Identification of Novel Sources of Resistance to *Ascochyta* Blight in a Collection of Wild *Cicer* Accessions

Toby E. Newman,¹ Silke Jacques,¹ Christy Grime,¹ Fiona L. Kamphuis,¹ Robert C. Lee,¹ Jens Berger,² and Lars G. Kamphuis^{1,†}

¹ Centre for Crop and Disease Management, School of Molecular and Life Sciences, Curtin University, Bentley, Western Australia, Australia

² Commonwealth Scientific and Industrial Research Organisation (CSIRO), Agriculture and Food, Floreat, Western Australia, Australia

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ABSTRACT

Chickpea production is constrained worldwide by the necrotrophic fungal pathogen *Ascochyta rabiei*, the causal agent of *Ascochyta* blight (AB). To reduce the impact of this disease, novel sources of resistance are required in chickpea cultivars. Here, we screened a new collection of wild *Cicer* accessions for AB resistance and identified accessions resistant to multiple, highly pathogenic isolates. In addition to this, analyses demonstrated that some collection sites of *C. echinospermum* harbor predominantly resistant accessions, knowledge that can inform future collection missions. Furthermore, a genome-wide association study

identified regions of the *C. reticulatum* genome associated with AB resistance and investigation of these regions identified candidate resistance genes. Taken together, these results can be utilized to enhance the resistance of chickpea cultivars to this globally yield-limiting disease.

Keywords: *Ascochyta* blight, *Ascochyta rabiei*, *Cicer echinospermum*, *Cicer reticulatum*, crop wild relatives, *Didymella rabiei*, disease resistance, genetics, germplasm collection, *Phoma rabiei*, resistance, wild chickpea

Chickpea (*Cicer arietinum*) is one of the most important legume crops and is produced and consumed worldwide. Chickpea represents an important part of the human diet, particularly on the Indian subcontinent where it is one of the major sources of protein and micronutrients. India is the largest chickpea producer and produced approximately 11.4 million tons in 2018 (Food and Agriculture Organization of the United Nations 2018). The second largest producer and largest exporter, Australia, produced approximately 1.1 million tons of chickpea in 2018 (Food and Agriculture Organization of the United Nations 2018; Merga and Haji 2019).

However, the yield potential of chickpea cannot be reached as a result of many biotic and abiotic stresses, one of the most damaging being *Ascochyta* blight (AB) disease caused by the fungal pathogen *Ascochyta rabiei*. *A. rabiei* is a necrotrophic pathogen, which kills and feeds off dead tissue. Given conducive environmental conditions, disease symptoms can affect all aerial parts of the plant. This can result in loss of photosynthetic capacity of leaves, stem girdling and breakage, pod abortion, seed staining, and plant death (Nene 1982). A hallmark of AB is the appearance of concentric rings of pycnidia on lesions (Pande et al. 2005). AB is a major disease of chickpea globally and severe infection can cause a yield loss of up to 100% (Nene and Reddy 1987). In Australia alone, AB costs an average of \$4.8 million annually (Murray and Brennan 2012).

Prior to 2015, there were multiple resistant and moderately resistant cultivars available for Australian growers, including PBA Slasher and PBA HatTrick. The outbreak of a more aggressive strain

in Curyo, Victoria, in 2015, however, has rendered the majority of current cultivars either moderately susceptible or susceptible to this new strain (Australian Grains Research and Development Corporation 2017). Highly aggressive isolates are now widely distributed across the chickpea growing regions of Australia and are described as those that cause high levels of disease on formerly resistant and moderately resistant Australian varieties, PBA HatTrick and Genesis 090, respectively, and moderate levels on the resistant landrace ICC3996 (Mehmood et al. 2017).

To improve chickpea cultivar resistance to this devastating disease, novel sources of resistance are required. The improvement of crop species is often limited by a lack of genetic diversity, as a result of genetic bottlenecks associated with domestication. This problem is particularly pertinent to chickpea because of four primary evolutionary bottlenecks. In brief, domesticated chickpea suffers from a lack of genetic diversity as a result of restricted distribution of the wild progenitor *C. reticulatum*, the founder effect, a change from autumn to spring sowing in the Early Bronze Age, and the replacement of landraces by elite cultivars (Abbo et al. 2003). It has been estimated that approximately 93.5 to 97.5% of the genetic diversity present in the wild progenitor, *C. reticulatum*, is absent from contemporary chickpea breeding programs (von Wettberg et al. 2018). Further genetic diversity exists in the gene pool of *C. echinospermum*, a sister species of chickpea, which can be crossed with chickpea to produce fertile offspring, although fertility issues may arise (Pundir and Mengesha 1995). Exploitation of this vast amount of additional genetic diversity may hold the key to chickpea improvement.

Until 2013, the world collection of wild *Cicer* species was very poor. The entire world collection of primary gene pool accessions was made up of only 18 and 10 original accessions of *C. reticulatum* and *C. echinospermum*, respectively (Berger et al. 2003). However, in 2013, a collection mission to the site of chickpea domestication in southeastern Turkey increased the collection significantly to a total of 371 accessions. This mission sampled a large part of the *C. reticulatum* and *C. echinospermum* distribution in southeastern Anatolia from Adiyaman in the west to Sirnak in the east, Urfa in the south, and Bingol in the north, covering a range of rainfall, temperature, altitudes, and soil types (von Wettberg et al. 2018).

[†]Corresponding author: L. G. Kamphuis; lars.kamphuis@curtin.edu.au

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Accessions were collected on an individual plant basis, facilitating within and between collection site analyses. This collection has been demonstrated to harbor traits of interest for chickpea improvement such as water use efficiency, nutrient content, pod borer (*Helicoverpa armigera*) resistance, heat stress tolerance, and root-lesion nematode (*Pratylenchus thornei*) resistance (Reen et al. 2019; von Wettberg et al. 2018).

We set out to screen this collection for novel sources of AB resistance, describing differences between species, collection sites within species, and accessions within collection sites within species. The results described herein demonstrate that this collection of wild *Cicer* constitutes a valuable source of genetic resistance to AB, as we have identified *C. reticulatum* and *C. echinospermum* accessions with resistance to multiple highly virulent Australian *A. rabiei* isolates from distinct phylogenetic groups. This study also identified collection sites with predominantly resistant accessions and genetic loci associated with AB resistance. Collectively, these results can contribute toward the development of future chickpea cultivars with enhanced resistance.

MATERIALS AND METHODS

Plant and fungal materials. A total of 197 annual wild *Cicer* accessions comprising 149 *C. reticulatum* and 48 *C. echinospermum* accessions were acquired from the Australian Grain Genebank in Horsham, Victoria. These accessions were collected from southeastern Turkey in 2013 by the Commonwealth Scientific and Industrial Research Organisation and the University of California, Davis (UCD) (von Wettberg et al. 2018). The accessions were derived from five Turkish provinces: Adiyaman, Mardin, Sirnak, Diyarbakir, and Urfa. These provinces constituted 21 distinct collection sites, with *C. reticulatum* accessions collected from 15 sites and *C. echinospermum* accessions from six sites. From each site, between 1 and 21 individual accessions were collected (Table 1). Australian desi *C. arietinum* cultivars PBA HatTrick, PBA Slasher, Howzat, Kyabra, and Moti were included in experiments as controls ranging in resistance to *A. rabiei*. Prior to 2015, PBA Slasher was rated as resistant, PBA HatTrick as moderately resistant/resistant, Howzat as susceptible, Kyabra as susceptible, and Moti as very susceptible (Australian Grains Research and Development Corporation 2017; Moore et al. 2015a).

