Highlights in BioScience
ISSN:2682-4043
DOI:10.36462/H.BioSci.202405

Research Article

Open Access

Exploring QTL genes contribute to chickpea ascochyta blight resistance across multiple environments using SSR, DArT and SNP assays

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Editor: Jean Legeay, *University Mohammed VI Polytechnic, Morocco.*

Reviewer(s):
Ahmed Sallam, *Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Stadt Seeland, Germany.*.

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Received: June 15, 2024
Accepted: September 9, 2024
Published: December 26, 2024

Citation: Hamwieh A, Muhammad I, Ahmed S, Kababeh S, Alsamman AM, Istanbuli T. Exploring QTL genes contribute to chickpea ascochyta blight resistance across multiple environments using SSR, DArT and SNP assays . 2024 Dec. 26;7:bs202405

Data Availability Statement: All relevant data are within the paper and supplementary materials.
Funding: This work was supported by Grains Research and Development Corporation (GRDC), Australia.
Competing interests: The authors declare that they have no competing interests.

Abstract

Chickpea (*Cicer arietinum* L.) occupies the third leading position among grain legumes in cultivated area around the world. Ascochyta blight (AB) caused by *Ascochytarabiei* (Pass.) Labr. is one of the most destructive foliar diseases of chickpea and can cause complete crop failure in many chickpea growing regions around the world. A recombinant inbred line (RIL) population, comprising 165 lines derived from the cross FLIP98-1065 (R) ×ILC1929 (S),were evaluated in six environments over three years (2008 – 2011) and three locations in Syria (field and greenhouse locations in Tel Hadya "TH" and a field location at Lattakia "Lat"). The greenhouse experiments were conducted against AB pathotype II. ANOVA analysis indicated significant differences both among the RILs and among the environments. We produced a total of 1398 (134 SSR, 652 DArTseq and 612 SNP) markers and developed a high-resolution genetic map (1244 markers spanning 2503 cM on eight linkage groups). Three major conserved quantitative trait loci (QTLs) that confer AB resistance were identified: two on linkage group 2 (indicated as LG2-A and LG2-B) and one on linkage group 4 (indicated as LG4). These explain, respectively, a maximum of 18.5%, 11.1% and 25% of the total variation. In total, 18 predicted genes were located in LG4, and 9 and10 predicted genes, respectively, were located in LG2-A and LG2-B. This study presents a first set of SNP markers located within genes associated with AB resistance in chickpea, which could be applied in marker-assisted selection programs for breeding AB-resistant chickpeas.

Keywords: Ascochyta blight, *Ascochytarabiei*, chickpea, AMMI analysis, QTL, SNP

Introduction

Ascochyta blight (AB) of chickpea, caused by *Ascochyta rabiei* (Pass.) Lab., is one of the most important diseases in many chickpea production areas. Four pathotypes have been identified within A.*rabiei* populations in Syria and other countries [1; 2; 3]. Chemical control of AB has proved inefficient and very expensive; therefore, new varieties with improved resistance are needed to manage the impact of the disease. Breeders using conventional methods have made considerable progress toward developing chickpea varieties with increased AB resistance [4; 5]. Research teams investigating AB of chickpea have identified 14 quantitative trait loci (QTLs) in eight linkage groups (LGs) that each contribute to *A. rabiei* resistance [6; 7; 8; 9; 10; 11; 12; 13; 14; 15]. This indicates that several mechanisms are likely responsible for AB resistance, but very little is known about the identity and functions of the genes involved. Two major QTLs, located on LG2 close to the markers GA16 and TA37, control resistance to AB pathotype I [10], while another QTL contributing to resistance to pathotype II is located on LG4 near simple sequence repeat (SSR) loci GAA47, TA130, TR20, TA72, TS72, and TA2 [16; 9; 10]. [10] traced the AB resistance of the chickpea line FLIP84-92C to regions on LG2 and LG4 associated with the resistance in FLIP84-92C to pathotype II. In that study, the TA46 marker explained a maximum of 69% of that lines total AB resistance; furthermore, additional markers (GAA47, GA24, and GA16) were identified on LG2, each explaining 10.4 – 19.3% of the line's total AB resistance [10]. Additional markers on LG1 (TS12b, STMS28, and TS45) have been reported to be associated with AB resistance under controlled conditions [7; 8].

A SCAR marker $SCY17_{590}$ was reported as linked to AB resistance [11], and this marker was applied successfully to selected resistant genotypes in Australian chickpea breeding materials [17]. This marker was further applied together with an allele-specific primer for the gene *CaETR-1* for genotyping QTLAR1 and QTLAR2 in chickpea germplasm [18; 19].

A study by [20] using an Fst genome scan and genome-wide association methods indicated that a 100 kb region on chromosome 4 was significantly associated with AB resistance. This region covered a large QTL interval of 7 Mb30 Mb, which had been genotyped at relatively low density with SSR or single nucleotide polymorphism (SNP) markers through previous mapping population studies. [20] and colleagues validated this QTL region on LG4 in a genome-wide association study (GWAS) using approximately 144,000 SNPs and a collection of 132 advanced breeding lines from the Australian chickpea breeding program. In total, 12 predicted genes were located in the region associated with AB resistance, including sequences annotated as encoding an NBS-LRR receptor-like kinase, a wall-associated kinase, a zinc finger protein, and serine/threonine protein kinases. One significant SNP located in the conserved catalytic domain of an NBS-LRR receptor-like kinase led to an amino acid substitution [20].

