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Nadim Tayeh, Nasser N. Bahrman, Isabelle I. Lejeune-Henaut, Rosemonde R. Devaux, Aurélie A. Bluteau, et al.. A high-density genetic map of the *Medicago truncatula* major freezing tolerance QTL on chromosome 6 reveals colinearity with a QTL related to freezing damage on *Pisum sativum* linkage group VI. *Molecular Breeding*, 2013, 32 (2), pp.279-289. 10.1007/s11032-013-9869-1 . hal-01004095

HAL Id: hal-01004095

<https://hal.science/hal-01004095v1>

Submitted on 28 May 2020

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A high-density genetic map of the *Medicago truncatula* major freezing tolerance QTL on chromosome 6 reveals colinearity with a QTL related to freezing damage on *Pisum sativum* linkage group VI

Nadim Tayeh · Nasser Bahrman · Rosemonde Devaux · Aurélie Bluteau · Jean-Marie Prosperi · Bruno Delbreil · Isabelle Lejeune-Hénaut

Received: 4 January 2013 / Accepted: 15 April 2013
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Abstract Freezing is one of the most serious abiotic stress factors that affect cool-season legumes. It limits species geographic distribution and causes severe yield losses. Improving tolerance to freezing has long been a main concern for legume breeders. *Medicago truncatula* Gaertn. has been selected as a model species for legume biology. Various studies have shown significant macrosyteny between *M. truncatula* and agronomically important crop legumes. A major freezing tolerance quantitative trait locus (QTL), herein referred to as Mt-FTQTL6, was previously identified on *M. truncatula* chromosome 6. The physical location of this QTL was determined in this study and its corresponding chromosomal interval was enriched with additional markers. Markers were first

developed using the draft sequence of *M. truncatula* euchromatin (release versions Mt3.0 and Mt3.5). Because Mt-FTQTL6 was found to coincide with an assembly gap, the *Glycine max* (L.) Merr. genome sequence was also used to generate markers. Five Mt-FTQTL6-linked markers were found to be common to a region on *Pisum sativum* L. linkage group VI harboring a QTL for freezing damage. A subset of markers was tested for transferability across 11 additional legume species. This study lays the groundwork for identifying the molecular basis of Mt-FTQTL6. Cross-legume markers will be useful in future efforts aiming to investigate the conservation of Mt-FTQTL6 in cool-season legumes and subsequently the existence of common mechanisms for response to freezing between *M. truncatula* and crop legumes.

Electronic supplementary material The online version of this article (doi:10.1007/s11032-013-9869-1) contains supplementary material, which is available to authorized users.

N. Tayeh · N. Bahrman · A. Bluteau · B. Delbreil (✉)
Université Lille 1, UMR 1281 SADV, Bâtiment SN2,
59655 Villeneuve d'Ascq Cedex, France
e-mail: bruno.delbreil@univ-lille1.fr

N. Bahrman · R. Devaux · A. Bluteau ·
I. Lejeune-Hénaut
INRA, UMR 1281 SADV, Estrées-Mons BP50136,
80203 Péronne Cedex, France

J.-M. Prosperi
INRA, UMR AGAP, 2 Place Pierre Viala,
34060 Montpellier Cedex, France

Keywords Freezing tolerance · Quantitative trait locus (QTL) · Synteny · *Medicago truncatula* · *Glycine max* · *Pisum sativum*

Introduction

The Fabaceae (Leguminosae) family is the third largest family of flowering plants, comprising more than 700 genera and 20,000 species. It ranks second to Poaceae in terms of economic importance. Most important food and feed legumes belong to the subfamily Papilionoideae. Species that originated from tropical regions are referred to as warm-season legumes; those from

temperate regions are designated as cool-season legumes (Doyle and Luckow 2003; Gepts et al. 2005).

Due to better moisture availability and longer growing season, fall-sown annual cool-season legumes are superior to spring types in grain or total forage yield (Sadeghipour and Aghaei 2012; Chen et al. 2006; Firincioğlu et al. 2009). However, harsh winter conditions in predominant production regions greatly limit the use of fall-sown annual crops. Winter stresses also have a crucial impact on the cultivation of perennial cool-season legumes (Bélanger et al. 2006). Improving winter hardiness is expected to expand cultivated areas and increase productivity and yield stability of cool-season legumes.

Winter hardiness of legumes is a complex trait due to its quantitative inheritance and the existence of genotype \times environment interactions (Kahraman et al. 2004; Klimenko et al. 2010; Castonguay et al. 2009). Freezing tolerance has often been used to predict winter hardiness. It is commonly defined as the potential of plants to minimize freezing effects upon a period of exposure to low non-freezing temperatures, known as cold acclimation, during which diverse physiological, molecular and metabolic modifications are induced (Guy 1990). Freezing tolerance is the most determinant factor affecting winter survival across a wide range of environments and genetic evidence of this statement has been provided from different legume species by reporting the co-localization of freezing tolerance quantitative trait loci (QTL) with QTL for winter hardiness (Brouwer et al. 2000; Kahraman et al. 2004; Dumont et al. 2009).

