



## **Genetic variability and QTL mapping of freezing tolerance and related traits in [i]Medicago truncatula[/i]**

Komlan Avia, Marie-Laure Pilet-Nayel, Nasser Bahrman, Alain Baranger, Bruno Delbreil, Véronique Fontaine, Céline Hamon, Eric Hanocq, Martine Niarquin, Hélène Sellier-Richard, et al.

### **► To cite this version:**

Komlan Avia, Marie-Laure Pilet-Nayel, Nasser Bahrman, Alain Baranger, Bruno Delbreil, et al.. Genetic variability and QTL mapping of freezing tolerance and related traits in [i]Medicago truncatula[/i]. TAG Theoretical and Applied Genetics, 2013, 126 (9), pp.2353-2366. <10.1007/s00122-013-2140-7>. <hal-01208635>

**HAL Id: hal-01208635**

**<https://hal.science/hal-01208635v1>**

Submitted on 6 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

**Title:**

**Genetic variability and QTL mapping of freezing tolerance and related traits in *Medicago truncatula***

**Authors:**

**Komlan Avia<sup>1,a</sup>, Marie-Laure Pilet-Nayel<sup>2</sup>, Nasser Bahrman<sup>1</sup>, Alain Baranger<sup>2</sup>, Bruno Delbreil<sup>3</sup>, Véronique Fontaine<sup>1</sup>, Céline Hamon<sup>2</sup>, Eric Hanocq<sup>1</sup>, Martine Niarquin<sup>1</sup>, Hélène Sellier<sup>1</sup>, Christophe Vuylsteker<sup>3</sup>, Jean-Marie Prosperi<sup>4</sup> and Isabelle Lejeune-Hénaut<sup>1,\*</sup>**

**Addresses:**

<sup>1</sup> INRA, UMR 1281 SADV, Estrées-Mons BP 50136, F-80203 Péronne, France

<sup>2</sup> INRA, UMR1349 IGEPP, Domaine de la Motte BP 35327, F-35653 Le Rheu, France

<sup>3</sup> USTL, UMR 1281 SADV, Bâtiment SN2, F-59655 Villeneuve d'Ascq, France

<sup>4</sup> INRA, UMR 1334 AGAP, 2 place Viala, F-34060 Montpellier cedex2, France

<sup>a</sup> Komlan Avia's present address: University of Oulu, Department of Biology, Plant genetics group, PO Box 3000, FIN-90014 Oulu, Finland

**\*Corresponding author : Isabelle Lejeune-Hénaut**

**e-mail : [Isabelle.Lejeune@mons.inra.fr](mailto:Isabelle.Lejeune@mons.inra.fr)**

**telephone number : 33 3 22 85 75 07**

**fax number : 33 3 22 85 69 96**

1   **Abstract**

2   Freezing is a major environmental limitation to crop productivity for a number of  
3   species including legumes. We investigated the genetic determinism of freezing  
4   tolerance in the model legume *Medicago truncatula* Gaertn (*M. truncatula*). After  
5   having observed a large variation for freezing tolerance among fifteen *M. truncatula*  
6   accessions, the progeny of a F6 recombinant inbred line (RIL) population, derived  
7   from a cross between two accessions, was acclimated to low above freezing  
8   temperatures and assessed for: a) number of leaves (NOL), leaf area (LA), chlorophyll  
9   content index (CCI), shoot and root dry weights (SDW and RDW) at the end of the  
10   acclimation period and b) visual freezing damage (FD) during the freezing treatment  
11   and two weeks after regrowth and foliar electrolyte leakage (EL) two weeks after  
12   regrowth. .Consistent QTL positions with additive effects for FD were found on LG1,  
13   LG4 and LG6, the latter being the most explanatory ( $R^2 \approx 40\%$ ). QTL for NOL, QTL  
14   for EL, NOL and RDW, and QTL for EL and CCI colocalized with FD QTL on LG1,  
15   LG4 and LG6 respectively. Favorable alleles for these additive effects were brought  
16   by the same parent suggesting that this accession contributes to superior freezing  
17   tolerance by affecting plants capacity to maintain growth at low above freezing  
18   temperatures. No epistatic effects were found between FD QTL but for each of the  
19   studied traits, 3 to 6 epistatic effects were detected between loci not detected directly  
20   as QTL. These results open the way to the assessment of syntenic relationships  
21   between QTL for frost tolerance in *M. truncatula* and cultivated legume species.

