Theor Appl Genet (2006) 113:1357–1369
DOI 10.1007/s00122-006-0390-3

ORIGINAL PAPER

Mapping quantitative trait loci in chickpea associated with time to flowering and resistance to *Didymella rabiei* the causal agent of Ascochyta blight

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Received: 7 March 2006 / Accepted: 4 August 2006 / Published online: 22 September 2006

Abstract Drought is the major constraint to chickpea (*Cicer arietinum* L.) productivity worldwide. Utilizing early-flowering genotypes and advancing sowing from spring to autumn have been suggested as strategies for drought avoidance. However, Ascochyta blight (causal agent: *Didymella rabiei* (Kov.) v. Arx.) is a major limitation for chickpea winter cultivation. Most efforts to introgress resistance to the pathogen into Kabuli germplasm resulted in relatively late flowering germplasm. With the aim to explore the feasibility of combining earliness and resistance, RILs derived from a cross between a Kabuli cultivar and a Desi accession were evaluated under field conditions and genotyped with SSR markers. Three quantitative trait loci (QTLs) with significant effects on resistance were identified: two linked loci located on LG4 in epistatic interaction and a third locus on LG8. Two QTLs were detected for time to flowering: one in LG1 and another on LG2. When resistance and time to flowering were analyzed together, the significance of the resistance estimates obtained for the LG8 locus increased and the locus effect on days to flowering, previously undetected, was significantly different from zero. The identification of a locus linked both to resistance and time to flowering may account for the correlation observed between these traits in this and other breeding attempts.

Communicated by J.-L. Jannink.

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Abbreviations

LOD	Logarithm of the odds of linkage between two loci
PEV	Proportion of explained phenotypic variance
QTL	Quantitative trait locus (QTLs for loci)
RDR	Relative response to *Didymella rabiei*
RIL	Recombinant inbred line (RILs for lines)
SSR	Simple sequence repeat
tRAUDPC	Arcsin transformed area under the disease progress curve

Introduction

Chickpea (*Cicer arietinum* L.) originated as a grain legume crop in the Near East Neolithic revolution dating some 10,000 years ago (Lev-Yadun et al. 2000). Currently it is a staple protein crop in the Indian sub-continent,

the Near East and across the Mediterranean basin (Ladizinsky 1995). Expansions of area under chickpea cultivation were recently reported in the USA, Canada and Australia (FAO 2005). In most of its growing systems chickpea is a dryland crop with a global average grain yield of 0.7 t ha^{-1}. In the Near East and Mediterranean-like environments the crop is sown either as a winter crop (rainfed), or as spring crop in which case it relies on residual soil moisture (Kostrinski 1974; Singh et al. 1997). Unlike the limited yield potential of spring-sown chickpea, ranging between 0.3 and 0.6 t ha^{-1} (Elazari-Volcani 1930; Kostrinski 1974), winter-sown crops may yield 2–4 t ha^{-1} (Singh et al. 1997). The higher yields of the winter-sown crops are attributed mainly to higher biomass accumulation resulting from improved water availability.

Because of their inherent long-day requirements, most Mediterranean chickpea stocks are relatively late to flower, even when sown as early as late November (Or et al. 1999; Kumar and Abbo 2001). Consequently, in the Mediterranean environments, podding and grain filling occur in the post rainy season, late April through June (Or et al. 1999; Singh and Reddy 1996; Singh et al. 1997). Lush vegetative growth following winter sowing may expose dryland chickpea to high evaporative demand and water stress during the critical stage of grain development, thereby restricting its grain yield (Turner et al. 2001).

Incorporating early flowering/podding into Mediterranean chickpea germplasm might assist in realizing the higher yield potential of winter-sown chickpea by extending pod-set duration (Kumar and Abbo 2001; Miller et al. 2002). However, winter sowing exposes chickpea to a high risk of Ascochyta blight caused by the fungus *Didymella rabiei* (Kovachevski) v. Arx. (anamorph *Ascochyta rabiei* (Pass.) Labrousse) and thus requires the development of resistant cultivars. Ascochyta blight is a major constraint of chickpea production worldwide (Kaiser 1992; Singh and Reddy 1996; Shtienberg et al. 2000; Pande et al. 2005). In the Mediterranean basin, the disease spreads rapidly when conditions are conducive to the pathogen (i.e., frequent rains and temperatures ranging from 15 to 20°C) and may result in total crop loss (Vir et al. 1975; Singh and Reddy 1996; Shtienberg et al. 2000). *Didymella rabiei* attacks all aerial parts of the plant causing necrosis and tissue collapse. Often, lesions girdle the stem or branches causing death of the parts above the lesion.

In the framework of a pre-breeding program aimed at incorporating early-flowering alleles into Israeli chickpea germplasm, we produced segregating populations derived from a cross between a cultivar of Kabuli type (large, white-cream thin coated seeds) and an accession of Desi type (small, dark seeds with thick irregular-shape coats). The Kabuli cultivar (Hadas) is high yielding, late-flowering and moderately resistant to *D. rabiei* while the Desi accession (ICC5810) is early-flowering and highly susceptible to the pathogen. The analysis of the phenotypic data obtained from the F_3 and F_4 progeny derived from 'Hadas × ICC5810' and the reciprocal cross showed that segregation of one (or a few) locus (loci) with major effect and possibly additional minor loci was the predominant mode of inheritance of the chickpea resistance in the field (Lichtenzveig et al. 2002). In addition, a negative correlation was found between resistance and early flowering (Lichtenzveig et al. 2002). This correlation is not unique to the mapping populations evaluated in this study, but is also apparent from the phenology of certain Ascochyta resistant selections from ICARDA (see review by Kumar and Abbo 2001). The association between resistance and flowering time is poorly understood, yet it is highly relevant for chickpea breeding to determine whether the correlation is caused by pleiotropy or tight genetic linkage. Understanding the genetic basis of the association between days to flowering and resistance to *D. rabiei* is of utmost importance for the development of early flowering varieties for dryland winter-cropping.

Quantitative trait locus (QTL) mapping by means of DNA markers is a highly effective approach for dissecting the genetic factors affecting complex traits (Young 1996). With QTL mapping the effect of specific loci can be assessed and the interactions between resistance genes, plant development, and the environment can be analyzed. The aim of this study was to determine the genetic basis of the association between flowering time and resistance to *D. rabiei*. This included assessing the number and chromosomal positions of loci associated with each of the traits, estimating their effect, and identifying molecular markers closely linked to these QTLs.