The cool-season legume, *M. truncatula*, has been chosen as a model molecular-genetic system for legume biology (Cook 1999). A draft sequence of its euchromatin, comprising approximately 94 % of all genes, is currently available (assembly version Mt3.5; Young et al. 2011). Three QTL for freezing tolerance at the vegetative stage were identified in *M. truncatula* after a cold acclimation period using the F83005-5 \times DZA045-5-derived population (LR3 population; Komlan et al. 2013). They were mapped on chromosomes 1, 4 and 6. The major QTL on chromosome 6 (Mt-FTQTL6) accounted for 40 % of the freezing tolerance variation. The simple sequence repeat (SSR) marker MTIC153, derived from an expressed sequence tag (EST) with a Bcl-2-associated athanogene annotation (Julier et al. 2003), was reported as the peak marker for this QTL but its sequence could not be

located on the *M. truncatula* chromosome 6 pseudo-molecule. In parallel, a QTL related to freezing damage was detected on *P. sativum* linkage group (LG) VI under both field (WFD 6.1; Lejeune-Hénaut et al. 2008) and controlled conditions (FD164.c; Dumont et al. 2009) using the Champagne \times Terese-derived population (Pop2 population). *M. truncatula* chromosome 6 and *P. sativum* LG VI were reported to share conserved macrosynteny (Aubert et al. 2006; Bordat et al. 2011). However, no common molecular markers were available to determine whether Mt-FTQTL6 and WFD 6.1/FD164.c are located on corresponding genomic regions.

“The objectives of the present study were to: (1) place Mt-FTQTL6 on the *M. truncatula* genome sequence; (2) enrich the QTL interval with markers; (3) compare the chromosomal location of Mt-FTQTL6 with that of *P. sativum* WFD 6.1/FD164.c; and (4) provide cross-species markers that would permit the examination of Mt-FTQTL6 conservation in other legume species.

Materials and methods

Marker development

Bacterial artificial chromosome (BAC)-derived markers

Two markers from the integrated genetic map of *M. truncatula* chromosome 6 (University of Minnesota 2006; <http://www.medicago.org/genome/map.php>), 004F08 and h2-166b10a, were transferred to the LR3 genetic map. These markers mapped on both sides of marker MTIC153 (Komlan et al. 2013). The 21 BAC clones spanning the interval between 004F08 and h2-166b10a on *M. truncatula* genome assembly version Mt3.0 were used to generate primer pairs for candidate BAC-derived markers. With the release of the Mt3.5 genome assembly version and thus updates of BAC/contig order and orientation, the interval was extended to include 11 additional BAC clones located down to mth2-9p17 [National Center for Biotechnology Information (NCBI) GenBank accession (GB acc.) AC146307]. Coding sequences from target BAC clones were submitted to BLAST (Basic Local Alignment Search Tool; Altschul et al. 1990) searches against the NCBI *M. truncatula* nucleotide and high-throughput

genomic sequence (HTGS) databases to avoid redundancy. Selected non-redundant sequences were searched for highly similar EST/messenger RNA from other legume species. Homologous sequences were aligned using MultAlin (Corpet 1988). Primers were designed on conserved exonic, preferentially intron-spanning, regions. When no primers for candidate gene-based markers could be obtained, markers were developed on non-repetitive intergenic microsatellite-flanking sequences. Microsatellite motifs were identified using Perfect Microsatellite Repeat Finder (<http://sgdp.iop.kcl.ac.uk/nikammar/repeatfinder.html>). The minimum number of repeats was set to 5.

Syntenic-based markers

MTIC153-corresponding EST (GB acc. AL369679) and coding sequences from BAC clones encompassing the boundaries of Mt-FTQTL6 were used as BLAST queries to identify similar sequences in the *G. max* genome. Candidate regions syntenic to Mt-FTQTL6 could thus be located on *G. max* chromosomes 5, 9, 10, 16, 17 and 20. In *M. truncatula*, homoeologous regions to Mt-FTQTL6 could be located on chromosomes 1 and 5 following *M. truncatula*-*M. truncatula* and *M. truncatula*-*G. max* BLAST and reverse BLAST searches. Among all blocks syntenic to Mt-FTQTL6 in *G. max* and *M. truncatula*, genes from the Mt-FTQTL6-containing region exhibited the highest similarity to those on *G. max* chromosomes 9 and 16. Both *G. max* regions are homoeologous and derived from the *G. max* lineage-specific paleotetraploidization (*ca.* 13 million years ago; Schmutz et al. 2010). Protein sequences from target *G. max* regions were used to search for putative homologous *M. truncatula* EST, HTGS, or nucleotide sequences using TBLASTN. NCBI legume EST databases and the *Lotus japonicus* (Regel) K. Larsen genome sequence (<http://www.kazusa.or.jp/lotus/blast.html>) were also regularly searched for highly similar counterparts (> 85 % sequence identity). All homologous sequences obtained by BLAST searches were translated in silico (ExpASY Translate Tool; <http://web.expasy.org/translate>), aligned and used for phylogenetic tree construction (phylogeny.fr; Dereeper et al. 2008). Orthologous sequences to those from *G. max* chromosomes 9 and/or 16 that were identified by the phylogenetic method were retained for primer design.