22

23   **Key words**

24   *Medicago truncatula*, freezing tolerance, QTL, epistasis, electrolyte leakage,  
25   chlorophyll content index.

## 26    **Introduction**

27    Freezing temperature is a major environmental limitation to the distribution of plant  
28    species and to crop productivity (Levitt 1980). Among cultivated plants, the legume  
29    crops are also often constrained by negative temperatures. This is particularly the case  
30    for cool season grain legumes (pea, faba bean, lentil, chickpea) for which autumn  
31    sowings are being developed in order to benefit from a higher biomass production as  
32    well as to avoid drought and heat stresses of late spring (Stoddard et al. 2006). Winter  
33    hardy cultivars, able to survive freezing temperatures and other associated winter  
34    stresses are thus desirable.

35    For legumes, as for other crops, selection for winter hardiness (WH) is generally done  
36    by evaluating breeding nurseries in the field for several years. This evaluation is based  
37    on the observation of winter damages and/or winter survival, which are largely  
38    attributable to insufficient freezing tolerance (FT). Screening tests in controlled  
39    conditions have also been developed to assess FT itself. Such experiments are  
40    complementary to field experiments, because they allow the independent assessment  
41    of freezing tolerance without the confounding effect of other winter stresses such as  
42    low temperature pathogens.

43    Considered as a valuable basis for marker assisted selection, mapping of Quantitative  
44    Trait Loci (QTL) has also been helpful to gain insight into the genetic determinism of  
45    both winter hardiness and freezing tolerance for grain legume species. Under field  
46    conditions, QTL affecting WH have been detected for lentil (Kahraman et al. 2004)  
47    and pea (Lejeune-Hénaut et al. 2008), and also for other important crops such as  
48    cereals (barley: Pan et al. 1994; Francia et al. 2004; wheat: Börner et al. 2002; oat:  
49    Wooten et al. 2009), forage grasses (ryegrass: Xiong et al. 2007; alfalfa: Brouwer et  
50    al. 2000) and rape (Asghari et al. 2008). Significant positive correlations between WH

51 and FT have been reported in various species and FT QTL with coincidental positions  
 52 with WH QTL have been identified (barley: Francia et al. 2004; wheat: Baga et al.  
 53 2007; oat: Wooten et al. 2009; ryegrass: Xiong et al. 2007; pea: Dumont et al. 2009).  
 54 Auxiliary traits have also been taken into consideration to help evaluating and/or  
 55 resolving FT into physiological related components. Although FT can be assessed at  
 56 the whole plant scale through a visual scoring of frost damages exhibited by the aerial  
 57 organs of the plants or by the proportion of plants surviving freezing exposure, it can  
 58 also be indirectly characterized by the measurement of tissues electrolyte leakage  
 59 (potato: Stone et al. 1993; rape: Teutonico et al. 1995; Arabidopsis: Jaglo-Ottosen et  
 60 al. 1998; alfalfa: Brouwer et al. 2000; barley: Zhu et al. 2000; ryegrass: Xiong et al.  
 61 2007; pea: Dumont et al. 2009). Quantitative Trait Loci controlling the variation of  
 62 electrolyte leakage have been identified for alfalfa (Brouwer et al. 2000), ryegrass  
 63 (Xiong et al. 2007) and pea (Dumont et al. 2009). In addition, QTL for other auxiliary  
 64 traits associated with cold acclimation have also been considered to elucidate the  
 65 genetic bases of FT. These include developmental stage (rape: Asghari et al. 2008;  
 66 pea: Dumont et al. 2009), fatty acid composition (faba bean: Arbaoui et al. 2008),  
 67 crown fructan content, accumulation of COR proteins or maximum quantum yield of  
 68 the Photosystem II photochemistry (barley: Hayes et al. 1993; Francia et al. 2004),  
 69 sugar content or RuBisCO activity (pea: Dumont et al. 2009). For all these traits,  
 70 overlapping confidence intervals or coincident positions with FT and/or winter  
 71 survival QTL have been reported. Whether these common positions are only due to  
 72 genetic linkage or reflect the effect of a pleiotropic locus is still unknown.