Materials and methods

Plant population

The plant population studied consisted of 120 recombinant inbred lines (RILs) derived from a cross between the Israeli cultivar Hadas (maternal parent) and an Indian accession ICC5810 (Lichtenzveig et al. 2002). Hadas is a high-yielding cultivar of Kabuli type with beige, relatively large (450 mg) seeds, moderately resistant to *D. rabiei*, late to flower and semi-erect

growth habit. ICC5810 is an Indian accession (with poor agronomic performance in Israel), highly susceptible to the fungus, early to flower, with typical Desi black small (150 mg) seeds and with a leaning growth habit. The progeny of the cross were propagated by single-seed descent up to F_6. Simple sequence repeat (SSR) genotypes and seed weight were assessed on F_5 plants and time to first flower and resistance to the pathogenic fungus were evaluated on F_6 lines derived from the same F_5 plants.

Field trials and inoculation

The RILs along with seven varieties (ICC5810, Hadas, ICC7344, ILC1929, ILC482, ILC3279 and Bulgarit) were evaluated in 2002 in different locations: Massuot-Yitzchak (hereafter Massuot), located near Ashkelon in central Israel (440 mm average annual rainfall) and Gilat Research Centre, located in the Northern Negev region (230 mm average annual rainfall). The experiments were laid out in the same way, each consisting of ten 125 m rows spaced 1 m apart. Of the ten rows, four (rows 1, 4, 7 and 10) were planted to a highly susceptible cultivar (Ayelet) and served as "spreader rows". The inclusion of spreader-rows served to ensure uniform dissemination of the disease across the field. The RILs and seven varieties were sown in the remaining six trial rows. The varieties served to assess random field trends and relative inoculum pressure; these are referred as the standard varieties hereafter. The 750 lineal meters in the six trial rows were divided into 1 m experimental units (plots). The seven standards were replicated 24 times (four plots in each row), and RILs were replicated five times and assigned randomly to the available plots. The six trial rows served as incomplete blocks. With the intention of measuring the reaction of single plants and maximizing the number of experimental units only three seeds per experimental unit were sown by hand

The spreader-rows at the Gilat site were inoculated with a *D. rabiei* suspension derived from a single conidiospore about 5 weeks after most of the genotypes had emerged. The suspension preparation and inoculation procedure were carried out as described by Lichtenzveig et al. (2002). Prior to inoculation of the spreader-rows, the trial rows were protected with systemic fungicides (alternative applications of Azoxystrobin 125 g ha^{-1} and Mancozeb, see Fig. 1b) to avoid premature infection caused by naturally occurring sources or the spore spray and to ensure epidemic uniformity throughout the experimental site as well. The experimental site in Massuot was treated in a similar way. However, even though the inoculation was done on a cloudy day and the site was heavily watered with sprinkler irrigation prior to inoculation, dry conditions immediately post-inoculation impeded the establishment of the disease in the site.

The nursery in Massuot was planted by the end of December 2001 and remained uninfected; the Gilat nursery was planted in mid-January and was successfully inoculated by mid-March. The first Ascochyta blight symptoms in Gilat were observed, as expected, 13 days post-inoculation in the spreader-rows and 23 days post-inoculation in the trial-rows (Fig. 1). By May 5, the percentage of disease severity in the spreader-rows reached 80–100%.

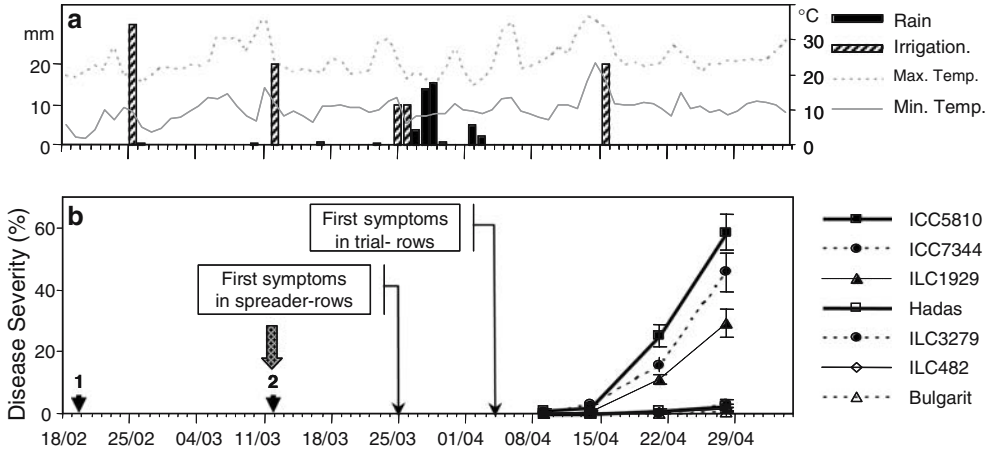

Fig. 1 Seasonal profile in the Gilat Research Centre 2002 (**a**) and Ascochyta blight severity in standard varieties in the experimental site therein (**b**). *Arrowheads* show fungicide application: *1* application to the entire site and *2* application to the experimental lines only. The *arrow* shows inoculation date. The appearance of first Ascochyta blight symptoms is indicated. *Vertical bars* represent the SE of the response

Phenotypic assessment

Each plant was scored individually. Except for those showing abnormal development, all plants within a meter-plot were scored. Disease severity (% of infected foliage area) was assessed visually at the Gilat experimental site. First scores were taken soon after noting the first Ascochyta blight symptoms in the trial-rows. Subsequent assessments were performed every 6–7 days. The relative area under the disease progress curve (RAUDPC) was calculated using the season long assessments and according to the epidemic duration. RAUDPC values were transformed (arcsin or angular transformation; Kuehl 2000) to minimize deviations from normality (Lichtenzveig et al. 2002). To simplify the representation of the plant response to the disease, the response is presented as the proportion (in percentage) of a given arcsin transformed RAUDPC (tRAUDPC) with respect to the highest averaged tRAUDPC RIL value (tRAUDPC = 0.34 = 100%, Fig. 2) and is referred to as the relative response to *D. rabiei* (RDR). Days to first flower were determined every 6–7 days at both sites. In addition, the seed weight of 100 $F_{5:6}$ seeds was recorded for each of the F_6 RILs prior to sowing; the F_5 maternal plants were grown in a non-infected nursery.

