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ORIGINAL PAPER

Use of synteny to identify candidate genes underlying QTL controlling stomatal traits in faba bean (*Vicia faba* L.)

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Abstract

Key message **We have identified QTLs for stomatal characteristics on chromosome II of faba bean by applying SNPs derived from *M. truncatula*, and have identified candidate genes within these QTLs using synteny between the two species.**

Abstract Faba bean (*Vicia faba* L.) is a valuable food and feed crop worldwide, but drought often limits its production, and its genome is large and poorly mapped. No information is available on the effects of genomic regions and genes on drought adaptation characters such as stomatal characteristics in this species, but the synteny between the sequenced model legume, *Medicago truncatula*, and faba bean can be used to identify candidate genes. A mapping population of 211 F_5 recombinant inbred lines (Mélodie/2 × ILB 938/2) were phenotyped to identify quantitative trait loci (QTL) affecting stomatal morphology and function, along with seed weight, under well-watered conditions in a climate-controlled glasshouse in 2013 and 2014. Canopy temperature (CT) was evaluated in 2013 under water-deficit (CTd). In total, 188 polymorphic single nucleotide polymorphisms (SNPs), developed from *M. truncatula* genome data, were assigned to nine linkage groups that covered ~928 cM of the faba bean genome with an average inter-marker distance of 5.8 cM. 15 putative QTLs were detected, of which eight (affecting stomatal density, length and conductance and CT) co-located on chromosome II, in the vicinity of a possible candidate gene—a receptor-like protein kinase found in the syntenic interval of *M. truncatula* chromosome IV. A ribose-phosphate pyrophosphokinase from *M. truncatula* chromosome V, postulated as a possible candidate gene for the QTL for CTd, was found some distance away in the same chromosome. These results demonstrate that genomic information from *M. truncatula* can successfully be translated to the faba bean genome.

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Introduction

Faba bean (*Vicia faba* L.), as one of the most important cool-season grain legumes (FAO 2012), is a valuable break crop in environmentally sustainable arable production systems across the world. It is a good source of highly nutritious and protein-rich grain for human consumption and animal feed (Duc 1997; Crépon et al. 2010). Global interest has recently increased in this crop, but it is reputed to be relatively sensitive to drought (Khan et al. 2010; Khazaei et al. 2013b) and yields can vary considerably from season to season.

Climate change is expected to lead to increased temperatures and changed precipitation patterns. Further, models predict that climate change, attributed to the emission of

greenhouse gases, will increase the frequency and intensity of droughts (IPCC 2012; Dai 2013). On a global basis, drought is considered the most important environmental constraint to crop productivity (Boyer 1982). Stomatal morphology and function play the central task in gas exchanges (H_2O and CO_2) between plant and atmosphere, so they are considered as key determinants of plant growth and water balance. Selection for better drought adaptation poses a challenge to breeders, due to the highly variable timing and severity of drought stress in natural environments. Thus, knowledge of the locations and the effects of genes that influence traits related to drought adaptation (e.g., stomatal and root characteristics) is urgently needed to reliably screen for and select targeted drought resistance traits using environment-independent molecular markers, especially in drought-sensitive species such as faba bean. Sizeable differences in stomatal morphology and function separated faba bean accessions adapted to dry regions from those adapted to wet regions (Khazaei et al. 2013a, b). Leaf temperature was the most informative of 16 measurements (see Khazaei et al. 2013a) to discriminate accessions from environments with contrasting seasonal moisture availability, and has been recommended for use as a cost-efficient surrogate for more expensive or sometimes unreliable morpho-physiological traits in this species (Khan et al. 2010) and in several other crops (Blum 2011a).

Faba bean is a diploid with six large chromosomes and the largest genome amongst diploid crop legumes (~13 Gbp), its genome being 26 times larger than that of *Medicago truncatula* L. (Sato et al. 2010). Marker-assisted selection (MAS) in faba bean is less advanced than in some other legume species, but significant efforts have been made to enrich genetic and genomic resources in this crop (Torres et al. 2010, 2012; Alghamdi et al. 2012), fortified by comparative genomics with *M. truncatula* and other legumes (Ellwood et al. 2008; Kaur et al. 2012; Torres et al. 2012; Khazaei et al. 2014). Genomic and transcriptomic approaches, now being applied in faba bean, open new opportunities for fine mapping or uncovering of candidate genes. Thus, considering the strong macrosynteny amongst these legumes, a set of expressed sequence tags (ESTs) from *M. truncatula*, pea (*Pisum sativum* L.), lupin (*Lupinus luteus* L.), lentil (*Lens culinaris* Medik.) and soybean (*Glycine max* (L.) Merr.) have been included in faba bean maps (Cruz-Izquierdo et al. 2012), and whole genome sequences of *M. truncatula* (Young et al. 2011) and chickpea (*Cicer arietinum* L.) (Varshney et al. 2013) offer further opportunities for translation to faba bean.

Large-scale transcriptome data, together with genomic markers based on single nucleotide polymorphisms (SNPs), now facilitate the development of cost-effective and highly saturated second-generation genetic maps (Saxena et al. 2012). Gene-based markers such as ESTs and SNPs have been recently developed in faba bean (Ellwood et al. 2008; Cottage et al. 2012a, b; Cruz-Izquierdo et al. 2012; Kaur et al. 2014). SNPs provide low genotyping cost per data point, high genomic abundance (high polymorphism), locus specificity (accuracy and reproducibility, Yan et al. 2010), codominance, simple documentation, potential for high-throughput analysis and common occurrence amongst elite germplasm (Cottage et al. 2012a, b), so they have emerged as powerful tools for genetic analyses and molecular breeding for crop improvement (Semagn et al. 2014).

Molecular approaches in faba bean breeding have been mostly limited to biotic stresses and anti-nutritional compounds (reviewed in Torres et al. 2010, 2012), but amongst abiotic stresses, some progress has also been made in identifying QTLs in frost tolerance (Arbaoui et al. 2008). Yield, its components, and flowering time (associated with drought avoidance) were fine mapped in faba bean (Cruz-Izquierdo et al. 2012).

Hence, this work had three main aims: firstly, to develop a gene-based linkage map for the Mélodie/2 × ILB 938/2 RIL population, and secondly, to identify QTLs controlling stomatal morphology and function and seed weight under well-watered conditions as well as canopy temperature in both well-watered and water-deficit conditions. The third aim was to exploit synteny between faba bean and *M. truncatula* to examine the putative gene content of the regions under detected QTLs for candidate genes whose homologues function in mediated drought responses in *M. truncatula* or *Arabidopsis*.

Materials and methods

Plant material

A population of 211 F_5 RILs was generated by single-seed descent from a cross between Mélodie/2 (*minor*, with beige seed coat and colourless hilum) as the pollen recipient and ILB 938/2 [*equina*, with green seed coat and black hilum, ICARDA (International Centre for Agricultural Research in the Dry Areas) accession number: ig 13987] as the pollen donor at the Department of Agricultural Sciences, University of Helsinki, Finland during 2009–2012. Mélodie/2 is an inbred line selected from the low vicine–convicine cultivar from INRA (Institut National de la Recherche Agronomique, France) with highly efficient use of water (in the sense of Blum 2009) and relatively high yield. ILB 938/2 is a selection from an Ecuadorian landrace with high water-use efficiency and low productivity (Khan et al. 2007, 2010; Khazaei et al. 2013b). In addition, the parental lines differed in a wide range of agronomic and morphological characters (supplementary Table 1) which confirms that they are genetically far enough from each other

and suitable for genetic map and genomic studies. Seed coat colour as a morphological marker was recorded during population development (F_1–F_5) and this was used as a quality check during the development of the population (supplementary Fig. 1).