73 *Medicago truncatula* Gaertn (*M. truncatula*) is a model legume that shows a high level  
 74 of synteny with pea (Choi et al. 2004b; Aubert et al. 2006) and has a genome which  
 75 sequence is under completion (Young et al. 2005; 2011). Despite origin and cropping

76 regions restricted to Mediterranean climates, freezing tolerance variability has already  
77 been reported in *M. truncatula* (Hekneby et al. 2001; Brandsæter et al. 2002;  
78 Pennycooke et al. 2008). However information is still lacking about the localization of  
79 the genome regions controlling this trait. Identifying such genetic factors for FT in *M.*  
80 *truncatula* should therefore enhance the understanding of FT in legumes and facilitate  
81 applications towards cultivated species.

82 The objectives of this project were to (a) determine the extent of genetic variability for  
83 freezing tolerance in a range of accessions of *M. truncatula* representing broad  
84 geographic origins and to (b) detect and relate QTL for FT and physiological traits  
85 known to be associated to cold acclimation among a population of recombinant inbred  
86 lines (RILs), in order to improve the understanding of the genetic basis of tolerance to  
87 subfreezing temperatures in this model legume.

88

## 89 **Materials and methods**

### 90 ***Plant materials: collection lines, parental lines and recombinant inbred lines***

91 A set of 15 *M. truncatula* accessions was tested for freezing tolerance. They included  
92 (a) 11 inbred lines, among which 8 constituting the *M. truncatula* core collection 8  
93 (CC8) that matches broad geographic origins (Ronfort et al. 2006), (b) the reference  
94 line Jemalong A17 and (c) three additional accessions chosen for particular  
95 characteristics (Table 1): DZA220-H is a population belonging to the tricycla  
96 subspecies of *M. truncatula*, ESP096-A is an inbred line which was collected from a  
97 high altitude site in the south of Spain and F34042-D is a population which was  
98 collected from one of the highest latitude site in the north of Montpellier (France). Two  
99 inbred line accessions, DZA045-5 (used as male), originating from Algeria and  
100 F83005-5 (used as female), originating from Salernes, France, were crossed to

101 generate a segregating population of 178 F6-derived recombinant inbred lines (namely  
102 LR3) produced by single seed descent. DZA045-5 was not tested among the  
103 collections lines but is closely related to DZA045-6. Both lines were derived from the  
104 same population and no molecular difference was found between them (Prosperi,  
105 unpublished results). The frost tolerance level of DZA045-5 can thus be inferred from  
106 that of DZA045-6. All the plant material was provided by INRA of Mauguio  
107 (Montpellier, France: <http://www1.montpellier.inra.fr/BRC-MTR/>).

108

### 109 ***Culture conditions***

#### 110 *Germination*

111 After scarification with sandpaper, seeds were placed in 2ml deionised water-  
112 containing Eppendorf tubes for 6-hours imbibition at room temperature. Seeds were  
113 then spread on wet filter paper-containing Petri dishes. To break dormancy and allow  
114 rapid and synchronized germination, the Petri plates were maintained at 4°C in the  
115 dark for 3 days. They were then transferred to 20°C under natural photoperiod and  
116 were planted after 2 days.