Researchers could use high-throughput genotyping systems to identify markers such as single nucleotide polymorphisms (SNP) markers and diversity array technology (DArT) markers. Therefore, we aimed to study the interaction between different environments and AB resistance, to produce a highly saturated genetic map, and to identify markers closer to potential AB resistance genes using a recombinant inbred line (RIL) population of chickpea, enabling practical application of marker(s) in chickpea breeding programs.

Materials and methods

Plant materials and DNA isolation

A mapping population consisting of 165 (F8) RILs derived from an intra-specific cross between an AB-resistant chickpea, FLIP98-1065, and an AB-susceptible chickpea, ILC1929, was used in this study. The RILs were developed using the single seed descent (SSD) method from F2 to F7. DNA was extracted from fresh leaves of six-week-old seedlings using the cetyltrimethyl ammonium bromide (CTAB) method [21; 22].

The sequences of chickpea SSR primers were obtained from published papers [16; 23; 24]. The PCR mixture (20 μL) contained 10 ng genomic DNA, 0.2 mM dNTP, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.), 10 pmol of each forward and reverse primer, and 1X PCR buffer (Invitrogen, Carlsbad, Calif.). Polymerase chain reaction (PCR) was conducted using a thermocycler (ABI GeneAmp2720) with the following program: a denaturing cycle of 94°C for 3 minutes, followed by 35 cycles of 94°C for 15 seconds (denaturation), a specific temperature depending upon the primer pair for 15 seconds (annealing), and 72°C for 30 seconds (extension), followed by a final extension at 72°C for 5 minutes. The PCR products were separated on an 8% polyacrylamide gel according to the electrophoresis user guide (Invitrogen).

DArT and DArTseq

DArTseq is a genotyping by sequencing (GBS) platform developed by DArT PL, Canberra, Australia (http://www.diversitarrays.com/dart-application-dartseq). DArTseq is a combination of complexity reduction methods that were initially developed for array-based DArT with sequencing of resulting representations on next-generation sequencing platforms.

Experimental Design and Locations

Four field experiments and two greenhouse experiments were performed at different locations in Syria, enabling us to examine disease susceptibility of the chickpea lines under six sets of environmental conditions **(Table 1)**. Field experiments were performed in Lattakia, Syria in 2009 (Lat09) and at the Tel Hadya station, Aleppo, Syria, in 2008, 2009, and 2010 (TH08, TH09, and TH10). These locations differed in the average rainfall they received during the experiments: in Lattakia, the average was around 750 mm, but in Tel Hadya, the average was 350 mm. Each experiment was laid out in an alpha lattice design with two replications using multiple row plots. The length of each plot was 5 m and rows were spaced 45 cm apart in all trials.

Two experiments were performed in the greenhouse at ICARDA headquarters in Aleppo, Syria in 2009 and 2010 (PII-2009 and PII-2010). In the greenhouse, five healthy seeds of each line were planted in a pot (15 cm diameter). The experiments were conducted in a growth chamber (temperature 22°C and 12 hours/12 hours light/dark). Locations, years, and planting dates for the six experiments are summarized in **(Table 1)**.

The AB cultures were obtained from the legume pathology laboratory at ICARDA. The experiments were laid out in a randomized complete block design with two replications. A spore suspension of AB pathotype II (concentration of 10^5 spores/mL^{-1}) was prepared from 14-day old *Ascochyta rabiei* culture grown on chickpea dextrose agar (4% chickpea flour, 2% dextrose, and 2% agar in 1 liter of distilled water) and used for seedling inoculations. The disease was scored when symptoms were observed on the susceptible chickpea control (ILC-263).

Scoring Plants for AB Symptom Severity

Ascochyta blight symptom scoring was based on a nine-point rating scale [25] in which plants are scored as follows: 1. immune, no symptoms of disease; 2. few, very small lesions (<2 mm) on leaves and stems (1 to 2% of the plant area infected); 3. many small lesions (6 to 10% of the plant area infected); 4. many small and large lesions (26 to 50% of the plant area infected); 5. many small lesions on the stem (51 to 75% of the plant area infected); 6. many large lesions, lesions coalescing, stem girdled (76 to 90% of the plant area infected); 7. many small and large lesions, lesions coalescing, girdling stem breakage (>90% of the plant area infected); 8. plant is almost dead; and 9. plant is dead.

Table 1. Rainfall "mm", temperature "ºC" (between brackets) and planting dates during 3 years (2008–2010) for experiments conducted in six environments in Syria (three in the field and two in the greenhouse at Tel Hadya TH; one in the field at Lattakia "Lat").