Field data analyses

All statistical analyses were performed with JMP-IN 4 (SAS Institute). The response of the standard varieties was analysed considering the plot mean as an experimental unit. Since some heterozygosity was expected to remain in the F_6-RIL population, the analyses were done on an individual-plant rather than on a plot basis; on average data from 8 (range 3–11) individuals were analyzed for each RIL. Days to first flower and tRAUDPC were examined independently for the presence of field trends using the General Mixed Model, including fixed (variety/RIL) and random (trial-row) effects; the Variety by Row interaction was estimated only for the standard varieties. The random effect estimates were determined to adjust the response scores in accordance with the significant field trends. Those adjusted scores were used in further analyses. Heritability estimates (h^2) for days to flowering and resistance to *D. rabiei*

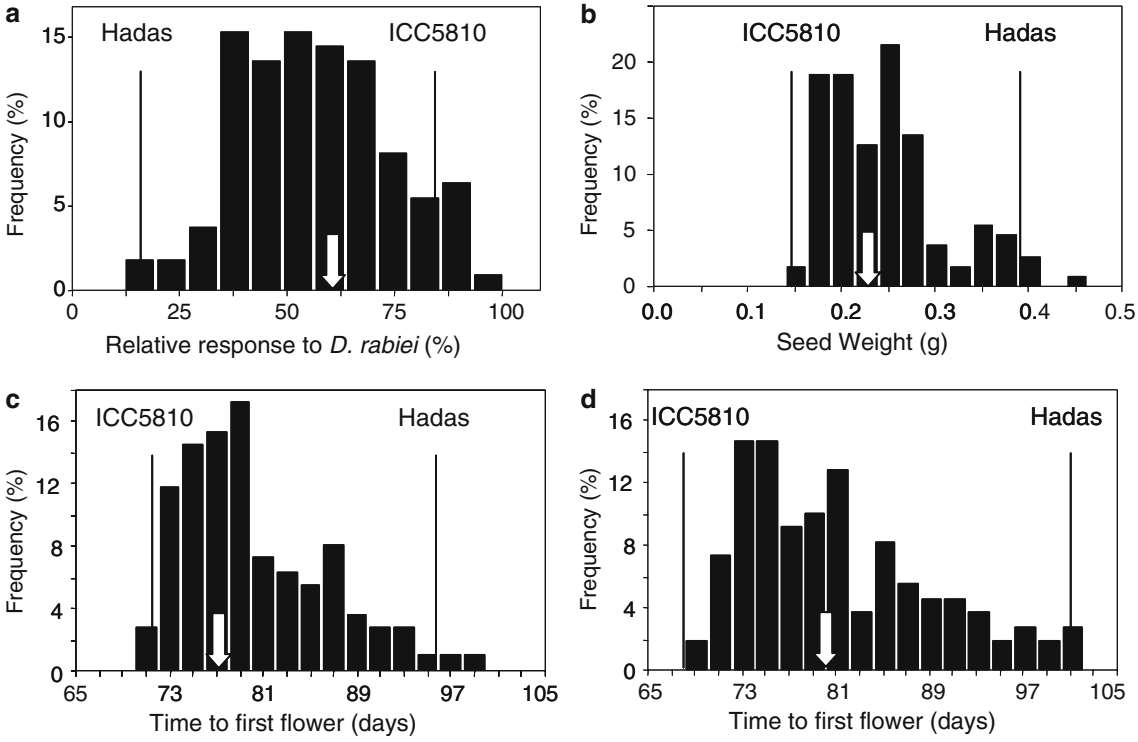

Fig. 2 Phenotypic distribution of the 'Hadas × ICC5810' RILs and the parental lines. All plots show F_6-RILs means adjusted in accordance with field trends, with the exception of **b** showing F_5 values. **a** Relative response to *Didymella rabiei* in Gilat, presented as the proportion of a given transformed (arcsin or angular transformation) relative area under the disease progress curve (tRAUDPC) with respect to the highest averaged tRAUDPC value (100% equals tRAUDPC = 0.34). **b** Seed weight of F_5 plants, predecessors of the F_6-RILs. **c** Time to first flower in Gilat. **d** Time to first flower in Massuot. *White arrows* indicate the populations' means

were calculated using between- and within-F_6 RIL variance components, estimated by one-way ANOVA with the F_6 RIL as the class variable.

Genetic correlations between the relative response to *D. rabiei* and time to flowering were estimated by calculating the correlation coefficient using the RILs' mean values for tRAUDPC and days to first flower (Lynch and Walsh 1998). Correlations between these traits and seed weight were determined using data obtained from different generations.

Genotyping and linkage analysis

The RILs were genotyped at F_5 using (SSR) markers as described by Lichtenzveig et al. (2005b). The primer sequences for marker loci starting with H were published by Lichtenzveig and co-authors (2005b); others were published by Winter and co-authors (1999) and served as anchors to deduce linkage group identity relative to published chickpea maps (Winter et al. 2000; Pfaff and Kahl 2003). Pearson Chi-square analysis was applied to test the observed segregation ratio of parental alleles against the expected 1:1 Mendelian segregation ratio for co-dominant inheritance in a RIL population. Residual heterozygous alleles were considered as missing data in the linkage analysis. Markers order and map distances were calculated with MAPMAKER V3.0 (Lander et al. 1987) for selfed RIL, using a LOD scores of 3 as the minimal threshold and a maximum recombination fraction of 0.25, using the Kosambi mapping function.