Growth conditions

The mapping population along with parental lines was evaluated in a climate-controlled glasshouse of the Department of Agricultural Sciences, University of Helsinki, Finland. The sowing dates were 7 October 2012, 1 February 2013 and 7 October 2013, which are called the 2012, 2013 and 2014 experiments. In 2012, the experiment was unreplicated and in the other 2 years there were three replicates of F_5 RILs in a completely randomised design. Seeds were inoculated with *Rhizobium leguminosarum* biovar. *viciae* (faba bean strain, Elomestari Oy, Tornio, Finland) before sowing in 4-L plastic pots containing a mixture of sand (size: 0.5–1.2 mm, Saint-Gobain Weber Oy Ab, Helsinki, Finland) and peat (White 420 W, Kekkilä Oy, Vantaa, Finland) (2:1 v/v) containing all essential nutrients. Each pot contained three plants. 15 additional plants of each parental line were distributed in the experiment as double control checks for measurements. Pots were filled with 2.48 kg of the mixture that had a water holding capacity of 24 % (w/w). Each pot was brought to water holding capacity by adding 595 mL of water. Pots were weighed three times per week and amounts of water equal to the loss in weight were added to maintain soil moisture level at field capacity. At 3, 6 and 9 weeks after sowing, 70 mL of nitrogen-free fertiliser (equivalent to 20 kg of P and 24 kg of K per hectare) was added to each pot. The photoperiod was 14 h light and 10 h dark, and the temperature was maintained at 21 °C day/16 °C night ± 2 °C. Photosynthetic photon flux density (PPFD) was approximately 300 µmol m^{-2} s^{-1} at the canopy level. Relative humidity was maintained at 60 %. To avoid shading and influence of other plants, pots were kept 20 cm apart at all times. The temperature, humidity and light conditions were automatically recorded throughout the experiments. The growth conditions were same for all experiments.

Measurements

Stomatal morphology

Stomatal density (SD) and length (SL) of the parental lines and RILs were measured on the middle part of the abaxial surface of the youngest, fully expanded leaflet of 8-week-old plants by the impression method as described previously (Khazaei et al. 2013a, b). Five microscope fields per pot were used for these traits.

Stomatal conductance

Three different leaflets of each 8-week-old plant were used for measuring g_s using a LI-6400 *xt* portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) equipped with a 2 × 3 cm leaf chamber with a LED light source (6400-02B, 90 % red and 10 % blue). PPFD was 1,000 µmol m^{-2} s^{-1}. A CO_2-injecting cartridge was attached to the system to control reference CO_2 concentration at 400 µmol mol^{-1}, a value close to that during plant growth. The flow rate was 400 µmol s^{-1}. Measurements were done between 9 and 11 a.m. using the youngest, fully expanded leaflet that was also used for stomatal morphology.

Canopy temperature

Canopy temperature was measured at flowering stage using an infrared thermometer (IRT, FLUKE® thermometer gun 574, Everett, WA, USA) from the fully expanded leaves used for the other measurements. Five leaflets per pot were used for this measurement. Canopy temperature was evaluated in 3 years, 2012, 2013 and 2014, under the same glasshouse conditions.

Seed weight

After plants were harvested, seeds were dried at 40 °C for 2 weeks, and 10 seeds per plant were weighed to determine the SW.

Induction of water-deficit

Immediately after the stomatal measurements were taken, a uniform water-deficit was initiated by reducing relative available water by 2 % (w/w) every day to bring the moisture level down from the field capacity (24 % w/w). Pots were weighed every day and where water use exceeded 2 %, irrigation was applied (Khan et al. 2007). When the soil moisture content had reached 2–4 %, 10 days after the induction of water-deficit was started, canopy temperature (CTd) was determined. Thereafter normal irrigation was continued until harvest.

Parental lines exercise

In the same conditions as described above, another experiment was designed to evaluate parental lines in 20 replicates under well-watered and water-deficit conditions. In this experiment, ten replicates were exposed to gradual and uniform water-deficit starting at beginning of flowering stage as described above. Canopy temperature and stomatal conductance were measured 10 days after initiating water-deficit, as described above. Plants were cut at 13 weeks and dry weight was determined.

DNA extraction

Nine discs (diameter 5 mm) from healthy, newly expanded leaves of the F_5 mapping population along with seedlings of the parental lines (Mélodie/2 and ILB 938/2) were taken when the plants were 3-week old and transferred to 96-well tube strong racks with lid, sealed with a perforated film and covered by 50 g desiccant sachet, and shipped to the LGC Genomics laboratory (LGC Genomics, Hoddesdon, UK) for high-throughput genomic DNA extraction with full quality control and sample normalisation support linked to services for SNP genotyping according to the manufacturer's instructions (http://www.lgcgenomics.com/nucleic-acid-extraction/services/dna_extraction_services/).

SNP discovery and selection

A total of 222 polymorphic SNPs between parental lines were chosen from the 756 SNPs that have been validated in an international panel of 35 accessions (Cottage et al. 2012b; Webb et al. in preparation), with priority given to those with higher frequencies in the validation panel. SNP sequences and specific alleles for parental lines used in this study are listed in supplementary Table 2.

SNP genotyping was carried out using the KASPar™ (Kompetitive Allele Specific PCR) assay (KBioscience, UK) platform, a single-plex SNP genotyping methodology using allele-specific amplification followed by fluorescence detection for genotyping (Cuppen 2007; http://www.kbioscience.co.uk/reagents/KASP_manual.pdf). On the basis of the fluorescence obtained, allele call data were observed graphically as a scatter plot for each marker assayed using the SNPViewer v 1.99 (http://www.lgcgenomics.com/services/instruments-and-software/software/?id=78).

Morphological marker

Green seed coat colour from ILB 938/2 was used as a morphological marker (supplementary Fig. 1) as it is a simple recessive trait in this species (Erith 1930; Sjödin 1971).

Statistical analysis

All measurements were tested for deviations from normality using skewness, kurtosis and the Shapiro–Wilk test. Non-normally distributed traits were transformed using the Box-Cox transformation (Box and Cox 1964). A two-way ANOVA (analysis of variance) was employed to test the effect of water treatments on canopy temperature of parental lines and RILs using IBM SPSS (IBM® SPSS® Statistics for Windows, v. 21, Armonk, NY, USA).