Environment code	Greenhouse		Field			
	PJI-2009	PJI-2010	Lat09	TH08	TH09	TH10
Location	Tel Hadya greenhouse	Tel Hadya greenhouse	Lattakia	Tel Hadya greenhouse	Tel Hadya greenhouse	Tel Hadya greenhouse
Year	2009	2010	2009	2008	2009	2010
Planting date	15 Nov. 2008	13 Nov. 2009	22 Mar. 2009	11 Dec. 2007	12 Dec. 2008	9 Dec. 2009
February	MI (21 řC)	MI (21 řC)	98.4 (13.2 řC)	22.0 (14.3 řC)	84.3 (14.2 řC)	50.6 (15.2 řC)
March	MI (21 řC)	MI (21 řC)	93.3 (20.3 řC)	27.9 (22.9 řC)	36.2 (17.1 řC)	12.4 (20.8 řC)
April	MI (21 řC)	MI (21 řC)	56.6 (23.4 řC)	1.9 (27.9 řC)	23.4 (24.0 řC)	12.7 (25.8 řC)
May	-	-	34.6 (25.1 řC)	10.9 (29.2 řC)	4.0 (30.1 řC)	0.0 (31.7 řC)
Date of score	-	-	22/5/2009	25/5/2008	17/5/2009	16/5/2010
Total rainfall (mm)	-	-	282.9	62.7	147.9	75.7

MI: Mist irrigation used to keep relative humidity around 80% under controlled environments.

Statistical Analysis

The disease rating data collected from the experiments were analyzed using GenStat 12th edition (VSNi, Hemel Hempstead, UK). To evaluate AB scores, analysis of variance (ANOVA) with multiplicative interactions analysis (AMMI) was performed. AMMI analysis was described by [26] based on the following formula:

$$Y_{ij} = \mu + g_i + a_j + \sum_{k=1}^{n} \lambda_k x_{ik} y_{jk} + r_{ij} + \bar{S}_{ij}$$

where μ is the overall mean of the test; g_i is the fixed effect of genotype i ($i = 1, 2, \ldots, g$); a_j is the fixed effect of environment j ($j = 1, 2, \ldots, a$); Y_{ij} is the mean response of genotype i in environment j; λ_k is the singular value of the k-th IPCA, ($k = 1, 2, \ldots, p$, where p is the maximum number of estimable principal components); x_{ik} is the singular value of the i-th genotype in the k-th IPCA; y_{jk} is the singular value of the j-th environment in the k-th IPCA; r_{ij} is the residue of the GEI or AMMI residue (data noise); k is the characteristic non-zero root, $k = [1, 2, \ldots, \min(g-1, e-1)]$. The plot genotype and environment interactions were analyzed against four interaction principal component axes (IPCAs). The ratio of the genetic variance to the total phenotypic variance was calculated to estimate heritability.

Genetic mapping was conducted using JoinMap 4® software, Kyazma [27]. Linkage groups were created based on a logarithm of odds (LOD) score greater than 3 and a recombination frequency below 0.45. Map distances in centimorgans (cM) were estimated using the Kosambi function [28]. QTL analysis was conducted using MapQTL 6® software, Kyazma [29], and the fraction of the variation explained by the QTL and the additive effects were calculated. The potential QTLs were estimated based on a 2,000 permutation test using LOD ≥2 as the significance threshold. Predicted genes within the QTL regions were identified across the chickpea genome using the BLAST package [30], and homologous protein-encoding genes were extracted from the chickpea genome sequence. The SnpEff tool was used to detect the genetic sequence effect for potential SNPs with a correlation to *Ascochyta* blight resistance.

Results

Phenotypic characterization

We collected data from three growing seasons (2008 – 2010) and from one greenhouse and two field locations; this allowed us to analyze plants exposed to six different environmental conditions. We observed significant differences ($P < 0.001$) in the AB severity scores (on a scale from 1 through 9) among the 165 RILs and among plants exposed to the six environments. However, the mean values of the AB severity scores showed a continuous distribution from 3 to 9 **(Figure 1 (A))** that fit the normal distribution. To estimate the interactions between AB resistance and the environments we tested, the AB scores were further analyzed using the AMMI model, which combines features of ANOVA and principal component analysis. The analysis indicated that 25% of the total sum of squares (SS) was due to environmental factors, and only 26.5% and 32.9%, respectively, was due to genotypic and gene-environment interaction (GEI) factors **(Table 2)**. The percentage attributed to GEI was about 1.2 times the percentage attributed to genotype, indicating substantial differences in genotypic responses across environ-