QTL detection

The QTL analysis was performed with the software-package MultiQTL (http://www.esti.haifa.ac.il/~poptheor/) using the general interval mapping and marker restoration options for a RIL-selfing population. Heterozygous alleles were not included in the analysis. The hypotheses that a single locus or two linked loci have an effect on one or two quantitative traits were evaluated similarly to Peng et al. (2003). First, by running 5,000 permutation tests (Churchill and Doerge 1994), the hypothesis that one locus on the chromosome has an effect on a given trait (H_1) was compared against the null hypothesis (H_0) that the locus has no effect on the trait. Subsequently, a genetic model was fitted to the QTL detected based on the proportion of total trait variance explained by the model; the simplest model, calculated by default, assumes equal residual variance for allelic groups, QQ and qq (Korol et al. 1996), while the alternative would be the 'general model', which assumes unequal residual variances. The models were compared to each other by running 3,000 permutation tests; in cases where the models differed significantly ($P < 0.05$), the model with the highest LOD score was fitted to the QTL; when the models did not differ significantly the simpler model was chosen. Once the genetic model was chosen, 5,000 bootstrap samples were run to assess the estimates and the standard deviation (SD) of the main parameters: locus effect, its chromosomal position, its LOD score and the proportion of explained variability (PEV). Second, the hypothesis that two-linked QTLs have a significant effect on one trait (H_2) was compared to the other two alternative hypotheses, H_1 and H_0 (Ronin et al. 1999), using the Monte Carlo test (5,000 simulations). As described above, a genetic model was chosen for the linked QTLs. In this case, the genetic model included the possibility of epistatic interaction between the loci and can be presented as: $\boldsymbol{x = \mu + 0.5(d_1 g_1 + d_2 g_2) + \varepsilon g_1 g_2 + \xi}$; where $\boldsymbol{x}$ represents phenotypic scores of individuals of the mapping population; $\boldsymbol{\mu}$ is the expected mean value of the trait; $\boldsymbol{d}_1$ and $\boldsymbol{d}_2$ are the substitution effects for Q_1/q_1 and Q_2/q_2, respectively; g_1, g_2 designate genotypes at QTLs Q_1/q_1 and Q_2/q_2 ($g_i = -1$ for *QiQi* if the RIL carries the Hadas alleles and $g_i = 1$ for *qiqi* if the RIL carries the ICC5810 alleles); the interaction between the QTLs is accounted for by the epistatic term ε; ξ is the residual variance, random and normally distributed with zero expectation.

Third, the hypothesis of a single locus having significant effects on a couple of traits was evaluated similarly to the described above by performing new genome scans. Finally, to evaluate the genome-wise significance of estimates obtained on a chromosome-trait basis the approach based on controlling the false discovery rate (FDR) (Benjamini and Hochberg 1995) was used to correct for multiple comparisons (Peng et al. 2003).

Results

Phenotypic assessment

General field trends

Days to first flower and resistance to *D. rabiei* were significantly affected by trial-row position in the field. The magnitude and pattern of the effect varied according to the trait and experimental site (Table 1). The row effect accounted for 4–7% of the random variance observed in days to flowering, while 35% of the total variance was explained by the row effect on the response to *D. rabiei* (Variance Component Estimate,

Table 1). Significantly ($P_t < 0.0001$) higher disease scores were recorded in the trial rows situated east of the spreader-rows (averaged tRAUDPC = 0.2511) than in the trial-rows situated west of the spreader-rows (averaged tRAUDPC = 0.1537). Similar trends were observed for the standard varieties (data not shown). It is reasonable to assume that the actual inoculum availability might have caused such a large effect on the disease response: the direction of the rows in Gilat was North-South and the wind direction during rain and irrigation events was usually West to South-East, causing most probably, a difference in the inoculum pressure spread to the trial-rows. Since no significant interaction between the standard lines and the row factor was observed for any of the traits ($P_F > 0.1622$), the responses were adjusted by subtracting the row effect from the assessed score for representation and further analyses. In addition to systematic field trends, considerable residual variances were observed for the evaluated traits (R^2, Table 1).

Resistance to D. rabiei

The standards' response to the pathogen is shown in Fig. 1 and Table 2. ICC5810 was the most susceptible (tRAUDPC = 0.286; RDR = 84.1%) and Hadas was among the most resistant to the disease (tRAUDPC = 0.053; RDR = 15.6%). The relative response of the other standard varieties was in line with previously published experiments (Lichtenzveig et al. 2002, 2005a); see order of varieties in Table 2. The standards' response to the disease validates the phenotypic assessment of the RILs.

The frequency distribution of the RILs' in their response to *D. rabiei* (Fig. 2a) was not significantly ($P_W = 0.2447$) different from a normal distribution. The frequency distribution was in accordance with the phenotypic range of the parental lines; only one family (0.9%) was significantly ($P_t < 0.0455$) more susceptible (RDR >96%) than ICC5810. The population mean (RDR = 59.1%, tRAUDPC = 0.2010) was significantly ($P_t < 0.0001$) different from the mid-parent value

Table 1 Analyses of variance, the proportion of variance explained by the general mixed model (R^2), and heritability values obtained from 'Hadas × ICC5810' F_6 RILs evaluated under field conditions

Site	Variable	Analysis of variance				R^2	Heritability[b]
		Source	df	Prob. of *F*	Variance component estimate (%)[a]		
Gilat	Response to *D. rabiei* [c]	RIL	110	<0.0001	–	0.56	0.33
		Row (random)	5	<0.0001	35.1		
	Days to flowering	RIL	110	<0.0001	–	0.48	0.38
		Row (random)	5	<0.0001	6.9		
Massuot	Days to flowering	RIL	119	<0.0001	-	0.62	0.54
		Row (random)	5	<0.0001	3.9		

[a] Variance component estimate (percentage of total random variance); not applicable to fixed terms

[b] Calculated based on relationships within a single generation (F_6)

[c] Analyzed as tRAUDPC

Table 2 Characterization of chickpea standard varieties

Variety	Relative response to *D. rabiei*[f] Gilat	Days to flowering Gilat	Days to flowering Massuot	Seed weight (g/seed) [e]
ICC5810	84.1 (23.8)[a]	71.5 (2.5)[d]	68.1 (3.2)[d]	0.146
ICC7344	69.1 (22.3)[b]	73.6 (4.4)[d]	69.5 (3.7)[d]	0.490
ILC1929	53.5 (18.5)[c]	87.9 (5.3)[c]	96.2 (5.4)[b]	0.174
Hadas	15.6 (6.7)[d]	95.8 (5.8)[b]	101.1 (3.4)[a]	0.389
ILC3279	9.7 (9.7)[d]	100.9 (3.9)[a]	101.5 (3.2)[a]	0.275
ILC482	7.6 (7.1)[d]	92.0 (8.6)[b,c]	92.4 (5.7)[c]	0.230
Bulgarit	6.8 (5.3)[d]	95.7 (5.0)[b]	99.5 (3.7)[a,b]	0.244

