherichia coli* DH10B thermo-competent cells selected with appropriate antibiotics. Positive clones (confirmed by size selection on electrophoresis gel and capillary sequencing) were transformed in *A. thaliana* via *Agrobacterium tumefaciens* strain GV3101. Transgenic seeds were selected under fluorescent microscope for expression of the FAST-Red selectable marker (Shimada et al., 2010).

### CRISPR adenine base editor

An single guide (sg)RNA targeting *WRR4A* stop codon in Col-0 (TTCTGAGaagcattcgaaag[nGA]) was assembled by PCR to a sgRNA backbone and 67 bp of the *U6-26* terminator. It was then assembled with the *AtU6-26* promoter in the Golden Gate compatible level 1 *pICH47751* (Engler et al., 2014). We designed a mutant allele of a plant codon optimized Cas9 with a potato intron (Addgene: 117515) with D10A (nickase mutant) and R1335V/L1111R/D1135V/G1218R/ E1219F/A1322R/T1337R, to change the PAM recognition from NGG to NG (Nishimasu et al., 2018). We assembled this Cas9 (golden gate compatible *Bpil*: GACA-GCTT) along with a barley codon optimized TadA module (golden gate compatible *Bpil*: AATG-GCTT) in a level 0 vector *pICH41308*. It was then assembled with the YAO promoter (Addgene: 117513) and the E9 terminator (Addgene: 117519) in a level 1 vector *pICH47811* (with expression in reverse orientation compared with the other level 1 modules). It was then assembled with a FAST-Red selectable marker (Addgene: 117499) and the sgRNA level 1 cassette into a level 2 vector *pICSL4723*, using the end-linker *pICH41766*. Level 0 vector was cloned using *Bpil* enzyme and spectinomycin resistance. Level 1 vectors were cloned using *Bsal* enzyme and carbenicillin resistance. Level 2 vector was cloned using *Bpil* enzyme and kanamycin resistance. It was expressed via *A. tumefaciens* strain GV3101 in Arabidopsis Oy-0. In the first generation after transformation, we did not detect any mutant from 24 independent transformants. It indicates an absence of activity of the

construct. It can be explained by the Cas9 mutations that were not tested before on this specific allele nor in combination with TadA.

### Transient expression in *Nicotiana tabacum* leaves

*Agrobacterium tumefaciens* strains were streaked on selective media and incubated at 28°C for 24 h. A single colony was transferred to liquid LB medium with appropriate antibiotic and incubated at 28°C for 24 h in a shaking incubator (200 r.p.m.). The resulting culture was centrifuged at 3000 *g* for 5 min and resuspended in infiltration buffer (10 mM MgCl<sub>2</sub>, 10 mM MES, pH 5.6) at OD<sub>600</sub> = 0.4 (2 × 10<sup>8</sup> cfu mL<sup>-1</sup>). For co-expression, each bacterial suspension was adjusted to OD<sub>600</sub> = 0.4 for infiltration. The abaxial surface of 4-week-old *N. tabacum* were infiltrated with 1 ml needle-less syringe. Cell death was monitored 3 days after infiltration.

### Resolution of *WRR4A<sup>Oy-0</sup>* cDNA sequence

RNA was extracted from Oy-0 using the RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free DNase Set (Qiagen, Hilden, Germany). Reverse transcription was carried out using the SuperScript IV Reverse Transcriptase (Thermo Fisher, Waltham, MA, USA). PCR was conducted using F: TCTGATGTCCGCAACCAAC (in the first exon) and R: GTCCTCTTCGGCCATATCTTC (in the last exon) with the Taq polymerase enzyme (NEB) following the manufacturer's protocol. The 2848 nt amplicon sequence, corresponding to the cDNA sequence (i.e. with already spliced introns) was resolved by capillary sequencing. It indicates that the splicing sites are identical between *WRR4A<sup>Oy-0</sup>* and the splicing sites reported in the database TAIR10 for *WRR4A<sup>Col-0</sup>*.

### Gene expression by reverse transcription-PCR

RNA was extracted from leaf tissue using the RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free DNase Set (Qiagen). Reverse transcription was carried out using the SuperScript IV Reverse Transcriptase (Thermo Fisher). PCR was conducted using primers indicated in Table S4 with the Taq Polymerase enzyme (NEB) following the manufacturer's protocol.

### Protein extraction and Western blot

Proteins were extracted from leaf tissue using TruPAGE LDS Sample Buffer (Sigma-Aldrich) following the manufacturer's recommendations. They were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and analysed by immunoblotting. After electrophoresis, separated proteins were transferred to Immobilon-P PVDF (Merck Millipore) membranes for immunoblotting. Membranes were blocked for 2 h in 5% non-fat milk, probed with horseradish peroxidase-conjugated antibodies overnight and imaged.