To newly evolved strains, the varietal resistance ratings of PBA Slasher, PBA HatTrick, and Howzat have changed to moderately susceptible, susceptible, and susceptible, respectively. The Iranian *C. arietinum* landrace ICC3996 was also included as a resistant reference line. Notably, ICC3996 has been used in breeding programs as a source of resistance to AB, including in the pedigree of the cultivars PBA Slasher and PBA HatTrick (Li et al. 2017).

Seeds of the wild *Cicer* accessions were scarified and pregerminated on wet filter paper prior to sowing. The wild *Cicer* germplasm was evaluated in a series of sequential experiments. In each of these experiments, the wild *Cicer* accessions and *C. arietinum* controls were arranged in a randomized complete block design (RCBD) with three plants per accession/cultivar per pot and three blocks (RCBD, $n = 3$). Plants were grown at 22°C under a 12-h light/12-h dark photoperiod in a controlled environment growth room. *A. rabiei* isolate ME14 was collected by Dr. Theo Pfaff from a chickpea crop grown in 2005 at the Department of Agriculture and Food Western Australia field station in Medina, Western Australia. Four highly aggressive isolates belonging to four distinct phylogenetic groups, as determined by Illumina sequencing, were also selected for infection assays: TR6417, 15CUR002, 15CUR005, and 16CUR017 (I. Bar et al., *unpublished data*; Mehmood et al. 2017). These isolates cause high levels of disease on PBA HatTrick and the kabuli cultivar Genesis 090 and moderate levels on the resistant landrace ICC3996 (Mehmood et al. 2017). In addition to these, another highly aggressive isolate, F17191-1, collected in 2017 from Port Broughton, South Australia, was included in our assays. F17191-1 has been reported to have overcome ICC3996 resistance (R. Ford, *personal communication*). The infection assays using these isolates allowed us to screen for resistance to multiple highly aggressive isolates. These isolates (15CUR005, TR6417, 15CUR002, 16CUR017, and F17191-1) are part of the extensive isolate collection maintained and kindly provided by Professor Rebecca Ford (Griffith University, Queensland, Australia) as single spore cultures (I. Bar et al., *unpublished data*; Mehmood et al. 2017). *A. rabiei* isolates were cultured on one-half-strength potato dextrose agar plates supplemented with streptomycin (100 µg/ml) at room temperature under a 12-h ultraviolet-A light/12-h dark photoperiod.

Infection assay and disease scoring. One hundred forty-six *C. reticulatum* and 44 *C. echinospermum* accessions were initially

TABLE 1. Collection site in Turkey and accession information on the wild *Cicer* accessions screened for Ascochyta blight resistance

Province	Collection site	Species	Accessions (n)	Prefix	Suffix and accession code number
Adiyaman	Oyali	<i>C. reticulatum</i>	11	Oyali	071, 073, 076, 081, 084, 085, 100, 101, 104, 105, 107
Mardin	Baristepe 1	<i>C. reticulatum</i>	8	Bari1	062, 063, 064, 068, 069, 091, 092, 093
	Baristepe 2	<i>C. reticulatum</i>	5	Bari2	062, 064, 067, 072, 074
	Baristepe 3	<i>C. reticulatum</i>	18	Bari3	064, 065, 067, 072C, 073, 074, 075, 079, 091, 092, 100, 101, 102, 103, 104, 106D, 110, 112
Sirnak	Besevler	<i>C. reticulatum</i>	8	Besev	061, 062, 065, 066, 074, 075, 079, 083
	Dereici	<i>C. reticulatum</i>	9	Derei	062, 065, 066, 069, 070, 072, 073, 074, 075
	Kayatape	<i>C. reticulatum</i>	7	Kayat	061, 063, 064, 066, 070, 077, 080
	Sarikaya	<i>C. reticulatum</i>	10	Sarik	061, 064, 065, 066, 067, 073, 074, 077, 078, 080
	Savur	<i>C. reticulatum</i>	1	Savur	063
	CudiB	<i>C. reticulatum</i>	12	CudiB	004, 005, 006, 008B, 009, 011, 016, 017, 018, 019, 022C, 023
	CudiA	<i>C. reticulatum</i>	14	CudiA	101A, 103C, 104, 105, 122, 124, 127, 128, 151, 152, 153, 154, 155, 221
Diyarbakir	Sirnak	<i>C. reticulatum</i>	21	Sirna	060, 063, 064, 066, 067, 069, 070, 071C, 081B, 082, 083, 085, 087, 088, 089B, 090, 101, 103, 105, 110, 111A
	Kesentas	<i>C. reticulatum</i>	11	Kesen	062, 065, 066, 067, 071, 073, 074, 075, 077, 101, 104
	Egil	<i>C. reticulatum</i>	6	Egil	063, 065, 066, 073, 074, 075
	Kalkan	<i>C. reticulatum</i>	7	Kalka	061, 064, 065, 066, 067, 070, 074
	Gunasan	<i>C. echinospermum</i>	3	Gunas	061, 062, 100
Urfa	Cermik	<i>C. echinospermum</i>	6	Cermi	061, 063, 071, 072, 073, 075
	Destek	<i>C. echinospermum</i>	12	Deste	061, 063, 064, 066, 071, 072A, 073, 075, 077, 078, 079, 080
	Siv-Diyar	<i>C. echinospermum</i>	10	S2Drd	061, 062, 065, 100 101, 102, 104, 105, 107B, 109
	Karabahce	<i>C. echinospermum</i>	15	Karab	062A, 063, 066A, 067, 081A, 082, 084, 085C, 086, 091B, 092, 093, 162, 164, 172
	Ortanca	<i>C. echinospermum</i>	2	Ortan	061, 066

screened for seedling resistance to two Australian *A. rabiei* isolates with differing aggressiveness, namely ME14 and 15CUR005, with the aim of identifying accessions with resistance to both isolates. A further three *C. reticulatum* accessions (Oyali_085, Oyali_087, and Sirna_083) and four *C. echinospermum* accessions (Deste_071, Deste_073, Karab_066A, and Ortan_066) were screened for resistance to just one of the isolates as a result of poor seed germination. Six desi chickpea (*C. arietinum*) lines with differential AB resistance were included as controls. These chickpea lines were the aforementioned Iranian landrace ICC3996 and the Australian cultivars PBA Slasher, PBA HatTrick, Howzat, Kyabra, and Moti.

Five-day-old cultures of *A. rabiei* were used to prepare spore suspensions for inoculation. In short, sterile purified water was poured on the plates and pycnidiospores were harvested by gentle rubbing with the end of a syringe plunger. The recovered suspensions were filtered through cotton wool and spores were diluted to the appropriate concentration using a hemocytometer. The aforementioned wild *Cicer* collection was screened with *A. rabiei* isolates ME14 and 15CUR005 at a concentration of 5×10^5 spores/ml. Then 0.05% Tween 20 was added to the spore suspensions before spray inoculation of 2-week-old seedlings with a handheld atomizer until runoff. Plants were misted using a commercial Holman misting system (product number MK21013) for 20 s every 2 h in order to provide leaf wetness and promote infection for the first 3 days postinoculation (dpi). The misting system provided a fine mist of water above the seedlings. From 3 dpi, plants were watered by hand. At 11 dpi, disease symptoms were scored as an approximate percentage of overall aerial tissue affected by observation (referred to as plant area damage). The wild *Cicer* germplasm was evaluated sequentially over six experiments without overlap between experiments, whereas the chickpea controls were present in all six experiments and over-replicated ($n = 6$ to 9) relative to the wild material ($n = 3$).