Map construction and QTL mapping

SNP segregation was subjected to the Chi square (χ^2) goodness-of-fit test to assess deviations from the expected Mendelian segregation ratio of 1:1. SNPs showing normal diploid segregation ($P \geq 0.05$) were chosen for the map construction. The linkage map was constructed using MapDisto v. 1.7.7.0.1 (Lorieux 2012) with logarithm of odds (LOD) score of 3.0 and recombination fraction of 0.3. The Kosambi function was used to calculate the map distance in centiMorgans (cM) (Kosambi 1943). Composite interval mapping (CIM) was used to detect the relationship between each linkage group and putative QTL locations of studied traits by Windows QTL Cartographer v 2.5_011 (Wang et al. 2012). Significant QTLs were analysed with CIM. Co-factors were determined using the forward and backward method in the standard CIM model with the probability in and out of 0.1. The number of control markers and window size were set to 5 and 10, respectively. The genome was scanned at a walk speed of 1 cM intervals. The 95 % significance threshold for QTL detection (at each trait) was determined by the phenotype permutation using 1,000 permutations at an experiment-wise $P < 0.05$ (Churchill and Doerge 1994). LOD profiles over each of the linkage groups and for each trait were drawn. LOD score value greater than the threshold was considered as evidence of a QTL in these profiles. We followed the convention that if two LOD peaks exceeded threshold in close proximity to each other, the smaller LOD peak was considered an artefact and omitted. Uncertainty of the QTL position around maximum LOD score was indicated by 1- and 2-LOD support intervals (Conneally et al. 1985; van Ooijen 1992). These intervals were calculated for the CIM run with five co-factors. Support interval threshold values were determined on the LOD score scale by subtracting one (two) LOD from the maximum LOD-score value. Linkage groups and QTL positions were drawn by MapChart v. 2.2 (Voorrips 2002).

Synteny and candidate genes

First, the overall level of collinearity between the selected QTL regions (using sequences of two flanking SNP markers) and *M. truncatula* was examined. Reciprocal best BLAST hit E values were used to determine the strength of the orthologous relationship. Gene family and phylogenetic context as well as tissue-specific gene expression profiles of the orthologous genes from model plant species were examined using the integrative database LegumeIP (http://plantgrn.noble.org/LegumeIP/) (*M. truncatula*, gene model, Mt3.5v3).

Results

Parental lines response to water stress

Mélodie/2 had a cooler canopy under well-watered conditions and a considerably greater increase in canopy temperature under water-deficit conditions than ILB 938/2. Stomatal conductance showed the opposite pattern (Figs. 1, 2). Considering plant biomass as an indicator of drought tolerance, water-deficit had threefold less effect on biomass production in ILB 938/2 than in Mélodie/2 (Fig. 1). All of these results show the ability of ILB 938/2 to maintain higher water status under drought conditions and obvious differences between two parental lines for these traits and their response to water stress. The genotype × treatment interaction was not statistically significant.

Linkage map

Of 222 SNPs, 3.6 % was monomorphic in the recombinant inbred population and 11.7 % was rejected on the grounds of excess missing data (>5 % missing data per marker) or excessive expected χ^2 goodness of fit ratio (1:1, $P < 0.05$) to facilitate production of an accurate linkage map. SNPs dropped from the experiment are marked with an asterisk in supplementary Table 2. Then, 188 SNPs and one morphological character (seed coat colour) were used to construct the linkage map and for future analysis. Four out of nine linkage groups (linkage groups 3–6) contained SNPs showing slightly distorted segregation, which are likely to be distributed throughout the faba bean genome, and the largest number of SNPs with distorted segregation was ten in linkage group 4. The map consists of eight main linkage groups (LGs 1-8) and a fragment (LG9), with lengths from 3.07 (LG9) to 223.02 cM (LG7) and a total length of ~928 cM (Table 1; Fig. 3). The number of SNPs and loci per linkage group ranged from 2 to 47 and 2 to 43, respectively. 52 SNPs co-segregated at 23 loci (Fig. 3). The average inter-marker distance was 5.8 cM. The two highest SNP distances were on linkage groups 7 and 8 (30 and 29.5 cM, respectively) (Table 1; Fig. 3). Linkage groups 4 and 6 were the most saturated, and the current linkage map is one of the highest saturated in faba bean. Segregation of seed coat colour, the morphological marker, fitted the expected ratio of 17:15 for an F_5 generation and also fitted a 1:1 ratio (supplementary Fig. 1), and the gene was assigned to LG6 (Fig. 3).

SNPs were named with "Mt" 1–8, referring to chromosomes I–VIII in *M. truncatula*, and a different colour is used for each of these Mt chromosomes on the faba bean map (Fig. 3). The macrosynteny between linkage groups and *M. truncatula* chromosomes for those SNPs that are not coded by "Mt" (e.g. HYPTE3SNP) was confirmed from the Ellwood et al. (2008) map. LGs 1 and 2 were syntenic to Mt-4 and Mt-7, respectively. LGs 3, 4 and 5 were strongly syntenic to Mt-1 (90 %), Mt-3 (91.5 %) and Mt-8 (91.7 %), respectively. LG6 was a combination of Mt-8 (51.6 %), Mt-4 (32.3 %) and Mt-3 (9.7 %). LG7 represented an assembly of Mt-5 (61.5 %) and Mt-2 (35.9 %) and LG8 covered Mt-2 (75 %) and Mt-6 (25 %) (Table 1; Fig. 3).

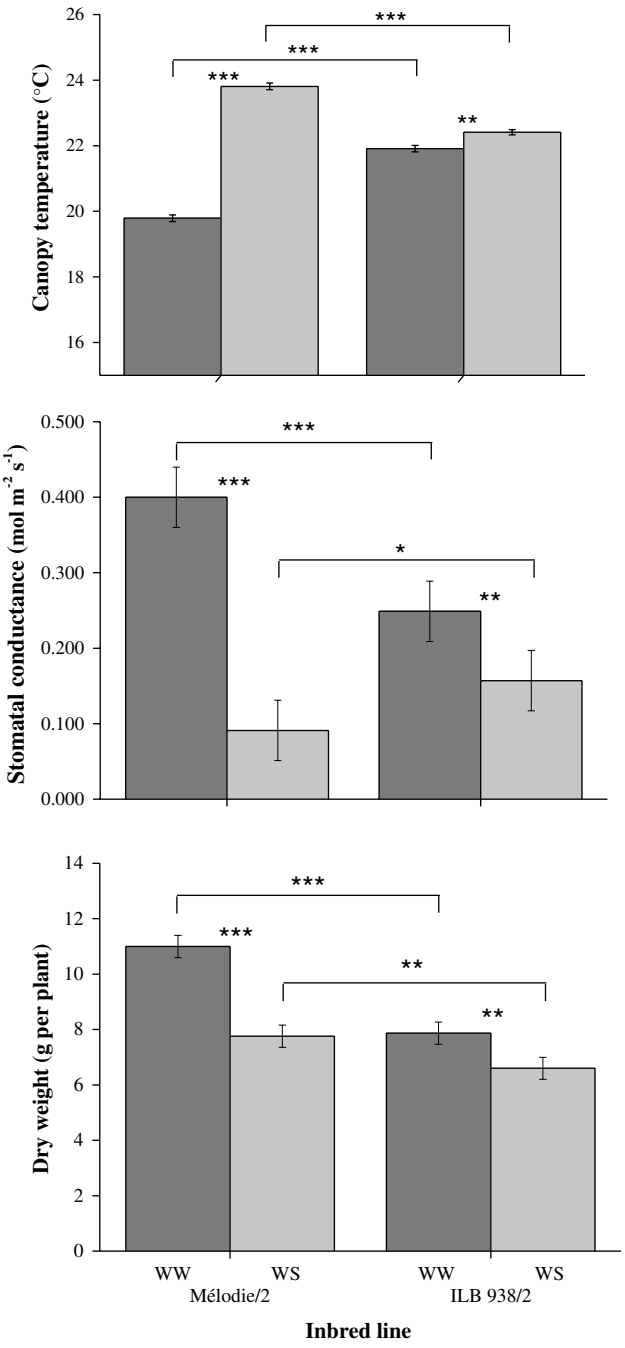

Fig. 1 Changes in canopy temperature, stomatal conductance and dry matter production in response to water stress in *parental lines*, Mélodie/2 and ILB 938/2. *, ** and *** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *WW* well-watered, *WS* water stress