In all cases, primers were designed using the Primer3 tool (Rozen and Skaletsky 2000) with default parameters. Maximum product length was generally limited to

500 bp. Degenerate nucleotides were included in some primer sequences to ensure cross-species amplification. The PrimerSearch tool, available at <http://www.legoo.org>, was used to check the specificity of the generated primer pairs for PCR amplification in *M. truncatula*.

Plant material

Markers developed in this study were first scored on recombinant inbred lines (RIL) from *M. truncatula* LR3 and *P. sativum* Pop2 populations. The LR3 population (178 F2:5 RIL) was developed by single-seed descent from a cross between the French freezing-tolerant line F83005-5 and the Algerian freezing-sensitive line DZA045-5 (INRA, UMR AGAP, Montpellier, France; <http://www1.montpellier.inra.fr/BRC-MTR>). This population was used to map QTL for freezing tolerance under controlled conditions (Kolan et al. 2013). Total genomic DNA of the 178 RIL from the LR3 population was provided by Dr. Marie-Laure Pilet-Nayel (INRA, UMR IGEPP, Rennes, France). The Pop2 population (164 F2:8 RIL) was derived from a cross between *P. sativum* lines Champagne and Terese (Loridon et al. 2005). QTL related to freezing damage were identified in this population both under field and controlled conditions (Lejeune-Hénaut et al. 2008; Dumont et al. 2009).

Large *M. truncatula* and *P. sativum* populations were used to determine the order of co-segregating markers on LR3 and Pop2 linkage maps, respectively. A population comprising 454 *M. truncatula* F6 plants was produced from the self-pollination of two residual heterozygous lines (RHL): 76-06 (237 plants) and 76-11 (217 plants). The parental lines 76-06 and 76-11 are sisters of LR3 RIL#76. All three have residual heterozygosity in the chromosomal segment harboring Mt-FTQTL6 and are largely homozygous throughout the rest of the genome (based on genotype data from 3–6 evenly-spaced microsatellite markers per chromosome). A *P. sativum* population of 1,438 individuals was obtained from the self-pollination of 132 parental plants heterozygous for WFD 6.1/FD164.c. These latter were selected among 189 total plants obtained from crosses between line Eden and eight progenies of a two-backcross plant that are heterozygous at WFD 6.1/FD164.c. The two-backcross plant was derived from a cross between Eden (female, recurrent parent) and Pop2 RIL#38 (male, donor parent). RIL#38 carries alleles from Champagne at WFD 6.1/FD164.c. Large

populations were grown in the greenhouse at an average temperature of 18/14 °C (day/night) and a 16/8-h light/dark photoperiod.

Eleven legume species belonging to the hologalegina, phaseoloid/millettioid and genistoid clades of the Papilionoideae subfamily (Doyle and Luckow 2003) were used for marker transferability tests (Online Resource 1), with one or two cultivars/accessions per species. Seeds were spread on wet filter papers contained in Petri dishes and left to germinate at room temperature in the dark.

Leaflets from the *M. truncatula* RHL-derived population and entire seedlings from the set of eleven legume species were collected on 1-month-old and 7–10-day-old plants, respectively, frozen in liquid nitrogen and stored at –80 °C until DNA extraction. Stipules from 1-month-old *P. sativum* plants were lyophilized and kept at –20 °C.

DNA isolation, PCR amplification and PCR product sequencing

Total genomic DNA extraction was performed using a Nucleospin 96 Plant II kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Buffer PL1 was used for cell lysis. Elutions were performed with 2 × 100 µL of pre-warmed ultrapure water (70 °C). DNA quality and quantity were assessed by agarose gel electrophoresis.

PCR amplification and sequencing were carried out as described in Online Resource 2. Briefly, PCR reactions were performed in a final volume of 15 µL with 10 ng of genomic DNA template. Two different thermal cycling protocols were employed and are referred to as touch-down or three-step protocol. PCR products were sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems, Foster City, California, USA) on a 3130xl Genetic Analyzer (Hitachi/Applied Biosystems). *M. truncatula* and *P. sativum* marker sequences are provided in FASTA format in Online Resource 12.

Polymorphism identification and genotyping techniques

Polymorphism tests were run using 8–16 individuals including parental lines and segregating progeny. Lines with residual heterozygosity in chromosomal

segments containing Mt-FTQTL6 and WFD 6.1/FD164.c were particularly valuable as they brought direct evidence for the linkage of markers to target regions. SSR and insertion/deletion (indel) polymorphisms were detected and genotyped using either agarose or capillary gel electrophoresis, depending on parental allele sizes. Detection of single nucleotide polymorphisms (SNP) was performed using a high-resolution melting technique (HRM). Genotyping of SNP markers was conducted either by HRM or competitive allele-specific PCR chemistry (KASPar; KBiosciences, Hoddesdon, UK) for larger-scale genotyping. Allele-specific and common primers for markers genotyped by KASPar are provided in Online Resource 8. Additional method details are given in Online Resource 2.