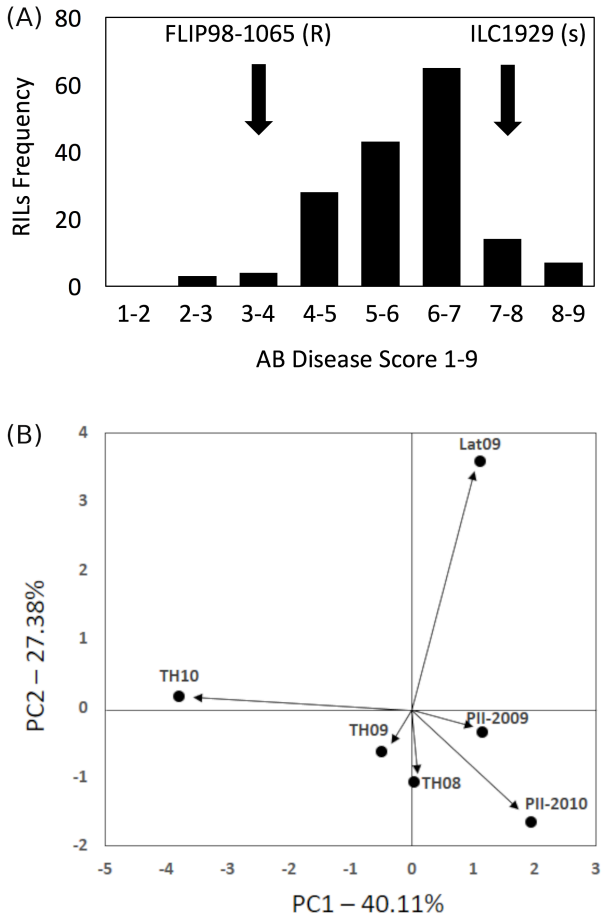

Figure 1. (A) Frequency distributions of AB disease scores for 165chickpea RILs derived from FLIP98-1065 (AB resistant) Œ ILC1929 (AB susceptible). Arrows indicate disease score for each parental genotype. (B) AMMI model 2 biplot of AB resistance scores for 165 RILs and 6environments.

ments. However, the large SS value for environmental factors indicated that diverse environments caused a significant portion of the variation in the AB values. The first principal component axis (PCA1) captured 40.11% of the GEI-SS, while the second (PCA2) captured only 27.4%. The sum of squares for PCA1 and PCA2 was 1484, which is smaller than the 1774 captured for genotype. Since mean squares for both PCA1 and PCA2 were significant at $P < 0.001$ and contributed about 67.52% of the total GEI, the postdictive evaluation suggested that two axes (PCA1 and PCA2) were significant for the model with 334 degrees of freedom **(Table 2)**.

The AMMI 2 biplot analysis for the six environments is shown in **(Figure 1 (B))**. The high rainfall environment in Lattakia (*Lat09*), which received 184.5 mm rainfall after planting, was located in quadrant II. The environments *TH08*, *TH09* and *TH10* were located in quadrants IV, III, and I, respectively. This variation is expected because the Tel Hadya location received different amounts and distributions of rainfall in the three growing

Table 2. ANOVA (AMMI model) for AB score of 165 RILs evaluated in six different environments in field and greenhouse.

Source	D.F.	S.S.	M.S.	F	Explained (%)
Genotypes	164	1774	10.82	10.37***	26.53
Environments	5	1673	334.58	95.99***	25.02
Interactions	819	2198	2.68	2.57***	32.87
IPCA 1	168	882	5.25	5.03***	40.13
IPCA 2	166	602	3.63	3.48***	27.39
IPCA 3	164	293	1.79	1.71***	13.33
IPCA 4	162	225	1.39	1.33	-
Error	978	1020	1.04	-	-
Total	1979	6686	3.38	-	-

*** Highly significant at the 0.001 probability level; D.F: degree of freedom, F: tabulated frequency.

seasons, notably during February and March when the temperature is around 20 ºC in Tel Hadya (*TH*). These conditions provide the best environment for AB disease development. The environments (PII-2009, PII-2010, *TH08* and *TH09*) were located close to the biplot origin. However, one environment, *TH10* (located in quadrant I) received 75.7 mm of rain in 2010, only 12.4 mm of which was received in March, the most important time for the AB disease development. This amount is 50% lower than the amounts received in 2008 and 2009.

Mapping and QTL analyses

Of the 650 SSR primer pairs we tested, only 134 (20.6%) of the primer pairs produced different PCR products for the two parental lines, *FLIP98-1065* and *ILC1929*. A total of 652 DArTseq markers and 612 SNPs obtained from DArT PL were merged with 134 SSR markers and used to develop the genetic map. The linkage map comprised 1244 markers spanning 2503 cM on eight LGs **(Table 3)**. The distance between the markers was 2 cM on average; however, 156 (11.4%) of the markers remained unlinked.

The QTL analysis identified three major QTL regions, one on LG4 and two on LG2 (referred to as LG2-A and LG2-B; **(Figure 2)**. The QTL on LG4 was consistently identified in all six environments used in this study and was considered a major QTL, explaining a maximum of 25% of the total variation at marker *drt-100004682*. The two QTLs on LG2 (LG2-A and LG2-B) were identified in only the three TH field environments (*TH08*, *TH09*, and *TH10*) and explained a maximum of 18.5% of the total variation at marker *sn-100021968*. On LG4, 36 markers were located within the QTL region, and fifteen of them were tightly linked to 15 genes within a span of 430,944 bp **(Figure 3 (A))**. The QTL LG2-A included 53 markers, and 12 of them were tightly linked to genes **(Figure 3 (B))**. The QTL LG2-B comprised 31 markers, seven of which were linked to genes. The most important genes identified in the three QTL regions are listed in **(Table 4)**, such as serine/threonine protein kinase BLUS1, coiled-coil domain-containing protein, transcription factor bHLH157, metal-

nicotianamine transporter YSL1, leucine-rich repeat receptor-like tyrosine-protein kinase PXC3, TMV resistance protein N-like, chitin elicitor receptor kinase 1-like, and SNF1-related protein kinase regulatory subunit beta-1. One SSR marker within LG2-B (*GA16*) was linked to an uncharacterized protein and explains a maximum of 16% of the total variation **(Figure 3 (C))**.

Table 3. Summary of the distribution of markers on different linkage groups in chickpea.