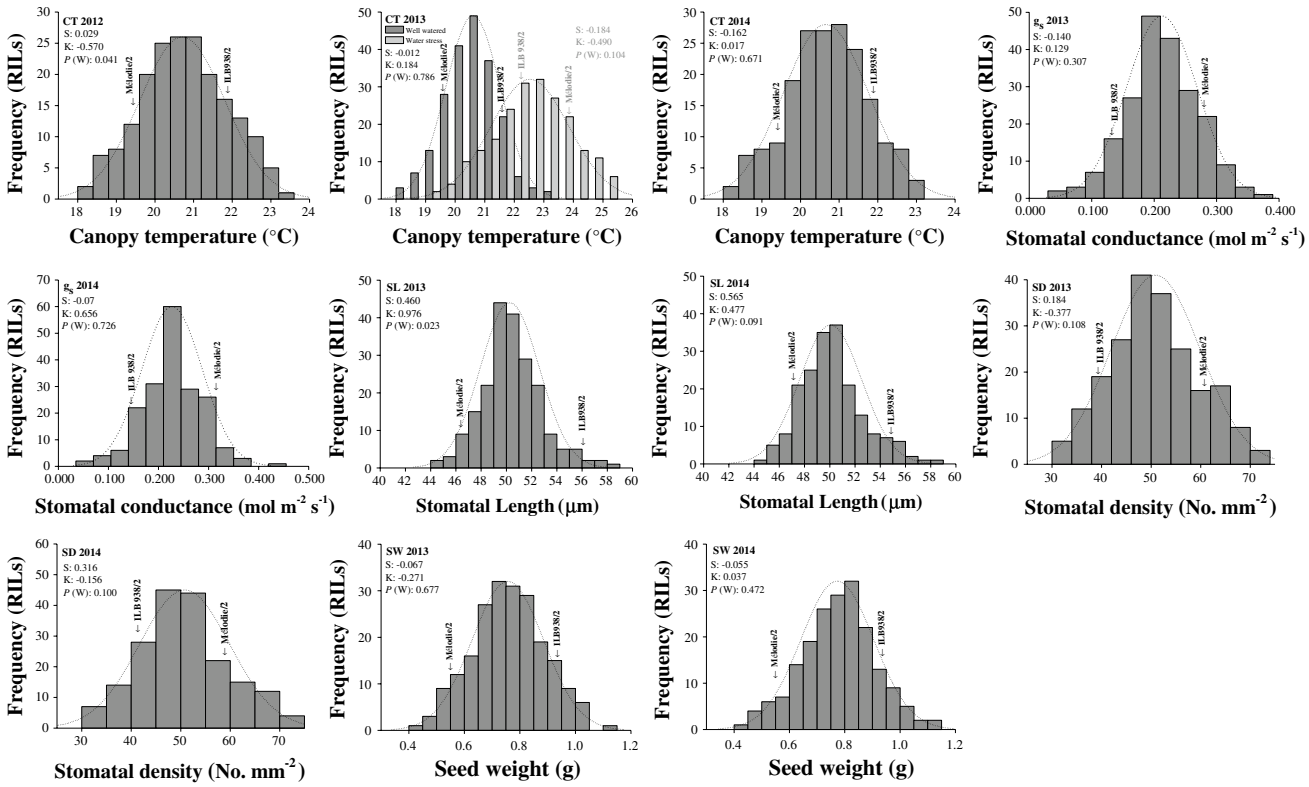

Fig. 2 Frequency distributions of drought adaptation-related traits in 211 RILs derived from a cross between Mélodie/2 and ILB 938/2. Each parental value is the mean of 15 replicates. *S* Skewness, *K* Kurtosis, *P(W) P* value of Shapiro–Wilk test for normality

Table 1 Characteristics of the genetic linkage map of Mélodie/2 × ILB 938/2

Linkage group	*V. faba* chromosome	*M. truncatula* chromosome	Number of SNPs	Number of loci	Length of linkage groups (cM[a])	Average SNP spacing (cM)	Largest distance between SNPs
1	VI	IV	6	5	31.02	7.8	10.4
2	V	VII	14	10	70.33	7.8	20.2
3	III	I	30	27	182.45	7.0	26.6
4	II	III	47	43	173.70	4.1	23.9
5	–	VIII	12	10	53.57	6.0	20.5
6	IV	VIII > IV > III	30	23[b]	106.63	4.8	15.9
7	I	V > II	39	33	223.02	7.0	30.0
8	I	II > VI	8	7	84.23	14.0	29.5
9	–	II	2	2	3.07	3.1	3.1
Total			188	160	928.02	5.8	

[a] *cM* centiMorgans

[b] Including seed coat colour (SC)

Assignment of linkage groups to faba bean chromosomes

The linkage groups were assigned to their respective chromosomes using markers that had been placed on identified chromosomes (Cruz-Izquierdo et al. 2012) and following the current consensus faba bean genetic map (Satovic et al. 2013). The assignments made here are consistent with those possible using the higher syntenic resolution possible using a 768-locus consensus genetic map (Webb et al. in preparation).