117

#### 118 *Sowing, temperature, light intensity and photoperiod settings*

119 Plantlets were placed in home-made polystyrene blocks, of 100 or 50 holes each, in  
120 order to protect roots from freezing injury based on previous observations of their  
121 greater sensitivity to frost than shoots (Chen et al. 1983). Each hole (diameter: 41mm;  
122 height: 80mm) was half filled with perlite, to promote the development of roots, topped  
123 with a well-moistened (water-saturated for up to 3 hours) Jiffy-7 pellet (diameter:  
124 41mm ; height when fully moisture: 40mm) made of sphagnum peat  
125 (<http://www.jiffygroup.com/jiffy/catalogue>). Lime and a special fertilizer with low

126 ammonium content were pre-added to the peat in order to stimulate growth. One  
127 germinated seed was inserted in each pellet and was lightly watered to allow a firm  
128 anchoring in the substrate. During the establishment phase, the seedlings were  
129 frequently watered with deionised water to avoid desiccation of the roots. To prevent  
130 a premature yellowing of the leaves, about 10 ml fertilizer was added in the pellets.  
131 This was performed about once a week by using the commercial liquid fertilizer NPK  
132 6:3:6 mixture supplemented with micronutrients (Substral®, SCOTTS), according to  
133 European Molecular Biology Organization practical course available at  
134 <http://www.isv.cnrs-gif.fr/embo01/manuels/index.html>.  
135 The different experimental phases, i.e. establishment, cold acclimation, freezing and  
136 rewarming, were conducted as presented in Table 2. The cold acclimation phase was  
137 carried out following two steps as inspired by Thapa et al. (2008) to prevent plants  
138 from a cold shock upon transfer from 20°C/14°C day/night to 8°C/2°C. Experiments  
139 were performed in a growth chamber (DAGARD; size: 3.2 x 3.2 x 2.4m; type: MA  
140 100, class M1) lighted by a combination of two types of PHILIPS lamps: HPI-T400W  
141 (metal iodide) and SON-TP400W (high-pressure sodium), with a mean photosynthetic  
142 active radiation (PAR) of 450  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ . Plant material was grown in controlled  
143 conditions during three series of experiments (and three individual experiments in each  
144 series), the first one including the collection lines and the second and third ones  
145 comprising the parental lines DZA045-5 and F83005-5, and their recombinant inbred  
146 progenies (LR3). For each of the three experiments dedicated to the evaluation of  
147 visual freezing damages of the collection lines, each line was represented by 50  
148 individuals allocated to 5 blocks. Three experiments were also carried out to evaluate  
149 visual freezing damages on the parental and recombinant lines, in which each line was  
150 represented by 15 individuals allocated to 3 blocks. Lastly, for each of the three



151 experiments conducted to observe physiological traits on the parental and recombinant  
152 lines, each genotype was represented by 15 individuals allocated to 3 blocks.

153

#### 154 ***Visual freezing damage scoring***

155 The levels of freezing damage (FD) were recorded twice during the freezing phase,  
156 after one and eight days of freezing (FD1D and FD8D, respectively), and once after  
157 two weeks under rewarming conditions (FD2W). Plants were individually given a  
158 score from 0 (no damage) to 5 (dead). The plants were scored 1 for frost burn limited  
159 to the leaf edges, 2 for frost burn on the majority of the leaf surface, 3 for frost burn  
160 on upper third of the stem, 4 for frost burn on upper three-quarters of the stem, as  
161 described for pea by Lejeune-Hénaut et al. (2008).

162

#### 163 ***Growth characterization***

164 Four traits characterizing plant growth were measured at the end of the acclimation  
165 phase. Plant developmental stage (number of leaves, NOL) was determined by  
166 counting all visible trifoliate leaves weighted by their folding decimal code (Moreau  
167 et al. 2006). Leaf area (LA) was determined by taking pictures of detached leaves  
168 spread out on a white paper sheet and analyzing them with the OPTIMAS 6.5 image  
169 processing program (Media Cybernetics, Silver Spring, MD). Shoot and root dry  
170 weights were determined after oven-drying at 70°C for 72h.

171

#### 172 ***Chlorophyll content assay***

173 A non-destructive measurement of the chlorophyll content was realized at the end of  
174 the acclimation phase with a hand-held chlorophyll content meter CCM-200 (Opti-  
175 Sciences, Hudson, NH, USA) which provides an index (chlorophyll content index,

176 CCI) estimating the leaf chlorophyll content (Richardson et al. 2002; Biber 2007). It  
 177 was performed on five leaves per plant and three times on each leaf.