LG	SSR	SNP	DART	Total	Length (cM)
LG1	11	214	274	499	1010.1
LG2	5	83	109	197	201.8
LG3	0	29	0	29	166.5
LG4	34	34	48	116	287.0
LG5	6	38	41	85	156.8
LG6	7	48	59	114	180.3
LG7	19	40	55	114	293.0
LG8	12	28	50	90	207.7
Total	94	514	636	1244	2503.0

Discussion

Ascochyta blight (AB), which is caused by *Ascochyta rabiei* (Pass.) Labr., is a major disease that limits chickpea production in cool and humid environments and is the largest contributor to high yield gaps in chickpea production in many countries [31; 4].

We expected to observe significant G × E in this study, as AB severity in chickpea is greatly affected by environmental conditions [32]. A study by Pande and colleagues [33] revealed significant genotypic effects and G × E interactions in AB severity. However, very few studies regarding G × E interactions in AB susceptibility of chickpea in multi-environments have been reported. [11] evaluated 106 F 6:7 RIL population over two cropping seasons under field conditions, and they identified a significant RIL × year interaction, which they attributed to differences between the two years in environmental conditions, fungus pathotype, or both.

For this study, we generated data from six different environments by conducting four field and two greenhouse experiments over the course of three years (2008 – 2010) in two locations (Tel Hadya and Lattakia). These environments provided different moisture conditions (rainfall varied in amount and distribution among the field experiments). Therefore, our results will contribute to the field's understanding of gene-environment interactions in AB disease susceptibility.

As indicated by [34] and [35], the results of the AMMI analysis can be predicted by using the first two PCAs. Conversely, the G × E-SS (sum of squares) in our study was about 1.2 times higher than that reported for the genotypes, and this indicates substantial differences in genotypic reactions across the environments we studied.

The results from our previous study on drought tolerance performance of 181 chickpea RILs in nine environments found that the GEI-SS was 2.3 times more than that obtained for genotype [22]. The AMMI analysis showed significant differences for all genotypes, environments, and GEI. Furthermore, the GEI revealed that the IPCA (1-3) together with the SS (1,777) was slightly larger than what was obtained for the genotypes (1,774), suggesting that the AMMI model excluded most of the actual data noise. The broad-sense heritability of AB resistance in the six environments in our study indicates high heritability (0.75), which shows that AB is controlled by major genes with large effect size in this study. However, this is much higher than the heritability values (0.38-0.43) calculated in a study of three F2 populations derived from crosses of genotypes that are moderately resistant to AB [13]. This may be attributed to our use of multi-environment data in our study.

Although several mapping populations have been developed, few maps have been constructed using single nucleotide polymorphism (SNP) markers in chickpea. In this study, 1244 markers spanning 2503 cM were mapped; this is much higher than the number of markers used by [36], who developed a high-density map using 150 RIL lines (*Lasseter* × *ICC3996*) and 504 polymorphic SSRs and SNPs.

We identified three major QTL regions in this study. One of these, the QTL on LG4, was consistent across the six environments used in this study and considered a major QTL/gene(s) that explained a maximum of 25% of the total variation at marker drt-100004682. A QTL on LG4 that confers AB resistance has been reported by several previous studies, all of which used relatively low-density maps with SSR or SNP markers [12; 13; 37; 36]. For example, [36] used SNP markers to identify a QTL conferring AB resistance; this QTL explained 14-45% of phenotypic variation and spanned around 13 Mb between markers SSR TA146 and SNP_40000185 on LG4.

We identified two QTL regions located on LG2 (LG2-A and LG2-B), but they were consistent only among the TH environments across three years (TH08, TH09, and TH10). This may indicate that they contain genes whose impact is dependent on environment (temperature/humidity), pathotype differences, or both, as the plants in our greenhouse experiments were inoculated with *Ascochyta rabiei* pathotype II. However, this may also explain why many research teams have failed to report the QTLs we found on LG2. In this study, LG2-A and LG2-B contained 33 and 23 markers spanning 28 cM and 63.6 cM, respectively. Based on GA13 as an anchor SSR marker, LG2-B corresponds with the QTL that was previously reported by [38], [10], and [9]. This QTL in this study could explain a relatively large percentage (18.5%) of the total estimated phenotypic variation in susceptibility to AB. Many other QTLs associated with AB resistance have been reported on linkage groups LG3 [13], LG5 [37], LG6 [13; 37], and LG8 [12] but were not identified in our study.

A total of eighteen predicted genes were located in LG4, and 9 and 10 predicted genes, respectively, were located in LG2-A and LG2-B. A co-dominant SCAR marker $SCY17_{590}$ tightly linked to QTLAR2 on LG4 was reported by [11] and was successfully used to characterize AB source [17]. However, no genes have