After initial evaluation of resistance to the *A. rabiei* isolates ME14 and 15CUR005, 24 wild *Cicer* accessions were selected to be screened with TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1 to identify accessions with resistance to multiple, highly virulent isolates. Selection was based on the two *C. reticulatum* accessions and two *C. echinospermum* accessions with the lowest average ME14- and 15CUR005-induced plant area damage in each of the six previous experiments. The same six *C. arietinum* lines as above were included as controls. 15CUR005 was already used in the initial screen; however, pycnidiospores of each of the isolates were prepared and spray inoculated at double the concentration of the initial screen in order to expose plants to a higher level of disease pressure. The five isolates were prepared and inoculated separately, as described above, except spores were inoculated at a concentration of 10^6 spores/ml. In these assays, leaf and stem damage were scored separately as a percentage of total area with visible disease symptoms.

Screening germplasm collections with single isolates is an efficient method to identify promising sources of resistance to diseases; however, crop varieties are exposed to multiple isolates when cultivated in the field. To more closely mimic this, we also infected the accessions with a cocktail of isolates mixed in equal spore concentrations with a total concentration of 10^6 spores/ml. The cocktail comprised the five highly aggressive isolates that had been inoculated individually in the earlier experiments described above: TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1. The infection assay was conducted as described above, and leaf damage and stem damage were scored separately as a percentage of total area with visible disease symptoms.

Mean values across biological replicates for each isolate were calculated for each accession. Mean values, box and whisker plots, and violin plots were generated for each species against each isolate and visualized using ggplot in R software. Statistical differences between mean values ($P < 0.05$ and 0.01) were determined by a one-way analysis of variance (ANOVA) and post hoc Tukey-Kramer test

or Student *t* test. The mean percentages of leaf and stem disease symptoms caused by TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1 on the resistant subset were subsequently used to assign the accessions to the ranking scale from 1 to 9 developed by Collard et al. (2001).

ANOVA. ANOVA routines in Genstat version 20 were used to analyze the data, using residual plots to check for normality and identify outliers within each experiment. Analysis of the balanced chickpea dataset demonstrated the absence of three-way interactions between cultivars, isolates, and experiments ($P = 0.772$), indicating consistent cultivar performance across experiments and suggesting that it was safe to pool the data. The pooled data were analyzed by nested unbalanced ANOVA partitioning variance between species, collection sites within species, and accessions within collection sites within species.

Genome-wide association study. Restriction site-associated DNA sequencing (RAD-seq) data were provided by UCD and can be found in the NCBI umbrella BioProject (PRJNA353637), which includes Illumina data from genotyping by sequencing of $>1,000$ wild *Cicer* accessions (PRJNA416006). In brief, the RAD-seq data were generated by digesting genomic DNA from each accession with *Hind*III and *Mla*III, ligating to adapters and sequencing as 100-bp reads on an Illumina HiSeq4000 system (von Wettberg et al. 2018). The sequencing data were first checked with fastqc (Andrews 2010). The Stacks module process_radtags was then used to check for an intact RAD restriction site and to discard reads with poor sequencing quality (average raw Phred score of <10 in a sliding window 15% of the read length). Reads were aligned to a *C. reticulatum* reference genome (accession PI489777 V2.0) using Bowtie2 where MAPQ scores were ≥ 30 (Gupta et al. 2017; Langmead and Salzberg 2012). The Stacks module gstacks was used to create loci using reads that aligned to the reference genome and to identify single nucleotide polymorphisms (SNPs). The Stacks module populations was then used to output a SNP file filtered for minimum minor allele frequency (>0.05) and minimum number of individuals required to process a locus (>0.95) (Rochette and Catchen 2017).

The STRUCTURE software package was used to output a population structure file ($K = 8$) (Pritchard et al. 2000). The mean plant area damage values of *C. reticulatum* accessions from the initial screen with ME14 and 15CUR005 were normalized to the mean plant area damage of the susceptible control Kyabra in each respective experiment to control for variation between experiments. The GAPIT R package was then used to conduct the genome-wide association study (GWAS) with the generated SNP file and the normalized AB disease data using a multilocus mixed model (MLMM), as a multilocus model may enable a higher power of detection of trait-associated SNPs and a lower false discovery rate compared with single-locus models (Lipka et al. 2012; Segura et al. 2012; Tang et al. 2016). STRUCTURE population analysis and a kinship matrix for the analysis generated using the VanRaden (2008) method were added as covariates. The significance threshold was set using a Bonferroni-corrected P value of 0.05. A mixed linear model (MLM) was then used to calculate heritability (h^2) and r^2 values for the trait-associated SNPs, as these are not produced using the MLMM.

Candidate resistance gene identification. Gene annotations within a 10-kb window from each trait-associated SNP were identified in the *C. reticulatum* PI489777 V2.0 genome using JBrowse on pulsedb.org (Skinner et al. 2009). The coding sequences (CDSs) were extracted. Using Geneious Prime version 2019.2.1, the CDSs were translated into amino acid sequences and the protein families (Pfam) database was searched for putative domains to infer function (Bateman et al. 2002; Kears et al. 2012). BLASTp (protein-protein BLAST) was also conducted to identify close homologs (Johnson et al. 2008).

Using 30-bp regions surrounding each SNP (15 bp on either side of each SNP), a BLAST search was conducted on the *C. arietinum*

CDC Frontier version 1.0 genome to identify corresponding positions in the chickpea genome for comparison with known AB resistance quantitative trait loci (QTLs) (Johnson et al. 2008; Varshney et al. 2013).

RESULTS

Wild *Cicer* species exhibit variation in AB resistance.

The collection of wild *Cicer* accessions were screened for their resistance to *A. rabiei* isolates ME14 and 15CUR005. Extensive variation was observed in disease symptom development after spray inoculation of *A. rabiei* ME14 and 15CUR005 pycnidiospores, with visible disease symptoms ranging from 0 to 100% of the aerial plant tissue affected (Fig. 1; Supplementary Fig. S1; Supplementary Table S1). Frequency distribution charts illustrate the range of plant area damage caused by ME14 and 15CUR005 (Fig. 1A and B).

To further investigate the effect of each isolate on the two wild *Cicer* species, a violin plot was generated to compare the damage caused by each isolate on each set of wild species (Fig. 1C). Inoculation with *A. rabiei* isolate 15CUR005 induced a mean plant area damage of 28% and a median plant area damage of 20% on *C. echinospermum* accessions. However, 15CUR005 induced a mean plant area damage of 42% and a median plant area damage of 34% on *C. reticulatum* accessions. A one-way ANOVA with post hoc Tukey-Kramer test demonstrated that damage caused by 15CUR005 on *C. echinospermum* accessions was significantly lower than damage caused by 15CUR005 on *C. reticulatum* accessions, indicating that *C. echinospermum* accessions are generally more resistant than *C. reticulatum* accessions to this isolate ($P < 0.01$). Inoculation with *A. rabiei* isolate ME14 induced a mean plant area damage of 35% and a median plant area damage of 22% on *C. echinospermum* accessions. The ME14 isolate induced a mean plant area damage of 42% and a median plant area damage of 39% on *C. reticulatum* accessions. Despite the lower median, there was

no significant difference between *C. echinospermum* accessions and *C. reticulatum* accessions for resistance to ME14 (Fig. 1C). This screen demonstrated that this novel collection of wild *Cicer* accessions harbors some accessions with promising levels of AB resistance for further analysis.

***C. echinospermum* accessions from specific collection sites are predominantly resistant to AB.** To dissect the results of the aforementioned AB resistance screen further, we used ANOVA and regression to partition variance between species, collection sites within species, and accessions within collection sites within species (Fig. 2; Supplementary Fig. S1). Chickpea controls were balanced across experiments. Analysis of the balanced chickpea dataset demonstrated the absence of three-way interactions between cultivars, isolates, and experiments ($P = 0.772$), indicating consistent genotype performance across experiments. Therefore, the data could reliably be pooled and analyzed together. Moreover, there was support for common variance among the six experiments, as evidenced by the narrow range of least significant difference (LSD) values returned by separate analyses (minimum to maximum LSD = 20.9 to 26.4).