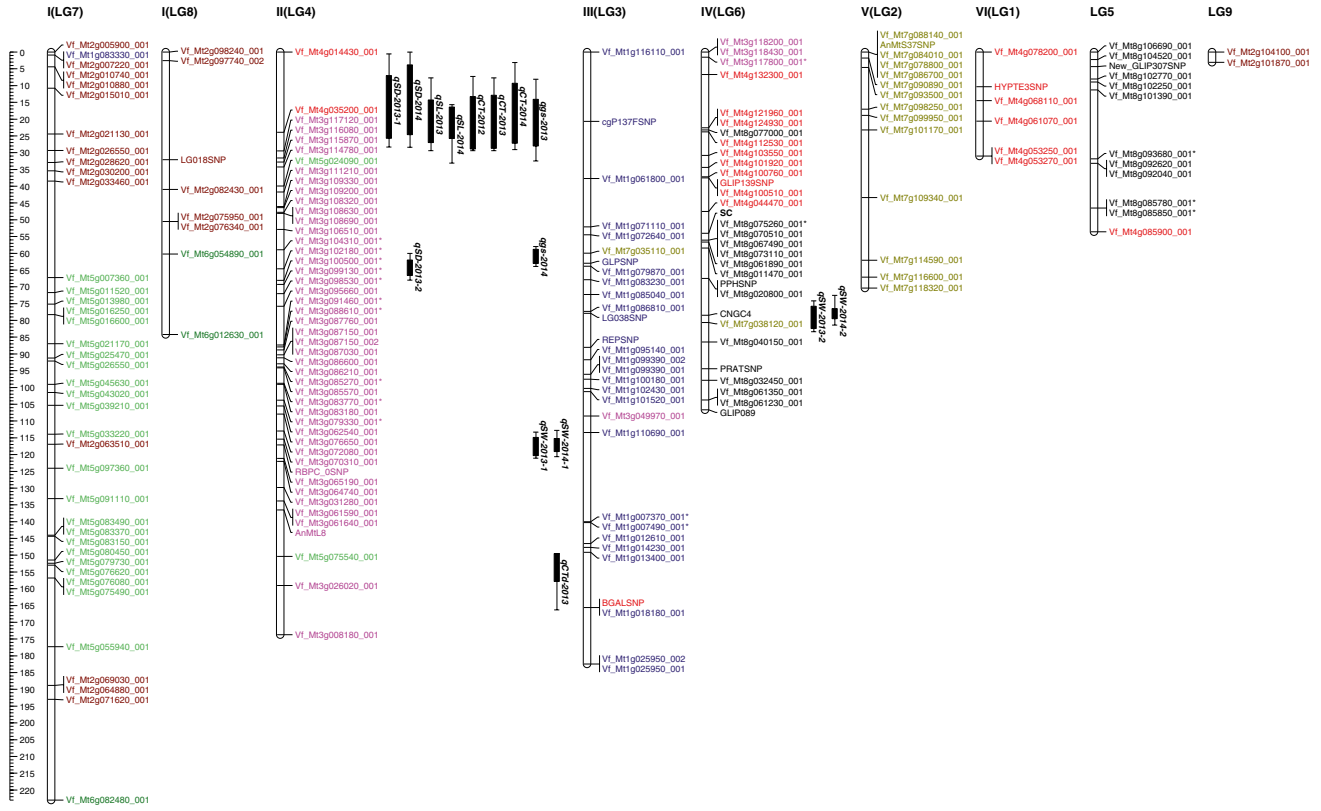

Fig. 3 The distribution and positions of QTLs on the genetic linkage groups and their chromosome assignments for stomatal density (SD), stomatal length (SL), canopy temperature (CT), canopy temperature under water-deficit conditions (CTd) and seed weight (SW). QTLs are represented by *boxes* extended by *lines* representing the LOD-1 and LOD-2 confidence intervals. *Asterisks* show SNPs with distorted distributions

Chromosome I

The largest linkage group, LG7, was assigned to the large, metacentric chromosome I and corresponded to *M. truncatula* chromosomes II and V (Khazaei et al. 2014; Webb et al. in preparation). LG8 was also assigned to chromosome I, since it included marker "LG018" that was already placed on this chromosome (Cruz-Izquierdo et al. 2012).

Chromosome II

The presence of the AnMtL8 (Ellwood et al. 2008) and RBPC (Cruz-Izquierdo et al. 2012) markers allowed a firm assignment of LG4 to faba bean chromosome II. This result was confirmed by the strong synteny between *M. truncatula* chromosomes III and IV and faba bean chromosome II (Webb et al. in preparation).

Chromosome III

The cgP137F, GLP, "LG038" and REP markers spreading along LG3 have been previously assigned to faba bean chromosome III (Cruz-Izquierdo et al. 2012; Satovic et al. 2013), and much of *M. truncatula* chromosome I has been shown to be syntenic with this faba bean chromosome. However, the BGAL marker at the end of this linkage group was previously placed on faba bean chromosome I (Cruz-Izquierdo et al. 2012; Satovic et al. 2013).

Chromosome IV

The chromosome tentatively indicated as "I.B" (Cruz-Izquierdo et al. 2012) was recently assigned to chromosome IV (Ruiz-Rodriguez et al. 2014). The presence of markers GLIP139, CNGC4, PRAT and GLIP089, previously assigned to chromosome I.B (Satovic et al. 2013), confirms that LG6 can be assigned to this chromosome. Green seed coat colour was also mapped to this chromosome.

Chromosome V

The marker AnMtS37 on LG2 identified it as a segment of chromosome V, and this was confirmed by the high synteny between chromosome VII in *M. truncatula* and V in faba bean (Webb et al. in preparation).

Chromosome VI

LG1 was assigned to a segment of chromosome VI by means of the HYPE marker (Cruz-Izquierdo et al. 2012) and the strong synteny with *M. truncatula* chromosome IV (Webb et al. in preparation).

Unassigned LGs

Linkage group 9 may belong to the faba bean chromosome I or V (Webb et al. in preparation). LG5 is saturated with markers from *M. truncatula* chromosome VIII and could belong to either chromosome VI or IV of faba bean. The GLIP307 marker in this linkage group was unassigned in previous works (Ellwood et al. 2008; Cruz-Izquierdo et al. 2012).

Phenotypic variation

There was a considerable variation in the evaluated phenotypes of the mapping population and huge differences between parental lines (Fig. 2). All traits showed normal Gaussian distribution patterns, which suggests the involvement of multiple genes. Skewness and kurtosis values were less than 1.0 in all traits, suggesting that data were normally distributed, but the Shapiro–Wilk test showed a non-normal distribution for stomatal length in 2013 and canopy temperature in 2012 ($P < 0.05$) (Fig. 2). Box–Cox transformation did not affect the QTL results, perhaps because of the low significance level of the Shapiro–Wilk test. When canopy temperature was evaluated under both well-watered and water-deficit conditions, the main effect of water treatment and its interaction effect with RILs were both highly significant ($P < 0.001$) (Table 2).

CT showed strong negative phenotypic correlations with g_s (Table 3). SD and SL were highly negatively correlated with each other, and SD was positively correlated with CT. SL showed a weak positive association with g_s and was negatively correlated with CT. CTd was positively correlated to CT. All of these results are in agreement with the germplasm survey of the same traits (Khazaei et al. 2013b). SW did not show a significant correlation with any other morpho-physiological measurements except CT in 2014 (Table 3). Furthermore a high positive relationship was found in each trait between years. The highly significant correlations found amongst some traits suggests that co-location of QTLs is not unexpected.

QTL mapping analysis

Information about the QTL analysis for five of the studied traits under well-watered conditions (SD, SL, g_s, CT and SW) and one trait under water-deficit conditions (CTd) in the RIL population is shown in Fig. 3, and a summary of QTL statistics is presented in Table 4. The phenotype permutation-derived LOD threshold at $P = 0.05$ varied from 2.8 to 3.0. 15 significant QTLs were identified for all studied traits through the treatments and years. Phenotypic variation explained by each individual QTL ranged from 5.7 to 9.3 %. QTLs were detected on two of the six chromosomes, II and IV, but there was a cluster of overlapping QTLs (8 out of 15) between the same flanking SNPs on chromosome II (Fig. 3).