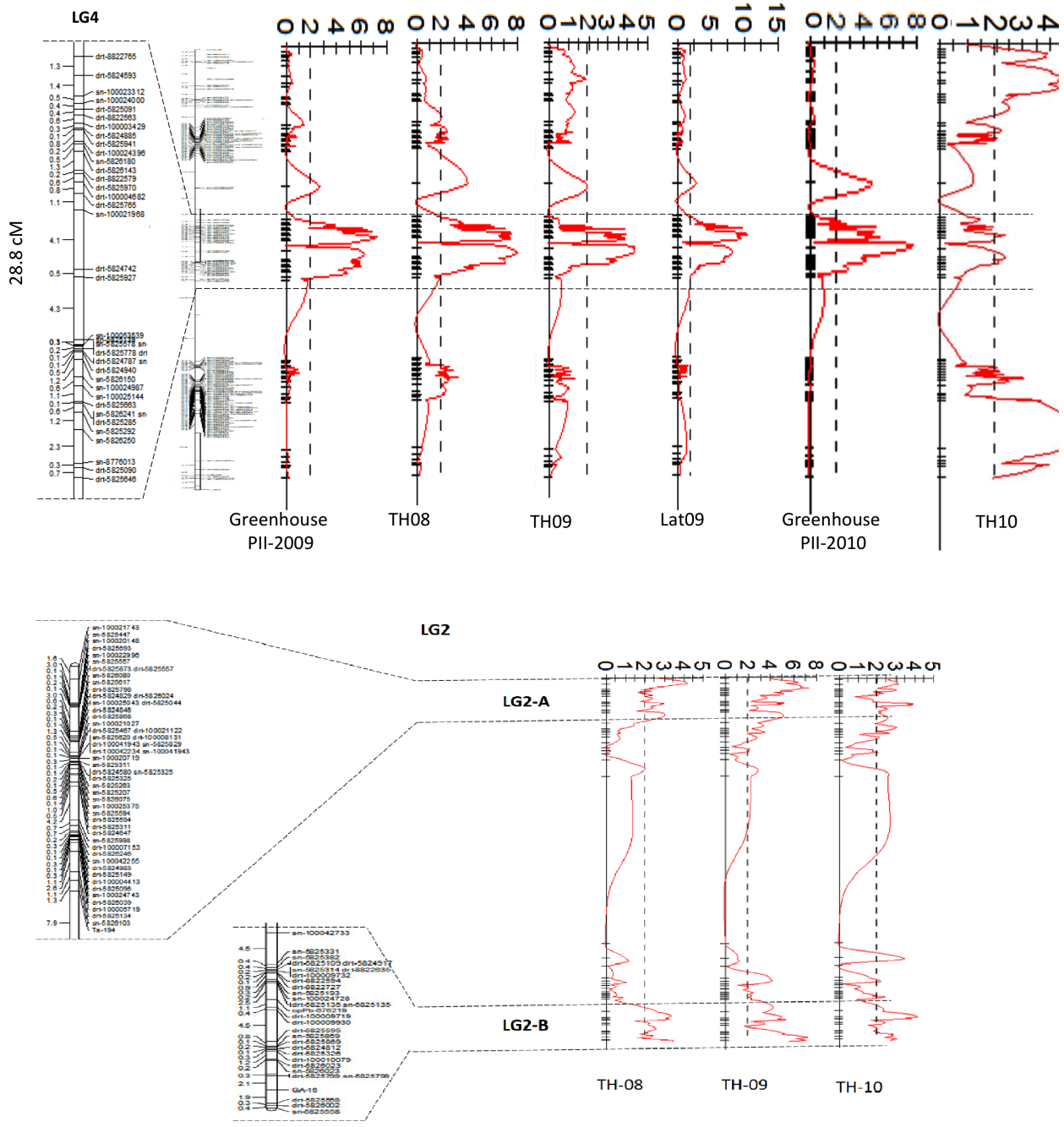

Figure 2. Linkage map of LG2 and LG4 depicting QTLs for AB resistance detected in a RIL (FLIP98-1065 Œ ILC1929) mapping population in chickpea. TH: Tel Hadya.