Means (standard deviation) are presented. For all responses except seed weight, least square means are presented. These were obtained from ANOVA of general mixed model with variety and row as fixed and random factors, respectively

[a–d] Multiple comparisons of means by Tukey–Kramer Honestly Significant Difference tests ($\alpha = 0.05$)

[e] A total of 150–250 seeds from several individuals were weighed together; the average of grams per seed was calculated

[f] ANOVA and comparisons of means were done using the tRAUDPC data and are presented herein as the proportion of the mean tRAUDPC relative to the highest tRAUDPC mean (tRAUDPC = 0.34)

(RDR = 49.8%, tRAUDPC = 0.1695) and closer to the average of the susceptible parent ICC5810 than to that of the resistant parent Hadas (Fig. 2a).

Time to first flower

Time to first flower was evaluated in Gilat and Massuot. The sites differed not only in their climatic conditions but also in sowing time (see Materials and methods). Although similar results were obtained at both sites, the residual variance of Massuot was of smaller magnitude (R^2 and heritability estimates, Table 1) and the differences between 'early' and 'late' lines were more pronounced (*e.g.*, Hadas and ICC5810 in Table 2). At both sites, the phenotypic distributions were significantly ($P_W < 0.0001$) different from normal distributions. A clear bi-modal distribution was evident for data obtained from Massuot (Fig. 2d), the bi-modality of the distribution of data from Gilat was more subtle (Fig. 2c).

Correlations between variables

A weak but highly significant negative correlation was obtained between days to first flower and susceptibility at Gilat ($r = -0.2653$, $P_r = 0.0049$, $n = 111$). Similar results were obtained when phenology data obtained from Massuot were correlated to disease severity data from Gilat ($r = -0.2344$, $P_r = 0.0133$). Correlation coefficient estimates obtained from data from the same experimental site (Gilat) might be biased by correlations among error residuals. However, estimates obtain from different sites (Gilat and Massuot) represent unbiased estimates of the genetic correlation. Both at Gilat and Massuot days to first flower were significantly correlated with seed weight ($r = 0.2488$, $P_r = 0.0088$ and $r = 0.4138$, $P_r < 0.0001$, respectively); no correlation was observed between resistance and seed weight.

QTL detection

One locus:one quantitative trait

The results obtained from analyzing each trait independently and assuming a single putative locus per linkage group are presented in Table 3. At the chromosome-trait level, all traits were found to be associated ($P < 0.05$) with at least one locus (Table 3). Most QTLs detected at the chromosome-trait level stood the genome-wise significance test (see asterisks representing the false discovery rate, Table 3). These included loci with relatively low LOD scores (LOD = 2–3) such as the LG8 locus associated with resistance (Table 3). The only locus which did not stand the genome-wise significance test was the LG1 locus associated with seed weight (false discovery rate >10%, Table 3).

The genetic model chosen for the putative QTLs associated with days to first flower and seed weight (Table 3) was the simplest model which assumes equal residual variance for the allelic groups, QQ (promoting) and qq (demoting). For the relative response to *D. rabiei*, the alternative model was chosen due to its significantly ($P < 0.05$) higher LOD score (6.92) and PEV (proportion of explained variability by a putative QTL = 0.42); the model assumes unequal residual variances (Table 3).

Linked loci:one quantitative trait

The hypothesis that two linked loci have an effect on a trait (H_2) was compared to the hypothesis that only one locus in the linkage group has an effect on a given trait (H_1). Only for the relative response to *D. rabiei*, the Monte-Carlo simulations resulted in a probability ratio favouring H_2 ($P = 0.016$), predicting two-linked loci in LG4. Four genetic models were evaluated for the putative QTLs (Table 4). Considering LOD scores and PEV values, the most suitable model assumes unequal residual variances of the allelic groups and epistasis between the two loci (model 4, Table 4). The estimates and standard deviations of the loci parameters were obtained by bootstrap tests (Table 5). Overall, the linked-loci analysis shows that most likely at least two loci on LG4 have an effect on the relative response to *D. rabiei*: one with a major effect in interval 2 close to marker TA2 (3.0 cM), and the other with a minor effect in interval 5 close to marker H1H13 (12.2 cM).

One locus:two quantitative traits

The assumption that one QTL has an effect simultaneously on two traits was evaluated for each pair of traits. In general, an increase in the estimation resolution (higher LOD scores and PEV values) was observed for the QTLs described in Table 4. The hypothesis that a single QTL has a significant effect on two traits was accepted only if the effect of each QTL was at least twofold higher compared to its standard deviation calculated across the bootstrap runs (Table 6). Interestingly, LG8 had a significant effect not only on resistance, as shown in Table 4, but also on days to first flower (Table 6), possibly accounting for the genetic correlation observed between these traits. To summarize the QTL detection results, loci with genome-wise significant effects and reliable estimates

Table 3 'One locus–one quantitative trait' hypothesis test

Locus estimates	Response to *D. rabiei*[h] Gilat		Days to first flower Massuot		Days to first flower Gilat		Seed weight[i]
LG [Interval] [a]	4 [2]	8 [1]	1 [2]	2 [13]	1 [2]	2 [13]	1 [3]
P[b]	0.0002 ***	0.0030 ***	0.0002 ***	0.0002 ***	0.0002 ***	0.0034 **	0.0206
LOD[c]	6.9 (2.2)	2.4 (1.3)	9.0 (3.1)	4.4 (1.8)	8.8 (3.0)	3.7 (1.6)	2.48 (1.36)
Position[d]	3.6 (2.7)	2.3 (0.7)	15.3 (4.4)	48.2 (16.4)	17.6 (4.9)	50.7 (18.2)	40.5 (5.0)
PEV[e]	0.42 (0.11)	0.11 (0.05)	0.56 (0.10)	0.22 (0.09)	0.53 (0.10)	0.18 (0.08)	0.11 (0.05)
Response Mean[f]	58.5 (1.7)	58.8 (1.6)	84.1 (1.0)	80.6 (0.8)	81.5 (0.8)	79.2 (0.7)	0.238 (0.006)
Effect[g]	−16.8 (3.2)	−10.3 (2.9)	14.5 (2.1)	7.4 (1.8)	10.1 (1.6)	4.9 (1.3)	0.041 (0.014)