Interestingly, there was some isolate by accession interaction within domesticated chickpea ($P < 0.001$) (Fig. 2, red squares). The most resistant genotypes (ICC3996, PBA HatTrick, and PBA Slasher) were damaged more by 15CUR005 than by ME14, while the opposite was the case for Moti, the most susceptible cultivar. Howzat and Kyabra had intermediate damage scores with no differences between isolates. Despite this interaction, there was a strong linear relationship between the two isolates in chickpea response, such that resistance to ME14 was proportional to resistance to 15CUR005 in the domesticated chickpea lines.

By contrast, there was much more deviation from the wild *Cicer* regression lines (Fig. 2), indicating significant isolate interactions. Both *C. echinospermum* and *C. reticulatum* contain accessions that are relatively resistant to 15CUR005 but susceptible to ME14 (Fig.

Fig. 1. Disease induced by *Ascochyta rabiei* isolates ME14 and 15CUR005 on wild accessions. **A**, Frequency distribution of plant area damage caused by *A. rabiei* isolate ME14. **B**, Frequency distribution of plant area damage caused by *A. rabiei* isolate 15CUR005. For A and B, the x-axis shows the percentage of plant area damage and the y-axis lists number of accessions. *C. arietinum* is shown in gray, *C. reticulatum* in black, and *C. echinospermum* in white. **C**, Violin plots displaying the plant area damage in *C. reticulatum* and *C. echinospermum* caused by ME14 and 15CUR005. The x-axis displays the species and isolate treatment and the y-axis displays the percentage of plant area damage. Black dots represent the mean values and within each violin plot is a box and whisker plot. Colored dots represent mean plant area damage for individual accessions. Letters above the plot (a, b) indicate significant differences in the mean values ($P < 0.05$, one-way analysis of variance with post hoc Tukey-Kramer test).

2), and a smaller number where the opposite is the case. Identifying accessions by collection site gives insight into the nested interactions of isolate by accession within collection site within species. In *C. echinospermum*, there is evidence for both differences between and within collection sites in different interaction patterns: Karatepe (Kara) and most Cermik (Cer) accessions have a wide range of plant area damage values where the two isolates are proportional, Ortanca (Kurt) and Siverek-Diyarbakir (Sivdiy) accessions are relatively resistant to 15CUR005 but not ME14 or vice versa, and most accessions from Destek (Dest) and Gunasan (Gun) were resistant to both isolates (Fig. 2A).

In *C. reticulatum*, however, the variance is largely distributed within collection sites and resistance to both isolates is broadly proportional, reflected in higher r^2 values for the *C. reticulatum* regression equation ($r^2 = 0.32$) compared with *C. echinospermum* ($r^2 = 0.17$) (Fig. 2). Thus, most collection sites return a wide range of plant area damage values, where damage caused by 15CUR005 is similar to that caused by ME14 (Fig. 2B). Specifically adapted outliers are the exception to this proportional trend but are not well defined by collection site. For example, Oyalı (Oya), Kesentas (Kes), and Besevler (Bes) collection sites all include outlying accessions resistant to 15CUR005 that are susceptible to ME14, whereas the remaining accessions screened follow the proportional trend. Sarıkaya (Sar) is arguably the exception, containing a high proportion of accessions that are susceptible to ME14 across a range of resistance to 15CUR005.

To summarize the key findings, two *C. echinospermum* collection sites harbor predominantly resistant accessions and *C. reticulatum* collection sites harbor accessions with a wide range of AB resistance.

Wild *Cicer* accessions exhibit resistance to multiple, highly virulent Australian *A. rabiei* isolates from distinct phylogenetic groups. A subset of wild *Cicer* accessions selected from the initial screen based on their resistance to ME14 and 15CUR005 were assayed for their resistance to additional aggressive isolates. Leaf and stem area damage of the resistant wild *Cicer* subset of 24 accessions and *C. arietinum* controls induced by TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1 inoculated individually and as a cocktail are presented in Tables 2 and 3. Values in the “rating” column represent the resistance rating using a modified 1 to 9 scale developed by Collard et al. (2001). There was a high correlation between average leaf and stem area damage and

between leaf and stem area damage caused by the cocktail ($r^2 = 0.76$ and 0.62 , respectively). Correlation graphs illustrate the positive correlations (Supplementary Fig. S2C and D). Notably, several accessions displayed better leaf resistance than the resistant Iranian landrace ICC3996 to individual isolates. For example, the *C. echinospermum* accession S2Drd_061 exhibited stronger leaf resistance to *A. rabiei* isolates 15CUR002 ($P < 0.05$) and F17191-1 ($P < 0.01$), whereas the *C. echinospermum* accession Karab_063 exhibited stronger leaf resistance to TR6417 ($P < 0.05$) and 15CUR005 ($P < 0.05$) (Table 2).

The disease severity induced by the cocktail of isolates showed moderate correlation with the average disease severity by single isolates for both stem and leaf area damage ($r^2 = 0.48$ and 0.46 , respectively). The correlation graphs for these data are provided in Supplementary Figure S2A and B. However, Gunas_100 was moderately resistant to individual isolates on average, but susceptible to the cocktail of isolates, based on leaf disease symptoms ($P < 0.05$) (Table 2).

The most resistant domestic chickpea line tested, ICC3996, exhibited moderate leaf susceptibility but strong stem resistance to the cocktail of five isolates. Three wild accessions exhibited leaf resistance to the cocktail: *C. echinospermum* accessions S2Drd_061 and Deste_064 and *C. reticulatum* accession Bari1_062. These three accessions had significantly less leaf area damage than ICC3996, as determined by a Student *t* test (S2Drd_061 and Deste_064: $P < 0.05$; Bari1_062: $P < 0.01$). These three accessions also exhibited strong stem resistance to the *A. rabiei* cocktail, although this was not significantly better than ICC3996 stem resistance. Accession Karab_063, which exhibited highly resistant stems and leaves resistant to individual isolates, could unfortunately not be assayed against the cocktail of isolates as a result of poor seed germination (Tables 2 and 3).

Four *C. reticulatum* genomic regions are strongly associated with AB resistance. To elucidate the genetic basis of resistance in the wild *Cicer* accessions, we undertook a GWAS. Considering the relatively small sample size of *C. echinospermum* accessions, we focused solely on *C. reticulatum*. We utilized RAD-seq data described by von Wettberg et al. (2018), which was filtered and aligned to a *C. reticulatum* genome (accession PI489777 V2.0), available in NCBI (Gupta et al. 2017). Using this alignment, a filtered genotype file was generated with 12,740 polymorphic loci

Fig. 2. Interaction between collection sites of wild *Cicer* accessions and plant area damage values. The x-axis shows the percentage of 15CUR005 plant area damage and the y-axis shows the percentage of ME14 plant area damage. Red dashed lines are the *C. arietinum* linear regression lines; 1:1 lines are shown in black for comparison with the linear regression lines. **A**, *C. echinospermum* plant area damage with each collection site assigned a different color. The green dashed line is the *C. echinospermum* linear regression line. LSD = least significant difference, Cer = Cermik, Dest = Destek, Gun = Gunasan, Kara = Karabaha, Kurt = Ortanca, and Sivdiy = Siv-Diyar. **B**, *C. reticulatum* plant area damage with each collection site assigned a different color. The yellow dashed line is the *C. reticulatum* linear regression line. Bar = Baristepe 1, Bes = Besevler, Cudi1 = CudiB, Cudi2 = CudiA, Der = Dereici, Eg = Egil, Kalk = Kalkan, Kay = Kayatape, Kes = Kesentas, Muk = Baristepe 2, Oya = Oyalı, Sal = Baristepe 3, Sav = Savur, Sir = Sirmak, and Sar = Sarıkaya.