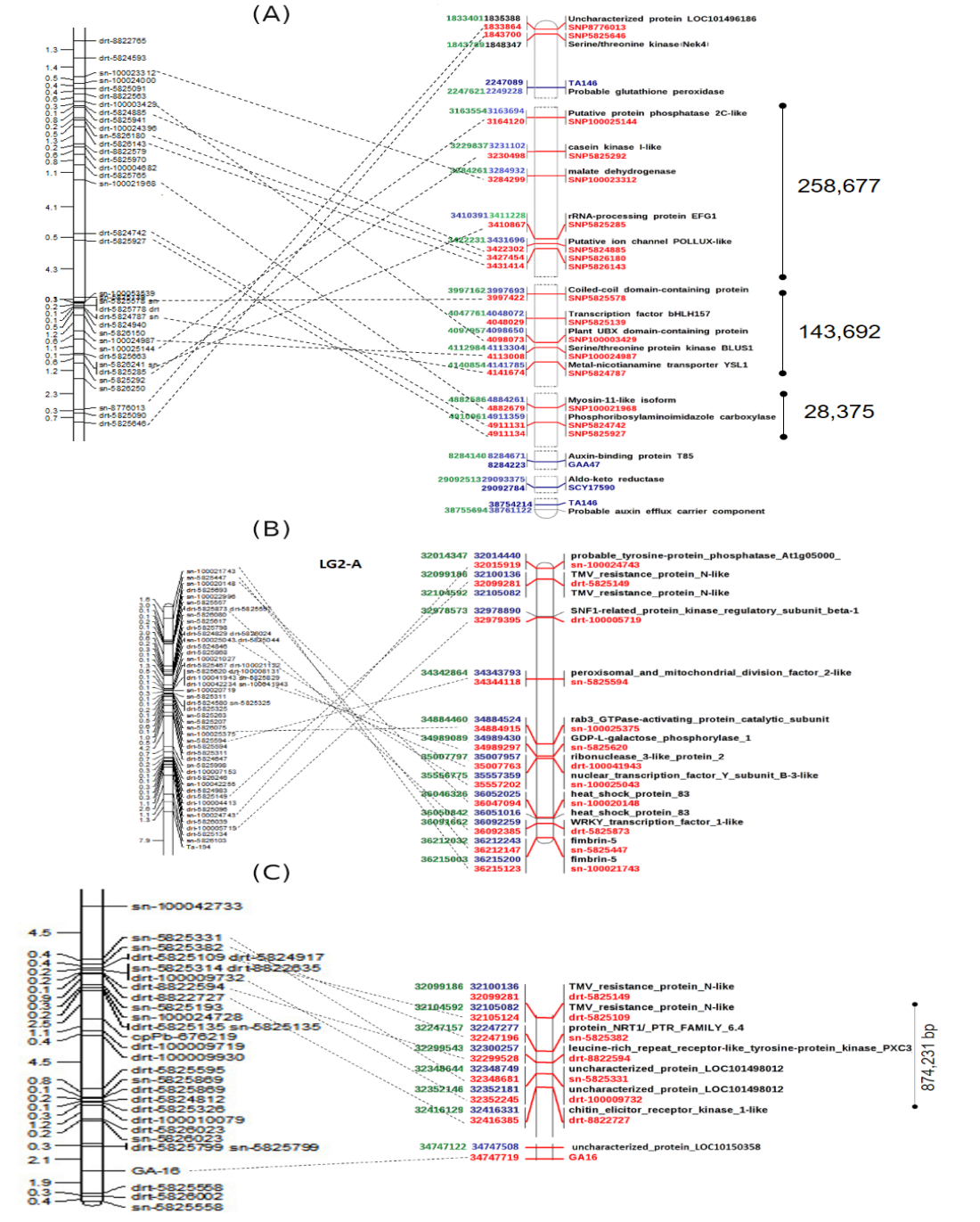

Figure 3. (A) Genetic and physical maps of markers on LG4. The estimated genetic distances are in centimorgans (cM) (left). The physical locations of mapped markers on chromosome 4 are shown in base pairs (bp) (right). (B) Genetic and physical maps of markers on LG2-A, one of the two major QTL regions conferring AB resistance in chickpea. (C) Genetic and physical maps of markers on LG2-B, the second major QTL region conferring AB resistance in chickpea.

Table 4. Markers on LG4 significantly associated with AB resistance across six environments.

Marker / sequence position	Env. Code	LOD	Variance	% Expl.	Additive	Marker gene linkage
sn-100024987	Lat-09	7.3	2.37	18.4	-0.73	Serine/threonine protein kinase BLUS1
	PII-2009	5.97	0.86	15.3	-0.40	(SNP is located within the gene)
	PII-2010	4.16	2.04	11.0	-0.50	
	TH-08	3.08	0.77	8.2	-0.26	
	TH-09	7.03	1.49	17.8	-0.57	
	TH-10	2.14	2.98	5.8	-0.43	
sn-5825578	Lat-09	7.28	2.37	18.4	-0.73	Coiled-coil domain-containing protein
	PII-2009	5.91	0.87	15.2	-0.39	(SNP is located within the gene)
	PII-2010	4.14	2.04	10.9	-0.50	
	TH-08	3.08	0.77	8.2	-0.26	
	TH-09	7.02	1.49	17.8	-0.57	
	TH-10	2.15	2.97	5.8	-0.43	
sn-5825139	Lat-09	7.12	2.38	18.0	-0.72	Transcription factor bHLH157
	PII-2009	5.12	0.89	13.3	-0.37	(SNP is located within the gene)
	PII-2010	4.02	2.05	10.6	-0.49	
	TH-08	3.29	0.76	8.8	-0.27	
	TH-09	6.94	1.50	17.6	-0.57	
	TH-10	2.36	2.96	6.4	-0.45	
drt-100004682	Lat-09	10.52	2.17	25.5	0.87	NA
	PII-2009	7.20	0.84	18.2	0.43	
	PII-2010	4.66	2.02	12.2	0.53	
	TH-08	3.92	0.75	10.4	0.30	
	TH-09	7.55	1.47	19.0	0.59	
	TH-10	2.38	2.96	6.4	0.45	
sn-5826150	Lat-09	6.95	2.39	17.6	-0.72	NA
	PII-2009	5.25	0.88	13.6	-0.37	
	PII-2010	4.10	2.05	10.8	-0.50	
	TH-08	3.44	0.76	9.1	-0.28	
	TH-09	6.90	1.50	17.5	-0.56	
	TH-10	2.17	2.97	5.9	-0.43	

NA: gene/position is not available

Table 5. Sequence and position on LG4 of SNP markers significantly associated with AB resistance.