Linkage analysis and QTL mapping

The genetic linkage map of the LR3 population was described in Komlan et al. (2013) and that of the Pop2 population was described in Loridon et al. (2005) and Aubert et al. (2006). The framework map of *M. truncatula* chromosome 6, used in this study, comprises four SSR, five random amplified polymorphic DNA (RAPD) and five amplified fragment length polymorphism (AFLP) markers (Online Resource 3a). Markers genotyped in the *P. sativum* Pop2 population were mapped on an 18-loci framework map of LG VI [nine SSR, four RAPD, one AFLP and three gene-based markers] (Loridon et al. 2005; Aubert et al. 2006). Markers having names starting with “NT” together with *P. sativum* markers MTIC153 and Ps92K09T were developed in this study. Marker Cabb was previously used by Bordat et al. (2011) to genotype multiple *P. sativum* segregating populations. A new primer set for Cabb was used in this study: forward primer 5'-TGTTCAATTTGTTGTGTTTGAAGC-3' and reverse primer 5'-AAATTATAGCTTTTGCCTTAACACG-3'. Full information regarding genetic distances between different *M. truncatula* markers is given in Online Resource 3. As described above, large *M. truncatula* and *P. sativum* segregating populations were derived from parental lines showing heterozygosity at Mt-FTQTL6 and WFD 6.1/FD164.c, respectively. They were thus treated as F2 populations and used to construct high-resolution linkage maps for target regions. In all mapping analyses, the goodness-of-fit to expected

segregation ratios, 1:1 or 1:2:1 accordingly, were assessed by the Chi squared test implemented in JoinMap 4.1 software (Van Ooijen 2006). Similar to MTIC153, BAC-derived and synteny-based markers developed in this study all exhibited distortion from the expected 1:1 segregation ratio (significant at $P < 0.01$) within the LR3 population showing a higher DZA045-5 allele frequency. No segregation distortion was noted for new markers in the other mapping populations. The grouping of markers into LG was evaluated using independent logarithm of odds (LOD). The map order was estimated using JoinMap 4.1 with the regression mapping algorithm (RM; default parameters: recombination frequency <0.4 , $\text{LOD} > 2$) and the Kosambi mapping function (Kosambi 1944). For comparison, maximum likelihood mapping (MLM) was also used. In all cases, the marker order was the same with both algorithms. Linkage groups were drawn using MapChart software 2.2 (Voorrips 2002).

QTL mapping analyses were performed using interval mapping implemented in MapQTL 6 software (Van Ooijen 2009). Freezing tolerance data, scored on the LR3 population by Komlan et al. (2013), were considered. For the Pop2 population, phenotypic data obtained under field experiments (Lejeune-Hénaut et al. 2008) and those from controlled conditions (Dumont et al. 2009) yielded the same QTL position. Only the LOD profile for data scored under controlled conditions is shown in this paper. LOD thresholds for QTL detection on *M. truncatula* chromosome 6 and *P. sativum* LG VI were estimated using permutation tests with a P value of 0.05 and 10,000 permutations.

Results

Location of Mt-FTQTL6 on *M. truncatula* chromosome 6 pseudomolecule

Twenty-five primer pairs for candidate gene-based (SNP/indel; 21 primer pairs) and intergenic SSR-flanking (four primer pairs) markers were designed based on full sequences of 16 *M. truncatula* BAC clones from eight different contigs (see Materials and Methods). Seventeen markers originating from 14 different BAC clones were found to be polymorphic between F83005-5 and DZA045-5 (Online Resource 4). All BAC-derived markers mapped close to marker MTIC153 in an 8.3-centiMorgan (cM) interval

(Online Resource 3b). The resulting genetic order is not completely concordant with the position of related BAC clones either in *M. truncatula* assembly version Mt3.0 or Mt3.5 (Online Resource 5). It agrees more closely with the BAC contig order/orientation proposed by version Mt3.5 for the upper part of the region containing Mt-FTQTL6; however, it agrees with the Mt3.0 version in the lower part. The orientation of BAC contigs could not be resolved due to the absence of recombination events. Interval mapping confirmed previous results regarding Mt-FTQTL6 position with marker MTIC153 as the closest marker to its peak. The 95 % confidence interval of Mt-FTQTL6 was located between markers NT6001 and NT6019, and thus between BAC clones mth2-156d20 (GB acc. AC157779) and mth2-48m20 (GB acc. AC225497) [Online Resources 5 and 6].

Construction of a gene-based linkage map for Mt-FTQTL6 using synteny between *M. truncatula* and *G. max*