(total of 138 accessions) (Supplementary Table S2). The marker density per megabase for each of the eight chromosomes ranged from 25.4 to 43.1 markers/Mb with a mean of 33.1 markers/Mb (Supplementary Table S3). The normalized ME14 plant area damage values had a mean of 0.81 (equivalent to 81% of the damage caused on the susceptible control Kyabra), a standard deviation of 0.43, and a coefficient of variation of 52.1%. The normalized 15CUR005 plant area damage values had a mean of 0.71 (equivalent to 71% of the damage caused on the susceptible control Kyabra), a standard deviation of 0.34, and a coefficient of variation of 47.6%. The heritability of the traits was 36.7 and 30.5% for ME14 and 15CUR005, respectively (Supplementary Table S4). The GAPIT software package in R was used to conduct the GWAS by identifying associations between the genotypes and resistance to ME14 and 15CUR005 in the *C. reticulatum* accessions using a MLM approach (Lipka et al. 2012). STRUCTURE software was used to generate a population structure Q matrix, which was added as a fixed covariate. The number of populations was set as $K = 8$, as the *C. reticulatum* collection was previously demonstrated to comprise eight well-supported populations (von Wettberg et al. 2018). A kinship matrix was also added as a covariate; this was generated using the VanRaden (2008) method (Supplementary Fig. S3). Using a stringent significance threshold of a Bonferroni-corrected P value of 0.05 (3.9×10^{-6}), three SNPs were significantly associated with resistance to *A. rabiei* isolate ME14. Furthermore, a distinct SNP was found to be associated with resistance to

15CUR005. The MLM accounted well for population structure and familial relatedness, as indicated by the quantile-quantile plots (Supplementary Fig. S4). Because the MLM cannot produce r^2 values, we used an MLM to estimate the proportion of variance explained (PVE) by each SNP using the r^2 of the model with and without the respective SNPs. The PVE by the three SNPs associated with ME14 resistance was 6.77, 7.79, and 8.72%, accounting for a total of 23.28% of the phenotypic variance. The PVE by the SNP associated with 15CUR005 resistance was 15.21%. The major alleles of the SNPs on chromosomes 1, 3, and 6 are favorable for AB resistance, whereas the minor allele of the SNP on chromosome 4 is favorable for AB resistance (Table 4).

The *C. reticulatum* SNPs associated with resistance to ME14 reside on chromosome 1 (position 37,902,568), chromosome 4 (position 10,375,894), and chromosome 6 (position 3,610,729) (Fig. 3A; Table 4). The *C. reticulatum* SNP associated with resistance to 15CUR005 resides on chromosome 3 (position 42,986,120) (Fig. 3B; Table 4). The *C. reticulatum* PI489777 V2.0 genome was investigated for candidate resistance genes using JBrowse on pulsedb.org (Skinner et al. 2009). Candidate resistance genes were identified within 10 kb from each SNP, as performed by Xie et al. (2018). Two to four genes were identified within this region for each SNP association (Supplementary Table S5). Based on their predicted Pfam domains and closest BLASTp homologs, the strongest candidate genes for each of the SNPs were selected. For the SNP associated with resistance to ME14 on chromosome 1

TABLE 2. Leaf area damage caused by aggressive *Ascochyta rabiei* isolates and a cocktail of the isolates on a subset of wild *Cicer* accessions and *C. arietinum* controls

Accession ^a	Species	Leaf ^b													
		TR6417		15CUR002		15CUR005		16CUR017		F17191-1		Mean ^c		Cocktail	
		<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating
S2Drd_061	<i>C. echinospermum</i>	33	5	25*	4	9	2	10	2	4**	2	16**	3	16*	3
Karab_063	<i>C. echinospermum</i>	21*	4	36	5	2*	2	10	2	26	4	19*	3	ND	–
Deste_064	<i>C. echinospermum</i>	41	6	15*	3	ND	–	6	2	16*	3	19	3	13*	3
Bari1_062	<i>C. reticulatum</i>	57	7	42	6	4	2	11	3	11**	3	25	4	11**	3
Deste_061	<i>C. echinospermum</i>	42	6	41	6	7	2	8	2	38	5	27	4	26	4
Deste_079	<i>C. echinospermum</i>	44	6	49	6	4	2	9	2	32	5	28	4	29	4
Gunas_062	<i>C. echinospermum</i>	34	5	33	5	7	2	37	5	36	5	29	4	42	6
Gunas_100	<i>C. echinospermum</i>	42	6	54	7	4	2	17	3	35	5	30	4	63	7
ICC3996	<i>C. arietinum</i>	49	6	58	7	10	2	19	3	44	6	36	5	43	6
PBA HatTrick	<i>C. arietinum</i>	52	7	54	7	13	3	33	5	38	5	38	5	55	7
Bari1_063	<i>C. reticulatum</i>	72	7	85	8	4	2	16	3	17*	3	39	5	46	6
Kayat_063	<i>C. reticulatum</i>	68	7	71	7	24	4	22	4	14*	3	39	5	21*	4
Bari1_064	<i>C. reticulatum</i>	79	8	80	8	9	2	13	3	19	3	40	5	29	4
Egil_074	<i>C. reticulatum</i>	75	7	76	8	13	3	22	4	28	4	43	6	38	5
Egil_073	<i>C. reticulatum</i>	68	7	57	7	11	3	29	4	50	6	43	6	38	5
PBA Slasher	<i>C. arietinum</i>	71	7	69	7	21	4	33	5	31	5	45	6	46	6
S2Drd_101	<i>C. echinospermum</i>	64	7	76	8	22	4	27	4	39	5	46	6	54	7
CudiB_008B	<i>C. reticulatum</i>	71	7	81	8	16	3	42	6	35	5	49	6	34	5
Deste_072A	<i>C. echinospermum</i>	66	7	76	8	ND	–	ND	–	11**	3	51	7	28	4
Karab_172	<i>C. echinospermum</i>	83	8	75	7	25	4	47	6	28	4	51	7	43	6
Howzat	<i>C. arietinum</i>	73	7	69	7	31	5	35	5	49	6	52	7	70	7
Kesen_101	<i>C. reticulatum</i>	74	7	84	8	19	3	24	4	56	7	52	7	81	8
Sirna_081B	<i>C. reticulatum</i>	88	8	83	8	19	3	44	6	52	7	57	7	65	7
Sirna_060	<i>C. reticulatum</i>	92	8	91	8	36	5	17	3	57	7	59	7	57	7
Sirna_110	<i>C. reticulatum</i>	91	8	86	8	26	4	41	6	60	7	61	7	42	6
Bari3_091	<i>C. reticulatum</i>	79	8	86	8	54	7	67	7	46	6	66	7	60	7
Kyabra	<i>C. arietinum</i>	86	8	89	8	58	7	59	7	51	7	68	7	49	6
Deste_066	<i>C. echinospermum</i>	70	7	68	7	ND	–	ND	–	ND	–	69	7	ND	–
Moti	<i>C. arietinum</i>	83	8	96	8	81	8	54	7	77	8	78	8	85	8
Deste_077	<i>C. echinospermum</i>	ND	–	80	8	ND	–	ND	–	ND	–	80	8	ND	–

^a Bold type indicates the *C. arietinum* controls included in this study. S2Drd = Siv-Diyar, Karab = Karabahce, Deste = Destek, Bari = Baristepe, Gunas = Gunasan, Kayat = Kayatape, Kesen = Kesentas, and Sirna = Sirnak.