Estimates of QTLs with significant effects (at the chromosome-wise level) on the response to *D. rabiei*; days to first flower—evaluated in two environments (Massuot and Gilat) and seed weight—obtained from analyzing each trait independently. SD are shown in parentheses

[a] Linkage group and interval within LG associated with the quantitative trait

[b] Probability values from permutations analyses at the chromosome-trait level testing the null hypothesis: the linkage group has no effect on the trait. Asterisks indicate significance at genome-wise level at false discovery rate:10 (*),5(**), and 1% (***)

[c–g] Estimated by bootstrap tests at chromosome-wise level

[c] Maximum LOD score for a given interval

[d] Position (cM) of maximum LOD value within interval measured from the first marker in the linkage group (0 cM). Estimates obtained with MultiQTL were corrected according to distance obtained from MapMaker

[e] Proportion of explained variability by the putative QTL

[f–g] In percentage for the response to *D. rabiei*, in days for days to first flower, and in grams for seed weight

[g] The estimated effect of the 'Hadas' allele

[h] Analyzed as tRAUDPC, presented as the proportion (in percentage) of a given tRAUDPC with respect to the highest averaged tRAUDPC (0.34)

[i] Unlike other traits that were evaluated on F_6 plants, seed weight was obtained from the F_5 parental plants

Table 4 The LOD score and the proportion of explained variability (PEV) for the putative QTLs on LG4 associated with the relative response to *D. rabiei* are given for the combined assumptions of equal/unequal residual variances among the allelic groups (QQ QQ, QQ qq, qq QQ, qq qq) and the epistasis effect between the loci

Genetic model		LOD	PEV
1 Equal variance	No epistasis	5.8	0.230
2 Unequal variance	No epistasis	7.9[a]	0.349
3 Equal variance	With epistasis	6.5	0.266
4 Unequal variance	With Epistasis	9.3[b]	0.375

[a] LOD of model 2 is significantly (3,000 permutations, $P = 0.049$) higher than that of model 1

[b] LOD of model 4 is significantly (3,000 permutations, $P = 0.050$) higher than that of model 2

were plotted along their respective linkage groups of the 'Hadas × ICC5810' population (Fig. 3).

Discussion

Genetic basis of resistance to *D. rabiei*

Three loci with significant effects on resistance were identified using a population derived from a *C. arietinum* intra-specific cross between a Kabuli cultivar (moderately resistant) and a Desi accession (highly susceptible). A locus with a major effect (14.4%) on the resistance response was found on LG4 (QTL4.1) delimited by the markers H3C041 and TA2. The second locus (QTL4.2), with a minor effect (3.8%) on the resistance response, was found about 9.2 cm apart delimited by the markers H1A12/H1H13 and H1G20. Together, these loci explained the largest proportion of the response variance (42%). A third locus, also with a major effect (10.2%), was found on LG8, explaining 16% of the total phenotypic variance.

Several groups have already reported an oligogenic inheritance of resistance to *D. rabiei* in chickpea (Santra et al. 2000; Collard et al. 2003; Flandez-Galvez et al. 2003). Although some confusion exists as to the number of loci and their location in the chickpea genome, a detailed examination of published maps (Winter et al. 1999, 2000; henceforth, the 'standard chickpea map') makes it possible to formulate a general conclusion. Using a cross between *C. arietinum* and *C. reticulatum*, Santra and co-authors (2000) identified three resistance QTLs by single-point (or marker) analysis. The linked markers explained 42, 20 and 10% of the estimated phenotypic variance for QTL1, QTL2 and QTL3, respectively. The loci were mapped to three independent linkage groups in a genetic map constructed with RAPD (Random Amplification of Polymorphic DNA)

Table 5 Linked QTL analysis of the response to *D. rabiei* on LG4

					Parameters of genetic model				Residual			
	LOD	L 1	L 2	PEV	M	D1	D2	E	QQ QQ	QQ qq	qq QQ	qq qq
Mean	11.5	3.0	12.2	0.419	61.5	−14.4	−3.8	−4.1	13.18	6.94	5.59	17.9
SD	2.68	1.3	2.6	0.123	2.6	4.7	4.4	2.9	2.06	2.72	1.76	1.47

Mean estimates and standard deviation (SD) of the two QTLs (1 and 2) obtained by running 10,000 bootstrap samples. The residual standard deviations of the response for every allelic group are given

LOD Maximum LOD score for a given interval. *L* Location in recombinant distance (cM) of maximum LOD value within interval the respective intervals (QTL 1 in interval 2; QTL 2 in interval 5) measured from the first marker in linkage group (at 0 cM). *PEV* Proportion of explained variability by the putative QTLs. *M* Response mean in percentage, analyzed as tRAUDPC and presented as the proportion of a given tRAUDPC with respect to the highest averaged tRAUDPC (0.34). *D* Effect of the 'Hadas' allele in the respective locus. *E* Effect of the interaction between loci, the epistatic interaction

Table 6 'One locus–two quantitative traits' analysis

Locus estimates	Response to *D. rabiei*[g]: days to first flower (Massuot)		Response to *D. rabiei*: days to first flower (Gilat)	
LG [Interval] [a]	8 [1]		8 [1]	
P[b]	0.0002 ***		0.0024	
LOD[c]	3.9 (1.6)		3.6 (1.5)	
Position[d]	2.3 (0.8)		2.1 (0.9)	
PEV[e]	0.17 (0.06)		0.16 (0.06)	
Response Mean	58.8 (1.6)%	79.4 (0.7) days	58.8 (1.6)%	78.7 (0.5) days
Effect[f]	−10.1 (3.2)%	4.7 (1.4) days	−10.3 (3.1)%	3.2 (1.1) days

Estimates of QTLs with significant effects (at the chromosome-wise level) on the response to *D. rabiei* and days to first flower, evaluated in two environments (Massuot and Gilat). Single-unlinked QTL analysis, permutation tests and false discovery rate approach were performed for pair of traits. Standard deviations are shown in parenthesis