The Mt-FTQTL6 confidence interval, bracketed by markers NT6001 and NT6019, coincides with an assembly gap on the *M. truncatula* chromosome 6 pseudomolecule (Online Resource 5). A synteny-based approach using *G. max* as a reference genome was carried out in order to develop close markers to Mt-FTQTL6. Candidate orthologs for genes underlying Mt-FTQTL6 were assumed to exist among genes contained in an approximately 867-kb genomic region on *G. max* chromosome 16 (35.17–36.04 Mb) between *Glyma16g31930* and *Glyma16g32950* and/or in its homoeologous region on chromosome 9 (*Glyma09g26080*–*Glyma09g28090*; 32.33–35.09 Mb). *Glyma16g31930* and *Glyma16g32950* have their *M. truncatula* counterparts directly adjacent to gene/BAC sequences corresponding to markers NT6001 and NT6019. Primer pairs corresponding to 17 distinct genes yielded successful amplification from F83005-5 and DZA045-5 while primers corresponding to five other genes did not produce any amplification (Online Resource 7). Twelve markers were found to be polymorphic between F83005-5 and DZA045-5 and mapped within a 2.1-cM interval around MTIC153 (Online Resource 3c). Six BAC clones could be identified as having potentially originated from the Mt-FTQTL6-containing region based on markers located within or in contiguous clones. These include

two distinct unanchored BAC clones [mth2-154j21 (GB acc. AC146807) and mth2-53124 (GB acc. AC229695)] and four others forming a single contig that was formerly placed nearer the distal end of *M. truncatula* chromosome 6 (clone name, GB acc.: mth2-12e18, AC229727; mth2-1515, AC130804; mth2-172p22, AC146818; mth2-50m10, AC174372).

Synteny between Mt-FTQTL6 and *P. sativum* WFD 6.1/FD164.c

Twenty-nine BAC-derived and synteny-based markers originating from 26 distinct genes yielded positive amplification in *P. sativum* lines, Champagne and Terese, nine of which were polymorphic (Online Resources 4 and 7). Seven markers were mapped in the WFD 6.1/FD164.c region while two others, viz. BAC-derived markers NT6039bis and NT6044, mapped approximately 40 cM apart at the top of *P. sativum* LG VI (Fig. 1b). Five anchor markers were obtained between Mt-FTQTL6 and WFD 6.1/FD164.c corresponding genomic regions, revealing QTL synteny (Fig. 1). MTIC153 and co-segregating markers NT6067, Ps92K09T and NT6092Ps are located at 0.3 cM from markers NT6012, NT6011 and Cabb, and at 2.6 cM from marker NT6032 (Fig. 1b).

In order to develop additional markers in the upper part of WFD 6.1/FD164.c, new primer pairs were designed in conserved genes between the upper region of Mt-FTQTL6 and corresponding *G. max* regions (Online Resource 7). Out of nine markers with positive amplification from Champagne and Terese, NT6083 and NT6078 were found to be polymorphic and to co-segregate with other synteny-based markers (Fig. 1b).

Colinearity between *M. truncatula*, *P. sativum* and *G. max*

To enable high-resolution comparative analyses, markers linked to *M. truncatula* and *P. sativum* syntenic freezing tolerance QTL-containing intervals were used to screen large segregating populations, comprising 454 and 1,438 plants, respectively. The marker order on *M. truncatula* and *P. sativum* LGs was found to be almost identical to that of the corresponding genes on *G. max* chromosome 16, indicating a high level of colinearity between the three species (Fig. 2). A unique local rearrangement in marker/gene order was observed between *P. sativum* and *G. max*. This

concerned markers NT6078 and NT6083 (Online Resource 9). Data concerning the comparison of *M. truncatula* and *P. sativum* LGs with the orthologous region on *G. max* chromosome 9 are provided in Online Resource 9.

Cross-legume transferability of synteny-based markers

In addition to *M. truncatula* and *P. sativum*, the transferability of 27 synteny-based markers was evaluated across 11 legume species (Online Resource 1). Online Resource 10 summarizes the results of PCR amplification. Seventeen to 24 markers (63–89 % of the total tested markers) yielded positive amplification from each cultivar/accession. Thirteen markers were transferable across all tested species, among which eight have been shown above to be located close to Mt-FTQTL6 and/or WFD 6.1/FD164.c. In contrast to markers targeting exons, intron-spanning markers exhibited differences in product sizes across species with seven out of 16 markers showing size variations exceeding 100 bp (Online Resources 10 and 11).

Discussion

Synteny as a potential tool for circumventing difficulties in *M. truncatula* genome assembly

With the advent of sequencing technologies, large genomic resources are being developed from a wide variety of plant species including legumes. Besides genome sequencing and assembly efforts in *M. truncatula*, the gene-rich genomic regions of the model legume *L. japonicus* (Sato et al. 2008) and the crop legume *Vigna unguiculata* (L.) Walp (Timko et al. 2008) together with the whole genomes of the economically important warm-season crop legumes *G. max* (Schmutz et al. 2010) and *Cajanus cajan* L. (Varshney et al. 2012) have been sequenced and assembled. Since Mt-FTQTL6 was found to coincide with an assembly gap, it was thus possible to turn to other sequenced legume species for marker development. The gene-space sequence from *L. japonicus* and whole-genome sequence from *G. max* were considered here. Macro- and microsynteny have already been reported between *M. truncatula* and *L. japonicus* (Choi et al. 2004a; Cannon et al. 2006; Young et al.