^b The following rating scale was used: 1 = immune (no symptoms of disease), 2 = highly resistant (infection on 1 to 10% of leaves), 3 = resistant (infection on 11 to 20% of leaves), 4 = moderately resistant (infection on 21 to 30% of leaves), 5 = tolerant (infection on 31 to 40% of leaves), 6 = moderately susceptible (infection on 41 to 50% of leaves), 7 = susceptible (infection on 51 to 75% of leaves), 8 (highly susceptible, infection on 76 to 98% of leaves), and 9 = plant death. * $P < 0.05$ (significantly more resistant than ICC3996); ** $P < 0.01$ (significantly more resistant than ICC3996). ND = not determined as a result of poor seed germination.

^c Mean represents the average leaf area damage from the individual inoculations with isolates TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1.

(position 37,902,568), the strongest candidate gene was *Cr_02657.1*. The protein encoded by *Cr_02657.1* has two predicted WRKY DNA binding Pfam domains and its closest homolog is probably a WRKY transcription factor (TF) in *C. arietinum* (LOC101496076, 99.25% amino acid identity). The strongest candidate gene for the SNP associated with resistance to ME14 on chromosome 4 (position 10,375,894) was *Cr_09847.1*. The protein encoded by *Cr_09847.1* has an auxin response factor (ARF) Pfam domain and a B3 DNA-binding Pfam domain. Its closest homolog is a *Medicago truncatula* putative ARF family TF (MtrunA17_Chr1g0199681, 81.05% amino acid identity) and no homologs for this gene were identified in the *C. arietinum* genome. For the third SNP associated with resistance to ME14 on chromosome 6 (position 3,610,729), the strongest candidate gene was gene *Cr_16402.1*. The

protein encoded by *Cr_16402.1* has a sugar transporter Pfam domain and its closest homolog is a *C. arietinum* polyol-transporter 5-like protein (LOC101496554, 100% amino acid identity). Finally, for the SNP associated with resistance to 15CUR005 on chromosome 3 (position 42,986,120), the strongest candidate gene was *Cr_08467.1*. The protein encoded by *Cr_08467.1* has a thioredoxin/disulphide bond reductase Pfam domain and its closest homolog is a *C. arietinum* spermatogenesis-associated protein 20 (LOC101503487, 99.50%) (Supplementary Table S5).

We analyzed the presence of the identified SNPs in the *C. reticulatum* accessions screened with additional isolates. All accessions with data available at the SNP site harbored the allele favorable for 15CUR005 resistance. One accession (Kayat_063) also harbored all favorable alleles for ME14 resistance

TABLE 3. Stem area damage caused by aggressive *Ascochyta rabiei* isolates and a cocktail of the isolates on a subset of wild *Cicer* accessions and *C. arietinum* controls

Accession ^a	Species	Stem ^b													
		TR6417		15CUR002		15CUR005		16CUR017		F17191-1		Mean ^c		Cocktail	
		<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating	<i>n</i>	Rating
Karab_063	<i>C. echinospermum</i>	12	3	22	4	1	2	3	2	1	2	8	2	ND	–
Deste_064	<i>C. echinospermum</i>	28	4	8*	2	ND	–	3	2	3	2	10	2	4	2
Deste_061	<i>C. echinospermum</i>	20	3	26	4	1	2	2	2	6	2	11	3	8	2
S2Drd_061	<i>C. echinospermum</i>	31	5	26	4	2	2	4	2	1	2	13	3	6	2
Deste_079	<i>C. echinospermum</i>	26	4	33	5	1	2	1	2	7	2	13	3	8	2
ICC3996	<i>C. arietinum</i>	21	4	35	5	2	2	10	2	2	2	14	3	6	2
Gunas_062	<i>C. echinospermum</i>	23	4	28	4	4	2	13	3	4	2	14	3	8	2
Bari1_062	<i>C. reticulatum</i>	41	6	34	5	1	2	2	2	0	1	16	3	5	2
Gunas_100	<i>C. echinospermum</i>	29	4	40	5	1	2	10	2	5	2	17	3	8	2
PBA HatTrick	<i>C. arietinum</i>	30	4	41	6	2	2	11	3	3	2	17	3	18	3
PBA Slasher	<i>C. arietinum</i>	35	5	38	5	6	2	9	2	1	2	18	3	3	2
Howzat	<i>C. arietinum</i>	39	5	36	5	9	2	11	3	5	2	20	3	30	4
S2Drd_101	<i>C. echinospermum</i>	32	5	52	7	4	2	5	2	17	3	22	4	33	5
Egil_073	<i>C. reticulatum</i>	35	5	43	6	6	2	15	3	11	3	22	4	11	3
Deste_066	<i>C. echinospermum</i>	24	4	29	4	ND	–	ND	–	ND	–	26	4	ND	–
Kayat_063	<i>C. reticulatum</i>	52	7	64	7	10	2	6	2	4	2	27	4	8	2
Karab_172	<i>C. echinospermum</i>	50	6	50	6	9	2	25	4	5	2	28	4	16	3
Bari1_063	<i>C. reticulatum</i>	47	6	83	8	1	2	7	2	5	2	29	4	21	4
Egil_074	<i>C. reticulatum</i>	62	7	62	7	7	2	12	3	12	3	31	5	16	3
Bari1_064	<i>C. reticulatum</i>	81	8	70	7	6	2	3	2	8	2	33	5	16	3
Bari3_091	<i>C. reticulatum</i>	48	6	56	7	27	4	28	4	8	2	34	5	23	4
CudiB_008B	<i>C. reticulatum</i>	56	7	78	8	7	2	20	3	12	3	35	5	19	3
Kesen_101	<i>C. reticulatum</i>	68	7	66	7	8	2	6	2	26	4	35	5	64	7
Deste_072A	<i>C. echinospermum</i>	45	6	58	7	ND	–	ND	–	4	2	36	5	14	3
Kyabra	<i>C. arietinum</i>	71	7	79	8	24	4	26	4	18	3	44	6	17	3
Sirna_081B	<i>C. reticulatum</i>	91	8	82	8	7	2	19	3	22	4	44	6	42	6
Sirna_060	<i>C. reticulatum</i>	97	8	89	8	16	3	3	2	35	5	48	6	39	5
Deste_077	<i>C. echinospermum</i>	ND	–	50	6	ND	–	ND	–	ND	–	50	6	ND	–
Sirna_110	<i>C. reticulatum</i>	96	8	79	8	18	3	18	3	43	6	51	7	26	4
Moti	<i>C. arietinum</i>	54	7	82	8	54	7	35	5	48	6	55	7	73	7

^a Bold type indicates the *C. arietinum* controls included in this study. Karab = Karabahce, Deste = Destek, S2Drd = Siv-Diyar, Gunas = Gunasan, Bari = Baristepe, Kayat = Kayatape, Kesen = Kesentas, and Sirna = Sirnak.

^b The following rating scale was used: 1 = immune (no symptoms of disease), 2 = highly resistant (infection on 1 to 10% of stems), 3 = resistant (infection on 11 to 20% of stems), 4 = moderately resistant (infection on 21 to 30% of stems), 5 = tolerant (infection on 31 to 40% of stems), 6 = moderately susceptible (infection on 41 to 50% of stems), 7 = susceptible (infection on 51 to 75% of stems), 8 = highly susceptible (infection on 76 to 98% of stems), and 9 = plant death. **P* < 0.05 (significantly more resistant than ICC3996). ND = not determined as a result of poor seed germination.