[a] Linkage Group and interval within LG associated with the quantitative trait

[b] Probability values from permutations analyses at the chromosome-trait level testing the null hypothesis: the linkage group has no effect on the trait. Asterisks show significance at genome-wise level at false discovery rate: 10 (*), 5 (**) and 1% (***)

[c-f] Estimated by bootstrap tests at chromosome-wise level

[c] Maximum LOD score for a given interval

[d] Position (cM) of maximum LOD value within interval measured from the first marker in linkage group (0 cM). Estimates obtained with MultiQTL were corrected according to distance obtained from MapMaker

[e] Proportion of explained variability by the putative QTL

[f] The estimated effect of the 'Hadas' allele

[g] Analyzed as tRAUDPC, presented as the proportion (in percentage) of a given tRAUDPC with respect to the highest averaged tRAUDPC (0.34)

and ISSR (Inter Simple Sequence repeat) markers. SSR markers were later incorporated into this map (Tekeoglu et al. 2002) and in a later study (Cho et al. 2004) the authors suggested that the loci explaining the largest estimated phenotypic variance, QTL1 and QTL2, reside on LG4 (of the standard map) more than 50 cM apart from each other. Cho et al. (2004) provided no information on the position of QTL3 nor on the relative effect of the three loci. Collard et al. (2003) used an F_2 population derived from a cross between *C. arietinum* and *C. echinospermum* and scored the resistance in a glasshouse at seedling stage. Two QTLs were found associated with seedling resistance by interval mapping, both on LG4 (standard map) with LOD scores of 2.5 and 2.6, about 8 cM apart from each other, with no information on loci effects or genetic model. Flandez-Galvez and co-authors (2003) studied the resistance response of a Desi × Desi F_3 population both under field and glasshouse conditions. By multiple-interval mapping a 25 cM region along LGIII (LG4 of the standard map) was found to have a significant major effect on the resistance response in both environments, explaining 60 or 29% of the phenotypic response obtained from the field or glasshouse, respectively. In addition, two loci with minor effects, one on LGI (corresponding to LG3 in the standard map) and another on LGII (corresponding to LG8 in the standard map) were associated with the resistance response assessed in the field or the glasshouse, respectively. An apparent epistatic interaction between the major and minor loci for each of the environments was observed (Flandez-Galvez et al. 2003).

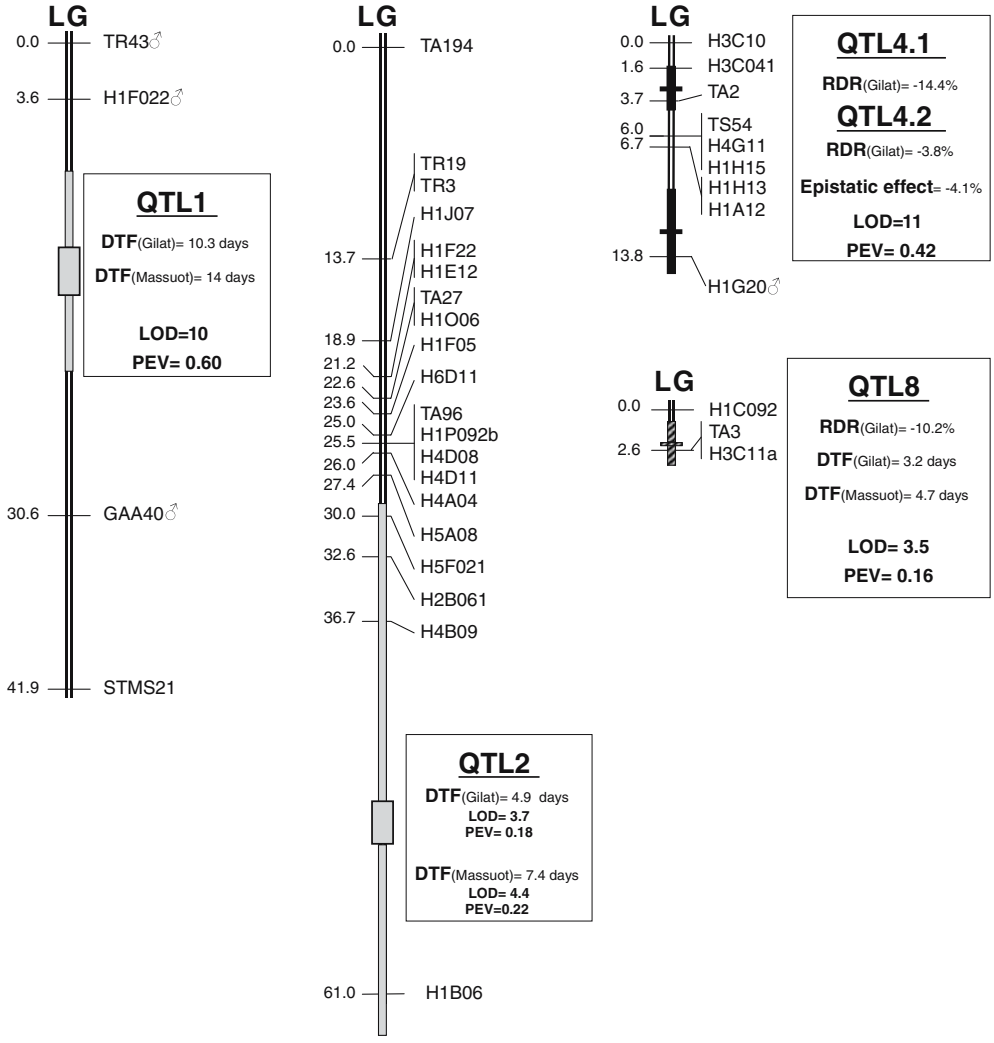

Fig. 3 Quantitative trait loci associated with resistance to *D. rabiei* (RDR) and days to flowering (DTF) in chickpea. Phenology data derived from two experimental sites, Massuot and Gilat. The estimated location of a given locus is shown by the position of the *wide bar*, while the *narrow bars* flanking it represent the standard deviation of the location. The names of the loci derived from their respective linkage groups. The parameter estimates for a given locus, *i.e.*, the locus effect, its LOD score and the proportion of explained phenotypic variation (PEV) are noted in the respective *boxes* which were derived from 'one locus–one trait analysis' (QTL2), 'linked loci–one trait analysis' (QTL4.1 and QTL4.2), and 'one locus–two trait analysis' (QTL 1 and QTL8). The effect magnitudes are given for the maternal allele (Hadas) for the respective trait. Marker loci ending with ♂ indicate a significantly ($P_{\chi 2} < 0.05$) distorted segregation in favour of the ICC5810 alleles. Marker loci prefixed with H were published by Lichtenzveig et al. (2005b); the others were published by Winter et al. (1999) and served as anchors to deduce the linkage group names in accordance to published chickpea maps (Winter et al. 2000; Pfaff and Kahl 2003)