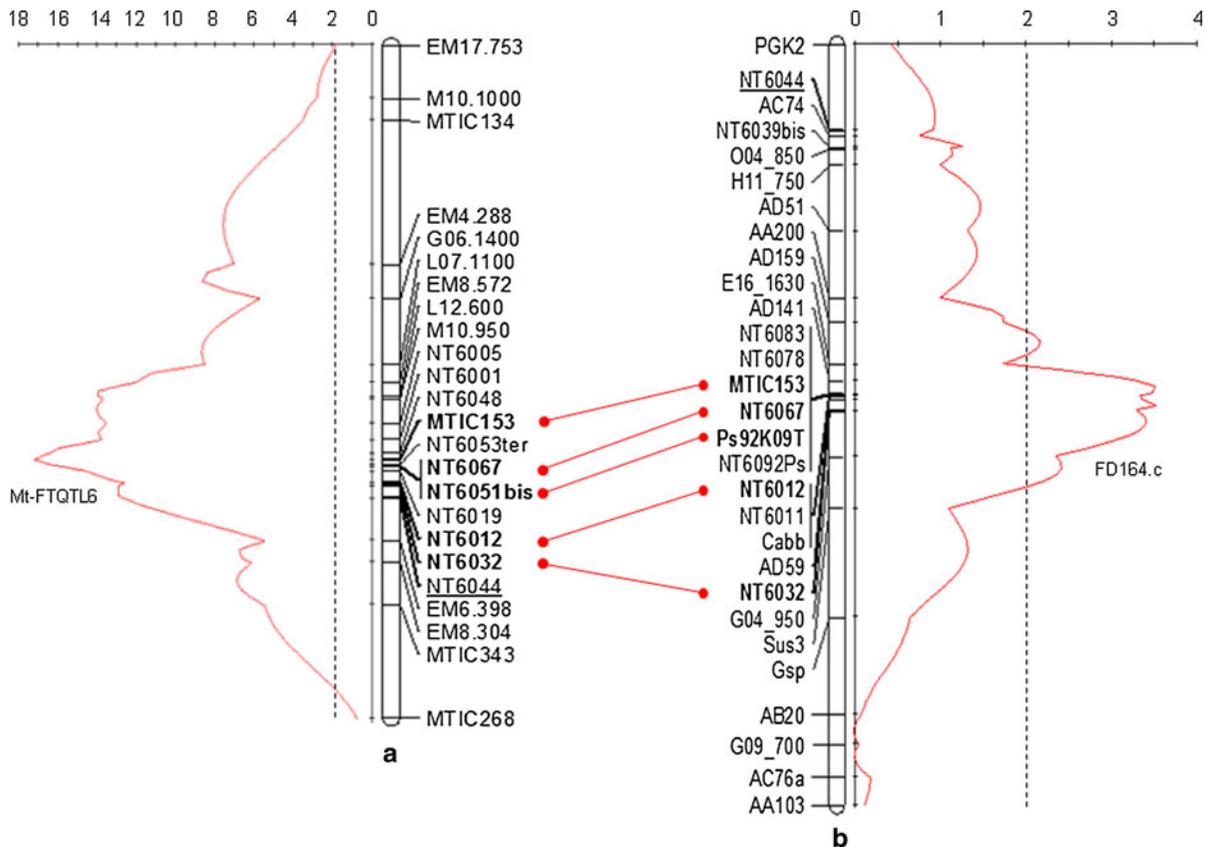


Fig. 1 Anchor markers between *M. truncatula* and *P. sativum* genomic regions harboring freezing tolerance-related QTL: Mt-FTQTL6 on *M. truncatula* chromosome 6 (a) and FD164.c on *P. sativum* linkage group VI (b). Solid red lines link anchor markers (in bold) between QTL-containing regions. The *M. truncatula* and *P. sativum* common marker, NT6044, is underlined on both linkage groups. It mapped outside the FD164.c confidence interval (41.3 cM distant from marker NT6083 or other coincident markers) to the top of *P. sativum* linkage group VI. For the sake of clarity, some of the tightly

linked or co-segregating markers on the *M. truncatula* chromosome 6 were excluded from the figure (see Online Resource 3 for full information). Horizontal axes show LOD values. Dashed black lines correspond to the significance thresholds estimated by permutation tests (LOD = 1.8 for *M. truncatula* chromosome 6 and LOD = 2 for *P. sativum* linkage group VI). Maximum LOD scores for Mt-FTQTL6 (LOD = 17.21) and FD164.c (LOD = 3.45) were obtained for both MTIC153 and co-segregating markers

2011) as well as between *M. truncatula* and *G. max* (Choi et al. 2004a; Mudge et al. 2005; Schlueter et al. 2008; Shin et al. 2008; Joseph et al. 2009; Young et al. 2011). The most similar genes to those from the target region on *M. truncatula* chromosome 6 were identified on *L. japonicus* chromosome 2 and *G. max* chromosomes 9 and 16. Because the assembly of the Mt-FTQTL6 putative orthologous region in *L. japonicus* is not complete, marker development was conducted using only *G. max* as a reference genome. High conservation of gene content and order between *M. truncatula* and *G. max* was observed based on the number of Mt-FTQTL6-linked markers that could be

obtained and their order on the genetic linkage maps constructed using either the LR3 or the RHL-derived population. These results extend previous observations on synteny conservation between *M. truncatula* and *G. max* and are particularly important since they concern *M. truncatula* chromosome 6, reported to be the shortest among all eight *M. truncatula* chromosomes, the most heterochromatic (Kulikova et al. 2001) and the richest in nucleotide-binding site-leucine-rich repeat genes (Zhu et al. 2002). Taking into account the effectiveness of this strategy, it can be argued that similar efforts may be helpful to provide markers linked to other residual gaps in the *M.*