^c Mean represents the average stem area damage from the individual inoculation with isolates TR6417, 15CUR002, 15CUR005, 16CUR017, and F17191-1.

TABLE 4. Information on the *Cicer reticulatum* single nucleotide polymorphisms (SNPs) associated with *Ascochyta* blight (AB) resistance

AB resistance	Chromosome	Position	SNP	<i>P</i> value	MAF ^a	Estimated allele effect size ^b	PVE (%) ^c	Corresponding position on <i>C. arietinum</i> genome ^d
ME14	1	37,902,568	C/T	1.10×10^{-6}	0.20	0.37	6.77	NW_004515897.1 ^e , position 70,453
15CUR005	3	42,986,120	C/T	1.54×10^{-6}	0.06	0.34	15.21	Chromosome 3, position 35,269,661
ME14	4	10,375,894	T/G	1.91×10^{-6}	0.14	–0.27	7.79	Chromosome 4, position 10,183,264
ME14	6	3,610,729	A/G	4.77×10^{-8}	0.27	0.30	8.72	Chromosome 6, position 3,423,700

^a MAF = minor allele frequency.

^b Allele effect size is relative to the minor allele.

^c Proportion of variance explained: PVE = (r^2 of model with SNP – r^2 of model without SNP) × 100.

^d *C. arietinum* CDC Frontier genome.

^e NW_004515897.1 is an unplaced scaffold.

(Supplementary Table S2). Kayat_063 exhibited significantly better leaf resistance to the highly virulent isolate F17191-1 and the cocktail of isolates. No firm conclusions can be made on the association of the SNPs with resistance to the other aggressive isolates considering the small sample size; however, this certainly warrants further investigation.

The corresponding positions of the *C. reticulatum* SNPs in the *C. arietinum* CDC Frontier genome are as follows: *C. reticulatum* chromosome 1, position 37,902,568 corresponds to *C. arietinum* unplaced scaffold NW_004515897.1, position 70,453; *C. reticulatum* chromosome 4, position 10,375,894 corresponds to *C. arietinum* chromosome 4, position 10,183,264; *C. reticulatum* chromosome 6, position 3,610,729 corresponds to *C. arietinum* chromosome 6, 3,423,700; and *C. reticulatum* chromosome 3, position 42,986,120 corresponds to *C. arietinum* chromosome 3, position 35,269,661 (Table 4). The aforementioned close *C. arietinum* homologs of *C. reticulatum* candidate genes on chromosomes 1, 3, and 6 all reside within 10 kb of the corresponding SNP in *C. arietinum*. The SNP on *C. reticulatum* chromosome 4 (10.4 Mb) corresponds to *C. arietinum* chromosome 4 (10.2 Mb), which resides within an identified QTL referred to as QTL_{AR1} (6.9 to 13.4 and 8.8 to 15.6 Mb) (Daba et al. 2016; Deokar et al. 2019; Sabbavarapu et al. 2013). Similarly, the SNP on *C. reticulatum* chromosome 6 (3.6 Mb) corresponds to *C. arietinum* chromosome 6 (3.4 Mb), which resides within a previously described QTL, QTL_{AR5} (0.7 to 12.4 Mb) (Deokar et al. 2019; Sabbavarapu et al. 2013). The only SNP significantly associated with resistance to 15CUR005 on *C. reticulatum* chromosome 3 (43.0 Mb) corresponds to *C. arietinum* chromosome 3 (35.3 Mb). This also lies within a reported QTL, QTL_{AR4} (30.1 to 37.9 Mb) (Tar'an et al. 2007). The SNP on *C. reticulatum* chromosome 1 (37.9 Mb) corresponds to an unplaced *C. arietinum* scaffold; therefore, we cannot conclude whether this lies within a known QTL or not.

DISCUSSION

AB is a major constraint to chickpea production worldwide. However, the narrow genetic diversity within the chickpea gene

pool is a limitation for the identification of sources of genetic resistance to this devastating disease. Therefore, close wild relatives of chickpea with far greater genetic diversity represent a promising solution to tackle this issue.

Some important inferences were drawn from the nested ANOVA and regression analysis. First, future screening of wild germplasm for AB resistance should prioritize *C. echinospermum* because it contains a much higher proportion of resistant material than *C. reticulatum* but has not been evaluated as extensively. The *C. reticulatum* results show that variance is largely distributed within, rather than between, collection sites, suggesting that there has been no strong regional or climatic selection for AB resistance. Accordingly, further *C. reticulatum* evaluation should target new accessions, regardless of collection site. On the other hand, the *C. echinospermum* results indicate a greater role for collection site, having identified locations with predominantly resistant accessions (Destek, Gunasan) and a high degree of interaction in others (Ortanca, Siverek-Diyarbakir). Interestingly, there was also some isolate by accession interaction within domesticated chickpea, with 15CUR005 causing more disease on the most resistant accessions ICC3996, PBA HatTrick, and PBA Slasher, whereas ME14 caused more disease on the most susceptible Moti. There was no difference in disease caused by ME14 and 15CUR005 on the accessions with intermediate resistance, Howzat and Kyabra. A plausible explanation is that 15CUR005 has evolved to overcome the genetic sources of resistance in ICC3996, PBA HatTrick, and PBA Slasher; however, this change in virulence does not confer any benefit on the more susceptible lines and may in fact have a fitness cost on the very susceptible Moti. These data support what has been observed in the field with PBA HatTrick and PBA Slasher ratings reclassified from moderately resistant/resistant to susceptible and resistant to susceptible, respectively, after the outbreak of a new strain in Curyo in 2015 (Australian Grains Research and Development Corporation 2017).

Wild relatives of chickpea have already been demonstrated to harbor genes that can improve disease resistance of chickpea varieties. For example, Knights et al. (2008) demonstrated that *C. echinospermum* accessions exhibit enhanced resistance to *Phytophthora medicaginis*, the causal agent of Phytophthora root rot, which constrains chickpea production in the clay soils of northern New South Wales and southern Queensland, Australia (Moore et al. 2015b). The genetic sources of resistance could be transferred to elite chickpea varieties through hybridization, resulting in progeny with comparable resistance to the *C. echinospermum* parent (Knights et al. 2008). Wild *Cicer* species also exhibit resistance to Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*), botrytis gray mold (*Botrytis cinerea*), cyst nematode (*Heterodera ciceri*), leaf miner (*Liriomyza cicerina*), bruchid beetles (*Callosobruchus* spp.), and the Pea streak virus (genus *Carlavirus*), as reviewed by Croser et al. (2003). Furthermore, the wild *Cicer* collection described herein has been demonstrated to harbor pod borer (*Helicoverpa armigera*) and root-lesion nematode (*Pratylenchus thornei*) resistance (Reen et al. 2019; von Wettberg et al. 2018).

Resistance to AB has also been described previously in wild relatives of chickpea. Singh and Reddy (1993) screened a large collection of 201 accessions of eight annual wild *Cicer* species and identified resistant to moderately resistant *C. judaicum* and *C. pinnatifidum* accessions. Interestingly, the *C. echinospermum* and *C. reticulatum* accessions screened were not resistant to AB. *C. judaicum* and *C. pinnatifidum*, however, cannot be readily crossed with the cultivated chickpea species, *C. arietinum*; therefore, introgression of this resistance to improve crop varieties would be particularly challenging (Ahmad and Slinkard 2004; Ahmad et al. 1988). Collard et al. (2001) identified AB-resistant *C. echinospermum* and *C. reticulatum* accessions; however, none were significantly more resistant than the Iranian landrace ICC3996. Here, we have identified wild *Cicer* accessions with

Fig. 3. Genome-wide association study of *Cicer reticulatum* resistance to Ascochyta blight using a multilocus mixed model. Single nucleotide polymorphisms (SNPs) are ordered by chromosome position on the x-axis (chromosomes 1 to 8 with unassembled scaffold SNPs on the right side). The negative logarithm of the association *P* values for each SNP is on the y-axis. The solid green line represents the significance threshold (Bonferroni-corrected *P* value of 0.05). **A**, Association of SNPs with resistance to isolate ME14. **B**, Association of SNPs with resistance to isolate 15CUR005.

superior AB resistance to the Iranian chickpea landrace ICC3996 to highly pathogenic Australian *A. rabiei* isolates. Incorporation of these accessions in breeding programs will facilitate the development of varieties with enhanced AB resistance. Furthermore, introgression of the genes underlying the resistance can be facilitated by molecular markers linked to the regions identified by the GWAS.