In conclusion, all above studies seem to agree that LG4 has a significant effect on the chickpea resistance to *D. rabiei* whereas other genomic regions have minor effects. In this study, robust statistical analysis strongly suggests the presence of at least two loci on LG4 associated with the response. This is in line with the results obtained by Muelhbauer's (Santra et al. 2000; Tekeoglu et al. 2002; Cho et al. 2004) and Taylor's groups (Collard et al. 2003; Flandez-Galvez et al. 2003). Our data suggest that these loci are in epistatic interaction (Table 4).

Genetic basis of time to flowering

By single-trait analysis of time to flowering, two QTLs were detected. Very similar results were obtained from the two experimental sites. The QTL found on LG1 between the markers H1F022 and GAA40 has a major effect and explains the largest proportion of the response variance: 14 and 10 days, 56 and 53%, for Massuot and Gilat, respectively. The second locus was found on LG2 between markers H4B09 and H1B06, with a minor effect and explaining

a smaller proportion of the phenotypic variance: 7 and 5 days, 22 and 18%, for Massuot and Gilat, respectively.

There is little information available on the genetic basis of time to flowering in chickpea. Data obtained from F_2 and F_3 of the 'Hadas × ICC5810' cross suggested a major photoperiod response gene (*Ppd*) affecting time to flowering (Or et al. 1999). Using interval mapping, Cho et al. (2002) identified a single QTL for days to 50% flowering on LG3 with a LOD score of 3.03. In our study, however, LG3 was not associated with any effect on time to flowering.

Cho et al. (2002) also identified a QTL for seed weight on LG4 accounting for 52% of the total phenotypic variance. Although the parental lines employed in our study differed in their seed weight and wide segregation was observed among the F_5 siblings (Fig. 2b), we could not detect a genomic region associated with seed weight. In this study, the third interval on LG1 was found to be associated with seed weight, but it did not stand the False Discovery rate test (Table 3). Taking this rejected seed weight locus into account, the fact that a QTL with a major effect for days to flowering is found in the neighbouring interval may perhaps explain the positive significant correlation found between seed weight and time to flowering in the 'Hadas × ICC5810' population in all generations tested (Or et al. 1999; Lichtenzveig et al. 2002). This interpretation, however, should be taken with considerable caution.

In this study, all loci associated with time to flowering also had a significant effect on the number of branches (data not shown). The developmental pattern of both mono- and dicotyledons plants determines that late flowering plants usually develop more branches, compared with their early flowering counterparts. A delay in commencement of flowering either because the days are still too short, or because the temperatures are too low, or because of slow leaf emergence (or any combination of these three factors) will allow more auxiliary meristems to develop into branches, while the opposite is true when day length and temperature requirements are satisfied early in the season. Therefore, it is not surprising that all loci of the time to flowering were associated with an effect on the number of primary branches. It is tempting to assume that one of our three tagged loci corresponds to photoperiod response, one to temperature response, and the third to inherent earliness per se (growth rate). To test this hypothesis, however, one would require an extensive crossing scheme between RILs possessing the sensitive or insensitive alleles for each of the flowering loci.

Association between resistance and time to flowering

The RILs data showed a weak but highly significant negative correlation between days to first flower and disease severity. This is in line with the results obtained using earlier generations of this population ('Hadas × ICC5810' F_3 and F_4 families, Lichtenzveig et al. 2002).

By using the MultiQTL software which enables analysis of correlated trait complexes, an increase in the resolution power of interval mapping of QTLs was achieved. For each locus-trait combination, higher LOD scores and PEV estimates were obtained when two traits were analyzed together compared to a single-trait analysis, even when the locus effect on the second trait was not significantly different from zero. Such was the case for loci associated with either flowering time (LG1 and LG2) or resistance to *D. rabiei* (LG4). These results are consistent with simulation analyses described by Korol et al. (1995) in which a higher resolution is provided by the two-trait analysis as compared with the single-trait analysis even when the traits do not depend on each other. In the case of the locus on LG8, when the traits were analyzed separately, the locus had a significant effect on the response to *D. rabiei* (LOD = 2.4, PEV = 0.11) with no significant effect on days to first flower. When resistance and time to flowering were analyzed together, the significance for the resistance estimates obtained for the LG8 locus increased to LOD = 3.9 and PEV = 0.17 and the effect on time to first flower, previously undetected, was significantly different from zero with magnitudes of 4.7 and 3.2 days to first flowers for Massuot and Gilat, respectively.

The identification of a locus linked both to resistance to *D. rabiei* and days to first flower may provide the mechanistic explanation for the genetic correlation observed in previous generations of this mapping population and corroborates our published biometric analysis (Lichtenzveig et al. 2002). However, whether this genetic correlation is due to pleiotropy or tight linkage still remains to be examined.

Integration of genetic mapping and phenotypic evaluation work of several research groups now provides a better understanding of the genetic basis of resistance to *D. rabiei* and time to flowering in chickpea. In addition, in their quest for early flowering and resistant germplasm, breeders are now better equipped with DNA markers linked to the loci associated with these traits.

Acknowledgments This research was supported in part by Research Grant Award US-3034-98R from The United States-Israel Binational Agricultural Research and Development Fund

(BARD) awarded to S. Abbo, D. J. Bonfil, and H.-B. Zhang. The authors thank Mrs. H. Van-Oss for her skilful assistance in SSR genotyping, Mr. David Carlebakh and Agr. Israel Shai (Massuot-Yitzchak), Mr. Israel Mufradi and Mr. Saker El-Atrash (Gilat Station) and Dr. Pfaff for their assistance in the field experiments. The authors would also like to thank Prof. Cahaner and Prof. Korol for their professional advice.

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