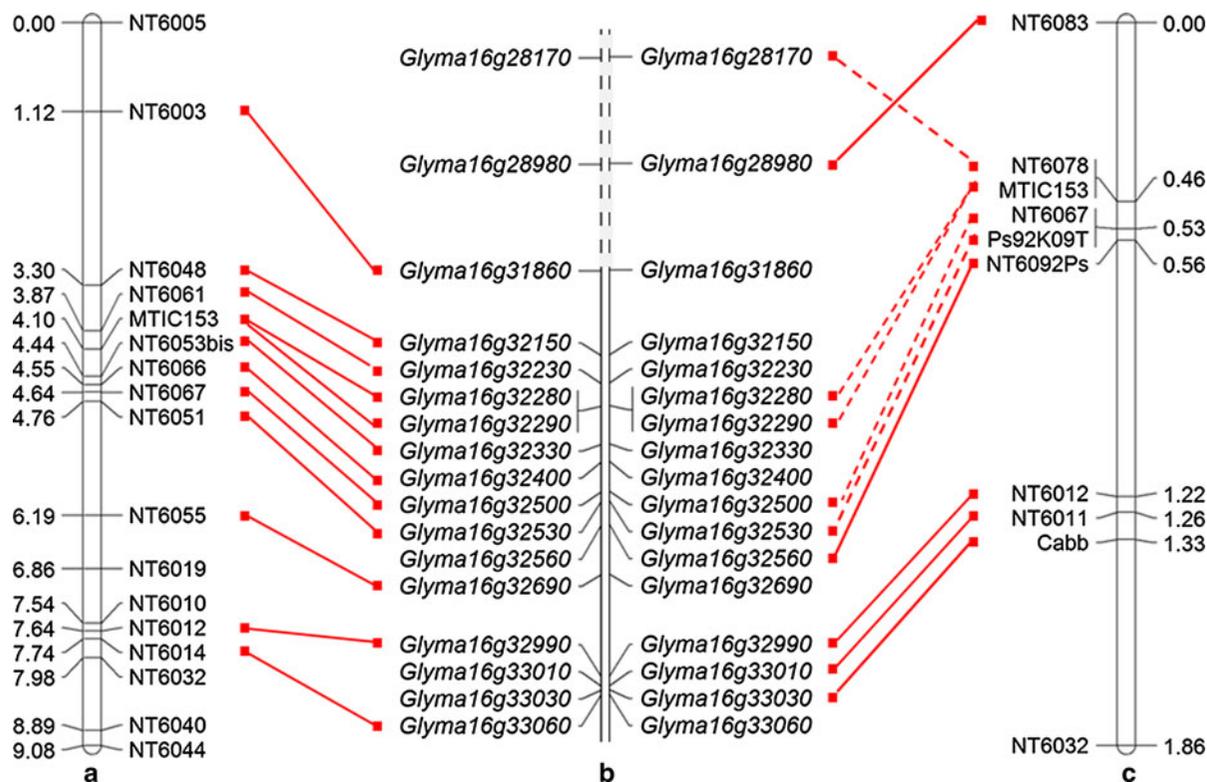


Fig. 2 Comparative linkage groups for *M. truncatula* (a), *G. max* (b) and *P. sativum* (c) showing conservation of marker/gene order on orthologous chromosomal segments. The *G. max*-corresponding linkage group represents the gene order on *G. max* chromosome 16 according to the genome assembly version 1.01. It was constructed using physical distances between different genes with 250 kb being equivalent to one scale unit. For presentation clarity, exceptions to gene spacing were made

for *Glyma16g28170* and *Glyma16g28980* (dotted bar). *Glyma16g28170* and *Glyma16g28980* are located at 3.01 and 2.23 Mb from *Glyma16g31860*, respectively. Solid red lines link markers from *M. truncatula* and *P. sativum* linkage groups to corresponding *G. max* genes. Dotted red lines are used in the case of co-segregating markers (where no certainty for order conservation with *G. max* genes exists at present)

truncatula genome and, as shown in this study, may help assign unanchored BAC clones to their corresponding genomic regions. The current *M. truncatula* genome assembly version (Mt3.5) still comprises 273 physical gaps and 146 unanchored BAC/BAC pools (Young et al. 2011).