It is unsurprising that we identified multiple SNPs associated with resistance to AB, as the quantitative nature of resistance in chickpea has been well documented. QTLs for AB have been discovered on chickpea linkage groups LG2 (Anbessa et al. 2009; Cho et al. 2004; Udupa and Baum 2003), LG3 (Anbessa et al. 2009; Tar'an et al. 2007), LG4 (Anbessa et al. 2009; Cho et al. 2004; Li et al. 2017; Lichtenzweig et al. 2006; Sabbavarapu et al. 2013; Stephens et al. 2014; Tar'an et al. 2007), LG5 (Sabbavarapu et al. 2013), LG6 (Anbessa et al. 2009; Sabbavarapu et al. 2013; Tar'an et al. 2007), and LG8 (Anbessa et al. 2009; Lichtenzweig et al. 2006). QTLs for AB resistance have also been identified in *C. echinospermum*. LG4 was also found to be a hotspot for AB resistance in *C. echinospermum*, with two QTLs on LG4 found to associate with seedling AB resistance. Moreover, markers on LG2 and LG3 were associated with stem resistance to AB (Collard et al. 2003).

To our knowledge, this is the first study in which the genetic basis of resistance in *C. reticulatum* has been dissected. Studies have used recombinant inbred lines derived from interspecific crosses between *C. arietinum* and *C. reticulatum* to map QTLs for AB resistance; however, these studies have used *C. reticulatum* as the susceptible parent and identified *C. arietinum* AB resistance QTLs (Aryamanesh et al. 2010; Cobos et al. 2006; Santra et al. 2000; Tekeoglu et al. 2000). Identifying the corresponding position of trait-associated SNPs in the *C. arietinum* CDC Frontier genome allowed us to investigate whether any of these reside within known AB resistance QTLs (Varshney et al. 2013). The corresponding position of the SNPs on *C. reticulatum* chromosomes 3, 4, and 6 were all within reported chickpea AB resistance QTLs. Whether the *C. reticulatum* loci confer resistance via different mechanisms to those identified in *C. arietinum* remains to be determined. The candidate resistance genes in close proximity to the *C. reticulatum* SNPs on chromosome 1, 3, and 6 all had close homologs in *C. arietinum*. It is possible that these homologs contribute to resistance in some chickpea accessions. However, the strongest candidate resistance gene in close proximity to the *C. reticulatum* SNP on chromosome 4 had no close homologs in *C. arietinum*; its closest homolog was in *M. truncatula* (81.05% amino acid identity). This may represent a gene that has been lost during domestication or selection, although we cannot rule out that the gene may be present in chickpea accessions other than CDC Frontier. Indeed, evidence suggests that *C. echinospermum* and *C. reticulatum* harbor different major or minor AB resistance genes to domesticated chickpea (Danehlouei pour et al. 2007). If these different resistance genes reside within syntenic regions to resistance loci in chickpea cultivars, pyramiding the resistance genes could be challenging for chickpea breeders.

Investigation into the regions of the *C. reticulatum* genome identified by the GWAS enabled identification of candidate genes responsible for the resistance phenotype, one of which was a probable WRKY TF (*Cr_02657.1*). WRKY TFs harbor at least one DNA-binding region with a highly conserved WRKYGQK motif and are required for the large-scale transcriptional reprogramming involved in responding to biotic stress (Eulgem 2005; Ryu et al. 2006). The protein encoded by the candidate gene identified here (*Cr_02657.1*) harbors two WRKY DNA binding domains. Another candidate resistance gene (*Cr_09847.1*) encodes a TF of a different family, ARF; members of the ARF family are primarily implicated in modulation of plant growth and development via regulation of genes responsive to the plant growth hormone auxin (Li et al. 2016). Evidence is emerging of a link between auxin and disease resistance

(Yamada 1993). In tomato (*Solanum lycopersicum*), several ARFs were differentially regulated in response to biotic stress and most *SlARF* promoters harbored biotic stress-responsive *cis*-acting elements, indicating a role of ARFs in the innate defense response to plant pathogens (Bouzroud et al. 2018). A TF of the AT-hook motif nuclear localized (AHL) family has previously been demonstrated to contribute to chickpea AB resistance in a major QTL on chromosome 4 (Kumar et al. 2018). Introgression of AB resistance-associated TFs into chickpea cultivars may be an effective approach to improve quantitative disease resistance attributable to the ability of TFs to modulate multiple resistance genes. A further candidate resistance gene identified here was a probable sugar transporter (*Cr_16402.1*). This protein may be involved in the movement of sugar from the extracellular apoplast to the host cell cytoplasm, thereby increasing the carbohydrate supply to mount a successful defense response and reducing the availability of extracellular sugar to *A. rabiei*, which primarily grows through the apoplast (Köhler et al. 1995). This mechanism has been demonstrated to occur in *Arabidopsis thaliana* via sugar transport protein STP13, which confers resistance to a bacterial pathogen, *Pseudomonas syringae* pv. tomato DC3000, and the fungal necrotrophic pathogen, *Botrytis cinerea* (Lemonnier et al. 2014; Yamada et al. 2016). The strongest candidate resistance gene for the locus associated with resistance to 15CUR005 was a probable thioredoxin (*Cr_08467.1*). Thioredoxin oxidoreductases regulate many defense signaling proteins and play an important role in the maintenance of cellular redox homeostasis, preventing reactive oxygen species (ROS) toxicity while enabling ROS-mediated defense signaling (Mata-Pérez and Spoel 2019). *Cr_08467.1* may be involved in signaling an immune response following recognition of *A. rabiei* infection. This study may contribute toward elucidating the molecular mechanism underlying resistance in *C. reticulatum*. However, further work is required to validate the causal genes. Genes neighboring the trait-associated SNPs within 10 kb were investigated, as conducted by Xie et al. (2018), although linkage disequilibrium (LD) may well extend beyond 10 kb. In the future, identification of LD blocks surrounding the trait-associated SNPs and subsequent gene expression analysis of genes within the LD blocks, including those discussed herein, would be an effective approach to validate the genes associated with AB resistance.

The use of 2-week-old seedlings enabled rapid screening of the accessions; however, the durability in the field and efficacy in adult plants of the identified resistance remains to be determined. Promisingly, Sharma et al. (2010) demonstrated that seedling and adult plant resistance for chickpea resistance against AB was highly correlated. Further experiments in field conditions will be required to support the durability of these novel sources of resistance in *C. reticulatum* and *C. echinospermum* in breeding programs.

Utilizing the identified AB-resistant *C. echinospermum* accessions (e.g., S2Drd_061 and Deste_064), we can now also identify resistance QTLs by generating biparental mapping populations to dissect the basis of resistance in this and other wild *Cicer* species. Once identified, there is the potential to pyramid resistance genes from *C. arietinum*, *C. reticulatum*, and *C. echinospermum* in order to generate highly resistant chickpea cultivars.

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