Marker	SNP	Position	Sequence
sn-100024987	50:T>G	4113008	TGCAGAACAAGAAGCAATATCTCAGGTATAACTTATATTCAATTTTTATC(**T/G**)GTCATAAAATGTGCTATA
sn-5825578	24:T>C	3997422	TGCAGTGCTTCCTAAATTCGAAGA(**C/T**)CCTGTTTCTGTTCCTGAGCCTGAACCTGAAACTCAACCTAAGGA
sn-5825139	55:G>A	4048029	TGCAGAAAGAAAGGAAATTCTCAAGTAATAAGTGAACTAAAAACAAGAG(**A/G**)AATTGAAATATAAAATTAG
sn-5826150	6:C>G	4113005	TGCAGA(**G/C**)GGCATTCTCTTCAGTGCTAGTTGTGCAGCATCTTTACAGATCGGAAGAGCGGTTTCAGCAGGA
sn-100053539	5:T>C	4168916	TGCAG(**C/T**)GGGTGACGCAGTTATAGTTTACAACGTTATGGTGTTAGTGAATGGTTGAAATTATTTTACAGA
sn-5825294	11:C>G	4167416	TGCAGCAAACT(**G/C**)TGAAAAAATAAAGTAAGAGAAGTTCTAAGGTTCTATGAAAAAATAATAATGTATATT

been reported as genes associated with AB resistance on LG4. In this study, we found the genes annotated as leucine-rich repeat receptor-like tyrosine-protein kinase PXC3, TMV resistance protein N-like, chitin elicitor receptor kinase 1-like, histidine kinase 2, probable L-type lectin-domain containing receptor kinase S.7, uridine kinase-like protein 4, and serine/threonine protein kinase BLUS1. Very recent research by [20] validated a QTL similar to the one we observed on chromosome 4 using GWAS with a collection of 132 advanced breeding lines from the Australian chickpea breeding program and 144,000 SNP markers. The study predicted 12 genes located on a QTL region in LG4, including those annotated as an NBS-LRR receptor-like kinase, a wall-associated kinase, a zinc finger protein, and serine/threonine protein kinases. However, only one significant SNP from that study, located in the conserved catalytic domain of an NBS-LRR receptor-like kinase, led to an amino acid substitution.

In our study, 12 markers on the QTL region on LG4 were consistent across environments and could be used for MAS in *Cicer arietinum*. These include the markers sn-100024987, sn-5825578, sn-5825139, and drt-5824787, which are located, respectively, within four genes (serine/threonine protein kinase BLUS1, coiled-coil domain-containing protein, transcription factor bHLH157,

and metal-nicotianamine transporter YSL1) **(Table 5)**.

Unfortunately, little research has been conducted on these genes in *Cicer arietinum*. However, [20] reported that NBS-LRR receptor-like kinase and several serine/threonine protein kinases on chromosome 4 were predicted genes associated with AB resistance. These genes are already known to have a role in plant disease resistance. For example, the network of protein serine/threonine kinases in plant cells appears to act as a central processor unit that plays a role in the resistance of tomato to disease caused by *Pseudomonas syringae* pv. tomato [39].

Yellow-leaf-specific 9 (YSL9 / NHL10) is a transmembrane gene related to plant defense response to virus. The gene product is localized to the chloroplast and the mRNA is cell-to-cell mobile. In *Arabidopsis*, the expression of this gene is induced by virus infection and in senescing leaves [40; 41]. On the other hand, BLUS1 (Blue Light Signaling1 or Ser/Thr protein kinase) mediates a primary step for phototropin signaling in guard cells and is essential for stomatal opening, and it also has a role in the stress-activated protein kinase signaling cascade [42]. The coiled-coil (CC) domain is implicated in specific interactions with other proteins that enhance plant disease resistance. For example, coiled-coil nucleotide binding site-leucine rich repeat (CC-NB-LRR) protein Rx in potato has been found to interact with the potato virus X (PVX) coat protein, conferring PVX resistance [43]. Transcription factor bHLH157 (basic/helix-loop-helix) is a master-switch gene in plants [44] and plays an important role in disease resistance, high temperature-mediated adaptations, and phytochrome signaling [45; 46; 47]. The DREB/CBF were also regulated by bHLH-type transcription factor, ICE1 [48], and recent studies demonstrate that the basic helix-loop-helix (bHLH) transcription factor AtMYC2 plays a role in multiple hormone signaling pathways [49]. The regulation of flavonoid biosynthetic gene expression by the cooperation of R2R3 MYB and basic bHLH transcription factors provides one of the best described examples of combinatorial gene regulation in plants [50]. The transcription activator-like effector (TALE) protein was shown to induce bHLH transcription factors that activate a pectate lyase and contribute to water soaking in bacterial spot of tomato [51]. *Cicer arietinum* genome annotation [52] has been used as an annotation database for the SnpEFF tool in order to detect the possible effect of sn-5825578, sn-100024987, sn-5826150, sn-100053539, sn-5825294, and sn-5825139. These SNPs have shown a modifier effect on YLS9, coiled-coil domain-containing protein 12, BLUS1, and transcription factor bHLH157. Some genes, such as BLUS1 and methionine gamma-lyase-like, are affected by two SNPs, while others are affected by only one SNP.

In summary, it is not yet clear how the predicted genes located in the QTL regions we identified play a role in AB resistance in *Cicer arietinum*. Therefore, further research into the potential roles of these genes is recommended. However, this study presents the first set of functional SNP markers in chickpea (Table 5), which could be converted to Kompetitive Allele Specific PCR (KASP) markers and applied in MAS breeding programs for AB resistance in chickpea.

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