Table 2 Summary analysis of variance (ANOVA) for canopy temperature across the water treatment in 2013

Source of variance	Degrees of freedom	Mean square
RIL	210	5.378***
Stress	1	934.210***
RIL × Stress	210	2.978***
Replicate	2	0.370**
Error	807	0.098

RIL recombinant inbred line

, * Significance at $P < 0.01$ and $P < 0.001$, respectively

Stomatal morphology

Two significant QTLs (*qSD-2013-1* and *qSD-2013-2*) were detected for stomatal density and the first one remained stable in 2014 (*qSD-2014*). The *qSD-2013-1* and *qSD-2014* showed a positive additive effect, suggesting that the positive allele came from Mélodie/2, whereas *qSD-2013-2* showed a negative additive effect, indicating that its positive allele came from ILB 938/2. Both significant QTLs (*qSD-2013-1* and *qSD-2013-2*) were mapped onto chromosome II in marker intervals Vf_Mt4g014430_001/Vf_Mt4g035200_001 and Vf_Mt3g102180_001/Vf_Mt3g100500_001, respectively, and explained 15 % in 2013 and 9.3 % in 2014 of the total phenotypic variation with LOD of ~2.9 (Table 4). A single QTL for stomatal length (*qSL-2013 and qSL-2014*) was found in both years, in the same interval as *qSD-2013-1* and with a negative additive effect, suggesting that alleles increasing stomatal size came from ILB 938/2. It explained ~6.0 % of the phenotypic variance (Table 4).

Stomatal function

For stomatal conductance, one significant QTL (*qgs-2013*) was identified in the same interval with *qSD-2013-1*, *qSL-2014*, *qSL-2013* and *qSL-2014* in chromosome II and explained 7 % of the phenotypic variance with a LOD value of 3.14. However, this QTL was not stable and *qgs-2014* co-located with *qSD-2013-2* in a region where there

Table 3 Simple pair-wise Pearson correlation coefficients amongst studied traits ($n = 211$)

	SD	SL	g_s	CT	SW	CTd
SD	**0.569*** **	−0.629***	−0.028^{ns}	0.241***	−0.107^{ns}	0.035^{ns}
SL	−0.347***	**0.511*** **	0.159*	−0.330***	0.135^{ns}	0.114
g_s	−0.081^{ns}	0.088^{ns}	**0.700*** **	–0.686***	0.027^{ns}	0.192**
CT	0.139*	−0.157*	−0.492***	**0.601*** **	0.105^{ns}	0.306***
SW	−0.112^{ns}	0.063^{ns}	0.083^{ns}	−0.221***	**0.722*****	−0.118^{ns}

Diagonal shows the correlation coefficients between 2013 and 2014 (in bold). Data above and below the diagonal are the correlation coefficients in 2013 and 2014, respectively

SD stomatal density, *SL* stomatal length, g_s stomatal conductance, *CT* canopy temperature, *SW* seed weight, *CTd* canopy temperature under water stress conditions

^{ns} Non-significant ($P > 0.05$); *, ** and *** significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

Table 4 Putative QTLs by composite interval mapping (CIM) for morpho-physiological traits related to drought adaptation of the Mélodie/2 × ILB 938/2 RILs population

Trait	QTL name[a]	Chromosome/ linkage group	Position (cM)	Flanking SNPs	LOD score	LOD at $P = 0.05$[b]	R^2	Additive effect
SD	*qSD-2013-1*	II (LG4)	18.01	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	2.93	2.8	0.087	2.430
	qSD-2013-2	II (LG4)	64.61	Vf_Mt3g102180_001/ **Vf_Mt3g100500_001**	2.91		0.057	–2.011
	qSD-2014	II (LG4)	15.01	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.01	2.9	0.093	2.818
SL	*qSL-2013*	II (LG4)	23.01	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.05	2.9	0.065	–0.701
	qSL-2014	II (LG4)	25.0	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.01	2.9	0.061	–0.591
g_s	*qgs-2013*	II (LG4)	23.91	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.14	2.8	0.068	–0.014
	qgs-2014	II (LG4)	59.90	**Vf_Mt3g104310_001**/ Vf_Mt3g102180_001	3.50	2.9	0.073	–0.203
CT	*qCT-2012*	II (LG4)	24.91	**Vf_Mt4g035200_001**/ Vf_Mt3g117120_001	3.68	2.9	0.084	0.329
	qCT-2013	II (LG4)	24.71	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.59	3.0	0.082	0.310
	qCT-2014	II (LG4)	22.01	Vf_Mt4g014430_001/ **Vf_Mt4g035200_001**	3.38	3.0	0.075	0.309
CTd	*qCTd-2013*	II (LG4)	150.31	**Vf_Mt5g075540_001**/ Vf_Mt3g026020_001	3.15	3.0	0.061	–0.364
SW	*qSW-2013-1*	II (LG4)	117.11	Vf_Mt3g070310_001/ **Vf_Mt3g065190_001**	4.82	3.0	0.093	–0.041
	qSW-2013-2	IV (LG6)	80.01	**CNGC4**/Vf_Mt7g038120_001	3.04		0.062	–0.033
	qSW-2014-1	II (LG4)	117.00	Vf_Mt3g070310_001/ **Vf_Mt3g065190_001**	3.42	2.9	0.068	–0.043
	qSW-2014-2	IV (LG6)	78.51	**CNGC4**/Vf_Mt7g038120_001	3.17		0.064	–0.036

SNPs that are in bold show the nearest marker to the QTL

R^2 represents the proportion of phenotypic variance explained by each QTL

SD stomatal density, *SL* stomatal length, g_s stomatal conductance, *CT* canopy temperature, *CTd* canopy temperature under water stress conditions, *SW* seed weight, *cM* centiMorgans

[a] QTLs are abbreviated by trait name and year

[b] Significant LOD at $P = 0.05$ with 1,000 times permutation

Table 5 *M. truncatula* and *A. thaliana* annotation within syntenic regions in the identified putative QTLs