### Generation of *WRR4A<sup>Oy-0</sup>* transgenic *Camelina sativa* line

We transformed a *WRR4A<sup>Oy-0</sup>* construct, under native promoter and terminator transcriptional regulation and with a FAST-Red selectable marker (Shimada et al., 2010) (see 'Gene cloning' section) in *C. sativa* cv. Celine using the floral dip method (Liu et al., 2012). We obtained four independent T1 lines (Figure S6). Transgenic expression was measured in T1 plants using reverse transcription-quantitative (q)PCR for *WRR4A<sup>Oy-0</sup>*, with *EF1a* as a housekeeping reference gene. We extracted RNA using the RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free DNase Set (Qiagen). Reverse transcription was carried out using SuperScript IV Reverse Transcriptase (Thermo Fisher). qPCR was

performed using a CFX96 Touch Real-Time PCR Detection System. Primers for qPCR analysis of *WRR4A<sup>Oy-0</sup>* are GCAAGATAGC-GAGCTCCAGA and GCAAGAAACATACAAGTCCTCCA. Primers for qPCR analysis of *EF1a* are CAGGCTGATTGTGCTGTTCTTA and GTTGTATCCGACCTTCTTCAGG. Data were analysed using the double delta  $C_t$  method (Livak and Schmittgen, 2001). We measured the segregation of FAST-Red in T2 seeds. Two lines are segregating 15:1 indicating a dual loci T-DNA insertion and two are segregating 3:1 indicating a single locus insertion. From the two single-locus insertion lines, we obtained five and four AcEx1 resistant lines, without any symptoms of infection. From these nine lines, one produced a bag of homozygous T3 seeds (the others producing no seeds or 3:1 segregating seeds). Twelve plants from this line were tested with AcEx1, 11 showed resistance (eight without symptoms and three with a chlorotic response) and one showed susceptibility (Figure 5).

### ACCESSION NUMBERS

Arabidopsis accessions used in this study are (NASC: N1436), HR-5 (NASC: N76514), Ws-2 (NASC: N1601), and Col-0 (NASC: N1092).

### ACKNOWLEDGEMENTS

We thank the Gatsby Foundation (UK) for funding to the Jones lab. We thank Mark Youles in TSL Synbio for his excellent support with Golden Gate cloning and for providing modules. This research was supported in part by the NBI Computing infrastructure for Science (CiS) group and Dan MacLean's group by providing computational infrastructure. BC, SF, OF, and VC were supported by Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/L011646/1. AR was supported by EMBO LTF (ALTF-842- 2015). BC, SW, and JDGJ were supported in part by ERC Advanced Investigator grant to JDGJ 'ImmunityByPairDesign' Project ID 669926.

### AUTHOR CONTRIBUTIONS

BC, SF, OF, AR, VC, EH, and JJ designed research; BC, SF, OF, AR, SW, and VC performed research; BC, SF, OF, AR, VC, EH, and JJ analysed data; and BC and JJ wrote the paper. All authors read and approved the final manuscript.

### CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

### DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials. The sequences of the genomic clones of *WRR4A<sup>Oy-0</sup>* and *WRR4A<sup>HR-5</sup>* are deposited at NCBI GenBank as MW533532 and MW533533 respectively.

### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Detailed map of candidate loci in Oy-0.

**Figure S2.** Fine mapping of chromosome 1 QTL.

**Figure S3.** Fine mapping of chromosome 3 QTL.

**Figure S4.** Detailed map of candidate loci in HR-5.

**Figure S5.** Phylogeny of *WRR4A* based on protein sequences.

**Figure S6.** Selection of a stable *WRR4A<sup>Oy-0</sup>* transgenic *Camelina sativa* line for resistance to AcEx1.

**Figure S7.** Map of Arabidopsis accessions used in this study.

**Table S1.** Summary of the *A. candida* isolates mentioned in this study.

**Table S2.** Phenotype of 283 Arabidopsis accessions in response to AcEx1 infection.

**Table S3.** Arabidopsis v1 RenSeq bait library (Arbor Bioscience, MI, USA) as described by (Jupe et al., 2013).

**Table S4.** Primers used in this study.

**Dataset S1.** Alignment of *WRR4A* nucleotide sequences used to generate the phylogenetic tree on Figure 4. File is on fasta format and was generated using the software MEGA10, with using the recommended parameters of MUSCLE.

**Dataset S2.** Alignment of *WRR4A* amino acid sequence used to generate the phylogenetic tree on Figure S5. File is on fasta format and was generated using the software MEGA10, with using the recommended parameters of MUSCLE.

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