Conservation of Mt-FTQTL6 in crop legumes

The conservation of genomic regions responsible for quantitative traits has been reported between different plant species and for multiple traits comprising, for instance, QTL for resistance to *Pyricularia grisea* between *Hordeum vulgare* L. and *Oryza sativa* L. (Chen et al. 2003), bud burst QTL in *Quercus robur* L. and *Castanea sativa* Mill. (Casasoli et al. 2006) and fruit quality QTL among *Fragaria* × *ananassa* and

Malus or *Prunus* populations (Zorrilla-Fontanesi et al. 2011). It is often assumed that genes with conserved functions may underlie syntenic QTL. Colinearity between chromosomal regions harboring grain protein content QTL in *H. vulgare* and *Triticum turgidum* L. ssp. *dicoccoides* suggested orthologous NAC transcription factors as responsible for the trait variation in both species (Distelfeld et al. 2008). In legumes, syntenic QTL have been identified among different species. In fact, one of the first evidences for plant orthologous QTL was provided by Fatokun et al. (1992) who reported that conserved regions of *Vigna radiata* (L.) Wilczek and *V. unguiculata* genomes contain seed weight QTL. These QTL from *V. radiata* and/or *V. unguiculata* were later reported to be conserved in *P. sativum* (Timmerman-Vaughan et al. 1996), *G. max* (Maughan et al. 1996), *Cicer arietinum*

L. and *L. japonicus* (Gondo et al. 2007). A genomic region responsible for the variation in days to flowering was suggested to be conserved in six legume species including *Vicia faba* L., *L. japonicus*, *P. sativum*, *Lupinus angustifolius* L., *C. arietinum* and *M. truncatula*, based on synteny between corresponding chromosomes (Cruz-Izquierdo et al. 2012). Almost at the same time, D'Erfurth et al. (2012) have shown that syntenic chromosomal regions containing seed weight QTL on *M. truncatula* chromosome 5 and *P. sativum* LG I harbor orthologous subtilisin-like proteases responsible for the QTL effects.

The present study provided the first evidence for Mt-FTQTL6 conservation in *P. sativum*. Colinearity between the genomic regions containing Mt-FTQTL6 and *P. sativum* WFD 6.1/FD164.c (Lejeune-Hénaut et al. 2008; Dumont et al. 2009) was determined based on five common gene-based markers. Attempts to compare Mt-FTQTL6's position with that of QTL for winter hardiness and/or freezing tolerance from *Trifolium pratense* L. (Klimenko et al. 2010), *Lens culinaris* Medik. (Kahraman et al. 2004) and *V. faba* (Arbaoui et al. 2008) were not successful. This is mostly due to the genomic markers used for linkage map construction in the respective studies not being suitable for comparative mapping. A survey of the literature yielded potentially important data from a study on *Medicago sativa* L. subsp. *sativa*. Castonguay et al. (2010) have reported that a sequence-related amplified polymorphism marker whose sequence matches *M. truncatula* BAC clone mth2-9p17 (GB acc. AC146307) is associated with freezing tolerance in recurrently selected populations of *M. sativa* subsp. *sativa* improved for their response to freezing. The fact that marker NT6016 developed from the mth2-9p17 sequence immediately flanks the Mt-FTQTL6 confidence interval (Online Resource 3b-c) and that the genomes of *M. truncatula* and *M. sativa* are highly syntenic (Julier et al. 2003; Choi et al. 2004b) make Castonguay et al.'s finding potentially important in the context of the present work.

Cross-legume markers (Online Resource 10) are useful to: (1) investigate whether the aforementioned freezing tolerance loci from *M. truncatula* and *M. sativa* subsp. *sativa* reside in corresponding genomic regions; (2) further examine Mt-FTQTL6 conservation in mapping populations and germplasm collections of agronomically important cool-season legumes by studying the association of markers with

phenotypic variation for freezing tolerance; and (3) assist the assembly of genomic contigs originating from Ft-MtQTL6 orthologous regions in different sequenced legume species allowing comparative genomic sequence analysis. In addition to synteny-based markers, some of the BAC-derived markers would also be successfully transferable across legumes and similarly used for the above-mentioned purposes.

Finally, in the light of the above results and discussion, it appears that one of the primary goals of future molecular studies on legume freezing tolerance should be to investigate the orthology of the genes underlying Mt-FTQTL6 and syntenic loci from different species. In line with this, and as a first step forward, fine mapping and map-based cloning of Mt-FTQTL6 are currently ongoing taking advantage of the markers reported here.

Acknowledgments The authors wish to thank Drs. Marie-Christine Quillet and Marie-Laure Pilet-Nayel for helpful discussions concerning this work, Grégoire Aubert for providing *P. sativum* RNA-seq data, Cécile Godé for assistance in PCR product sequencing and Frédéric Depta for plant care in the greenhouse. They acknowledge Eric Hanocq for providing the initial plant material for the construction of the large *P. sativum* segregating population. They would also like to thank Pascal Marget and Jean-Bernard Magnin-Robert of GRC of grain legumes, INRA, Dijon, France; Stéphane Fourtier of GRC of forage and turf species, INRA, Lusignan, France; Frederic Ottosson of NordGen, Sweden and Dave Stout of WRPIS, Pullman, Washington, USA for supplying legume seeds. They also thank the GENTYANE platform (INRA UBP, UMR 1095, Clermont-Ferrand) for genotyping. This work was partly financially supported by the Région Nord Pas-de-Calais, France (FEDER ARCIR PLANTEQ3 program). N. Tayeh was the recipient of a Ph.D. fellowship from the Ministère de l'Enseignement supérieur et de la Recherche, France.

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