QTL	Faba bean SNP	*M. truncatula* loci	Annotation	
			M. truncatula	*A. thaliana*
qSD-2013-1, *qSD-2014*, *qSL-2013*, *qSL-2014*, *qgs-2013*, *qCT-2012*, *qCT-2013*, *qCT-2014*	Vf_Mt4g035200_001	Medtr4g035200	Receptor-like protein kinase 5	Putative leucine-rich receptor-like protein kinase
qSD-2013-1, *qSD-2014*, *qSL-2013*, *qSL-2014*, *qgs-2013*, *qCT-2013*, *qCT-2014*	Vf_Mt4g014430_001	Medtr4g014430	Unknown protein	Expressed protein
qCT-2012	Vf_Mt3g117120_001	Medtr3g117120	BZIP transcription factor ATB2	–
qCTd-2013	Vf_Mt5g075540_001	Medtr5g075540	Ribose-phosphate pyrophospho-kinase 4	Ribose-phosphate pyrophospho-kinase
qCTd-2013	Vf_Mt3g026020_001	Medtr3g026020	Unknown protein	AT5g08050/F13G24_250
qSD-2013-2, *qgs-2014*	Vf_Mt3g102180_001	Medtr3g102180	Disease resistance protein	Leucine-rich repeat receptor-like protein kinase
qSD-2013-2	Vf_Mt3g100500_001	Medtr3g100500	Aspartic proteinase nepenthesin-1	F21M12.13 protein
qgs-2014	Vf_Mt3g104310_001	Medtr3g104310	NADH–ubiquinone oxidoreduc-tase subunit-like	–
qSW-2013-1, *qSW-2014-1*	Vf_Mt3g070310_001	Medtr3g070310	Arginyl-tRNA-protein transferase 1	Arginine-tRNA-protein trans-ferase 1 homologue
qSW-2013-1, *qSW-2014-1*	Vf_Mt3g065190_001	Medtr3g065190	Splicing factor 3B subunit 4	–
qSW-2013-2, *qSW-2014-2*	CNGC4	–	Days to flowering[a]	–
qSW-2013-2, *qSW-2014-2*	Vf_Mt7g038120_001	Medtr7g038120	Mechanosensitive ion channel domain	Mechanosensitive ion channel domain

[a] Cruz-Izquierdo et al. (2012)

are distorted SNPs markers, so there was more uncertainty in the marker map and the exact position of the QTL. The negative additive effect indicated that the QTL allele decreasing g_s was derived from ILB 938/2 (Table 4).

A significant QTL was found for canopy temperature under well-watered conditions in 2012 in chromosome II (*qCT-2012*) and it remained stable in 2013 and 2014 (*qCT-2013* and *qCT-2014*). All three QTLs co-located in the same region with *qSD-2013-1*, *qSL-2013*, *qSL-2014* and *qgs-2013*. For canopy temperature under water-deficit conditions, one significant QTL was detected (*qCTd-2013*) in another part of the same linkage group, in the interval Vf_Mt5g075540_001/Vf_Mt3g026020_001 and a LOD value of 3.15. The additive effect was positive for QTLs identified across years for CT, but negative for CTd, indicating that the alleles conferring warmer leaves under well-watered conditions were probably derived from Mélodie/2 and those conferring cooler leaves under stress conditions were from ILB938/2.

Seed weight

Two QTLs were detected for seed weight in 2013 and both were confirmed in 2014. The first QTL (*qSW-2013-1* and *qSW-2014-1*) was located on chromosome II in the marker interval Vf_Mt3g070310_001/Vf_Mt3g065190_001. The second QTL (*qSW-2013-2* and *qSW-2014-2*) was located on chromosome IV in the marker interval CNGC4/Vf_Mt7g038120_001 (Fig. 3; Table 4). Both of the QTLs showed negative additive effect, so alleles for higher seed weight at both loci were associated with ILB 938/2.

Epistatic interaction between different QTLs controlling the same trait was analysed for traits with more than one QTL (stomatal density and seed weight) using CIM analysis in QTL Cartographer or genome wide using QTL network. No significant epistatic interaction was observed amongst the QTLs.

Synteny

Annotated gene information on positional candidate genes in *M. truncatula* and *Arabidopsis thaliana* databases corresponding to the putative detected QTL regions of faba bean is listed in Table 5. Candidate genes for *M. truncatula* and *A. thaliana* were almost identical. The genes/gene families were mostly related to growth and development, stress induction and transport (Table 5). A segment of *M. truncatula* chromosome IV that harbours receptor-like protein

kinase was consistently associated with several detected QTLs, including *qSD-2013-1*, *qSD-2014*, *qSL-2013*, *qSL-2014*, *qgs-2013*, *qCT-2012*, *qCT-2013* and *qCT-2014*. The candidate gene for *qCTd-2013* was ribose-phosphate pyrophosphokinase 4 in *M. truncatula* chromosome V, on which most drought-induced genes are found.

Discussion

This study focused on some of the morpho-physiological traits in faba bean that affect gas exchange and hence drought adaptation, identifying QTLs and demonstrating how successfully translational-integrated genomic research can be utilised for identifying candidate genes. This report presents the first map of faba bean that uses its extensive synteny with *M. truncatula*. Eight of 15 detected putative QTLs clustered in the same genomic region, on chromosome II, corresponding to chromosome IV of *M. truncatula*, in a zone including receptor-like protein kinase 5.

Variation and association between studied traits

Though both parental lines were considered drought-adapted (Abdelmula et al. 1999; Khan et al. 2007), they differed in several morpho-physiological measurements and showed different responses to water-deficit. The RILs showed a wide range of response and transgressive segregation in all studied traits. ILB 938/2 had lower g_s, lower stomatal density, and hence lower productivity, and when exposed to drought conditions it had less reduction in g_s and less change in canopy temperature than Mélodie/2. In chickpea, genotypes that maintained higher g_s and kept cooler canopy temperature under drought stress conditions during the reproductive period performed better than those that did not (Rehman et al. 2011). In faba bean, it was shown that accessions with higher stomatal density had less resistance to water stress (Ricciardi 1989). In rice, improved drought adaptation was associated with a more extensive root system, reduced stomatal density, and higher water-use efficiency (Yu et al. 2013). Water loss can be regulated by both stomatal density and function (Hetherington and Woodward 2003; Lake and Woodward 2008). Stomatal density and size showed a strong negative correlation in faba bean (Khazaei et al. 2013b) as in several other species (Hetherington and Woodward 2003). Low stomatal density improves adaptation to drought-prone environments (Doheny-Adams et al. 2012). Stomatal size is also associated with drought adaptation, as faba bean accessions with larger stomata were better adapted to arid zones (Khazaei et al. 2013b). Canopy temperature in leaves depends heavily on environment and transpirational cooling. To avoid air currents (wind) and control CO_2 level, air humidity and air temperature, all of which strongly influence canopy temperature, our study was conducted in a climate-controlled glasshouse, ensuring that genetic factors alone would be responsible for differences amongst RILs. Rapid response of stomatal function to water-limited conditions is important for maintaining plant water status. The use of canopy temperature as an indirect selection criterion under drought conditions has been shown in several crops (Blum 2011a) including faba bean (Khan et al. 2010; Khazaei et al. 2013b) because of its convenient and quick measurement by a handheld infrared thermometer. Our results showed a strong and negative association of canopy temperature with both stomatal conductance and stomatal length, indicating the important roles of both stomatal function and morphology.

Linkage map

The large genome size of faba bean has complicated the development of saturated genetic linkage maps and the identification of important genes. High-throughput SNPs have greatly accelerated the development of high-density linkage maps in faba bean (Cruz-Izquierdo et al. 2012; Kaur et al. 2014) and some other legume species (Hiremath et al. 2012). The present linkage map spanned ~928 cM of the faba bean genome in nine linkage groups at an average inter-marker distance of 5.8 cM, which is an increase on the previous resolution of 7.26 cM by Cruz-Izquierdo et al. (2012). Seven of the nine linkage groups were assigned to the six faba bean chromosomes. Whilst this work was in progress, Kaur et al. (2014) released a high-density map (2.3 cM) of this crop, covering 1217 cM in 12 linkage groups. Such differences are expected, since each linkage map has been constructed from RILs differing in generations and in number of originating strains and thus may differ in degree of map expansion in estimated distances (Broman 2006). So far, all such maps are smaller than the existing gene-based genetic linkage map (Ellwood et al. 2008; Cruz-Izquierdo et al. 2012) and have generated more than the expected six linkage groups (Satovic et al. 1996; Ellwood et al. 2008), because of the limited number of markers and the large genome. Our map is the first to use a subset of SNP markers exclusively designed from single-copy faba bean coding sequences with explicit orthology relationships to single-copy *M. truncatula* genes in the model legume giving a comprehensive consensus genetic map of faba bean which has clarified the macrosynteny between the genomes of faba bean and *M. truncatula* genome (Webb et al. in preparation). Green seed colour was mapped to chromosome IV, near the junction between parts of *M. truncatula* chromosomes IV and VIII, which confirms the suitability of this monogenic Mendelian trait as a morphological marker in these sorts of studies (Cruz-Izquierdo et al. 2012).

QTL analysis

To our knowledge, no previous report has been made on loci controlling stomatal morphology and function in this species. In this study, five genomic regions were shown to be associated to morpho-physiological traits on chromosomes II and IV. The most important of these nine QTL intervals was in a region of faba bean chromosome II, which aligned to *M. truncatula* chromosome IV, where eight of these traits that showed strong correlations with each other (*qSD-2013-1*, *qSD-2014*, *qSL-2013*, *qSL-2014*, *qgs-2013*, *qCT-2012*, *qCT-2013* and *qCT-2014*) were co-located. Whilst gas exchange is clearly causally related to canopy temperature, the tight association with stomatal density indicates a lack of other variation for stomatal control in this cross. The stability of canopy temperature as a trait was confirmed by its co-location in the 3 years of assessment (*qCT-2012*, *qCT-2013* and *qCT-2014*). Although we found only two QTLs controlling canopy temperature, the second being detected under water-deficit, four significant QTLs were found in maize (*Zea mays* subsp *mays*) (Liu et al. 2011), six in chickpea (Rehman et al. 2011), 18 in rice (*Oryza sativa* L.) (Liu et al. 2005) and over 40 in wheat (Rebetzke et al. 2013). In common bean (*Phaseolus vulgaris* L.) no significant QTL was detected for canopy temperature under normal and drought stress conditions (Asfaw et al. 2012). The number of QTL detected for this trait depends, as always, on the existence of allelic differences between parents, with polyploids offering more opportunities than diploids, and the presence of a low enough environmental variance to allow the genetic variance to be detected. Concerning stomatal morphology, several significant QTLs were detected in *Quercus robur* (Gailing et al. 2008) and rice (Laza et al. 2010). Although drought adaptation is usually considered a complex quantitative trait, some authors question this (Blum 2011b), and studies like this one that breaks it down into its components will allow resolution of this supposed complexity.

Synteny

Model plants facilitate the study of complex biological processes in plants, and in the case of legumes, *M. truncatula* is the model providing understanding in development, responses to biotic and abiotic stresses and evolutionary biology. *M. truncatula* is phylogenetically close to several cultivated forage and grain legume species (Zhu et al. 2005) and has been completely sequenced and annotated (http://www.medicagohapmap.org/?genome). Macro- and microsyntenies were observed between *M. truncatula* and several cultivated species, such as faba bean (Ellwood et al. 2008; Kaur et al. 2012; Cruz-Izquierdo et al. 2012), lentil (Sharpe et al. 2013), pea (Tayeh et al. 2013) and chickpea (Varshney et al. 2013). Such syntenic relationships can be used to identify the homologous regions amongst legume species, which is useful in potentially translating discoveries regarding gene function from one (usually model) species to generate testable hypotheses in the less-studied species. Comparing putative QTL regions in faba bean with their syntenic regions in *M. truncatula* highlighted several valuable outcomes. Surprisingly, most of the detected QTLs in faba bean (e.g., *qSD-2013-1*, *qSD-2014*, *qSL-2013*, *qSL-2014*, *qgs-2013*, *qCT-2012*, *qCT-2013* and *qCT-2014*) were in chromosome II and harboured the same candidate gene, receptor-like protein kinase (RLK) 5 in *M. truncatula* chromosome IV as well as in *Arabidopsis*. RLKs regulate stomatal density and morphology (Nadeau and Sack 2002; Hara et al. 2009; Hunt and Gray 2009; Abrash and Bergmann 2010; Kondo et al. 2010; Sugano et al. 2010), probably by tuning cell division and expansion, and also transpiration efficiency and stomatal function (Masle et al. 2005; Shpak et al. 2005) by controlling stomatal morphology and leaf photosynthetic capacity, and as a result canopy temperature. In addition, RLKs are the most well-known gene family related to environmental stress-related phenotypes in *Arabidopsis* (Morris and Walker 2003). Furthermore, several studies show that RLKs play an important role in optimising plant responses to drought adaptation, including in rice (Ouyang et al. 2010), *Brassica rapa* L. and maize (Marshall et al. 2012), *Gossypium barbadense* (Zhao et al. 2013) and *Arabidopsis* (Kilian et al. 2007; Osakabe et al. 2010; Hua et al. 2012; Tanaka et al. 2012).

The *qCTd-2013* QTL, associated with the response of canopy temperature to water-deficit, included the gene for ribose-phosphate pyrophosphokinase 4. We suggest this as a candidate gene in this region, as it has been shown to be related to the cell membrane in one of the first steps of adaption to drought and is an indicator for drought response (see Chaves et al. 2003). In *Aquilegia*, ribose-phosphate pyrophosphokinase 4 was one of the most strongly expressed genes after water was withheld for 6 days (Henry 2009), indicating a role in response to short-term drought, as was observed in the current study. Gene ontology for both intervals with *qSW-2013-1* and *qSW-2014-1* was related to seed characters, regulation of seed germination (Vf_Mt3g070310_001) and embryonic development ending in seed dormancy (Vf_Mt3g065190_001) (http://www.medicagohapmap.org/?genome).

All of these results confirm that genomics information from *M. truncatula* can be translated to the large faba bean genome as was also shown when nominating candidate genes for stipule spot pigmentation (Khazaei et al. 2014).

Conclusion

Regions in chromosome II that showed relatively large effects on several stomatal traits should be explored more

deeply in future studies. The availability of expressed sequences from model or closely related species constitutes an important source of markers physically associated with coding regions, and is now extensively exploited for gene discovery and translational genomics amongst crops. The availability of DNA markers is no longer a bottleneck, but there is still a need to construct dense genetic maps facilitating QTL fine mapping and gene discovery. Subsequently, development of transferable and cost-effective markers, targeting important traits of interest in faba bean, will allow greater efficiency in breeding programmes. Parental lines showed different root morphology as well, and there is potential to extract more information from these parental lines and populations in the future.

Author contribution Conceived and designed the experiments: HK FLS. Performed the experiments: HK. Analysed the data: HK DOS MJS. Contributed reagents/materials/analysis tools: HK DOS MJS FLS. Wrote the paper: HK FLS MJS DOS.

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Conflict of interest The authors declare that they have no conflict of interest.

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