178

### 179 *Electrolyte leakage assay*

180 This assay was performed on foliar organs that underwent the freezing period,  
 181 excluding newly expanded leaves, if any. Three trifoliate leaves of each plant sampled  
 182 after 2 weeks of the rewarming phase were detached and placed in a 50ml tube  
 183 containing 25 ml of ultra-pure water. The tubes were kept at room temperature for  
 184 about 20h; they were shaken before measuring the conductivity of the solution (C1)  
 185 with an ION check30 conductivity meter (Radiometer Analytica). The tubes were then  
 186 frozen for a minimum of 1 day at -80°C and thawed over night at room temperature.  
 187 Thereafter they were shaken and the total potential conductivity (C2) was measured.  
 188 The mean electrolyte leakage (EL) was calculated as a percentage of the total leakage  
 189 from frozen samples (C1/C2 x 100).

190

### 191 *Statistical and quantitative genetic analyses*

#### 192 *Quantitative genetic analyses*

193 Statistical analyses were performed with SAS statistical package v8.01 procedures  
 194 (SAS Institute Inc., Cary, NC, USA; 2000). For each trait, the normality of residual  
 195 distributions was tested with Skewness, Kurtosis and Shapiro-Wilk statistics displayed  
 196 by the “univariate” procedure. For QTL mapping, we estimated RILs best linear  
 197 unbiased predictors (BLUPs) for each trait, and from the data recorded from the 3  
 198 replicated experiments, using the SAS “mixed” procedure.

199 Broad-sense heritabilities were assessed for the mapped traits using the linear model:

$$200 \quad Y_{ijk} = \mu + E_i + G_j + rep_{(i)_k} + GE_{ij} + \varepsilon_{ijk}$$

201 where  $Y_{ijk}$  represents the value of the trait under investigation for the genotype  $j$  at the  
 202 replication  $k$ ,  $E_i$  represents the fixed effect of experiment replicate  $i$ ,  $G_j$  represents the  
 203 random effect of genotype  $j$  and  $rep_{(i)k}$  the mean fixed effect of block  $k$  within  
 204 experiment  $i$ .  $GE_{ij}$  represents the random interaction effect between experiment  $i$  and  
 205 genotype  $j$ , and  $\varepsilon_{ijk}$  is the random error term of the model. Estimates of variances were  
 206 obtained from the model and heritability was calculated as  $H^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_e^2 / J)$   
 207 where  $\sigma_G^2$  represents the genetic variance,  $\sigma_e^2$  the environmental variance and  $J$  the  
 208 number of replications. Heritabilities and their standard errors were calculated using  
 209 the SAS “mixed” and “iml” procedures, according to Holland et al. (2003). Phenotypic  
 210 correlations were estimated between all traits BLUPs using the SAS “corr” procedure.  
 211 Genotypic correlations and their standard errors were calculated using multivariate  
 212 restricted maximum likelihood estimation with SAS “mixed” procedure, according to  
 213 (Holland 2006).

214

#### 215 *Genetic linkage map*

216 Types of markers and their genotyping procedures have been described in Gorton et  
 217 al. (2012). Genetic linkage analyses were performed using MAPMAKER/EXP,  
 218 version 3.0 (Lincoln et al. 1992), with a LOD score threshold of 3.0 and a  
 219 recombination frequency  $\leq 0.4$ , as described in Hamon et al. (2010). Marker order was  
 220 established using the ‘order’ command. CentiMorgan distances were calculated with  
 221 the Kosambi function.

222

#### 223 *QTL and epistasis analyses*

224 QTL analyses were performed with Windows QTL Cartographer 2.5 (Wang et al.  
 225 2012). QTL were initially detected with composite interval mapping (CIM) using

226 model 6. For each trait, the LOD significance threshold was determined after running  
 227 1000 permutations test ( $\alpha=0.05$ ). Cofactors were selected with the Forward and  
 228 Backward regression method.  
 229 QTL were subsequently refined using multiple interval mapping (MIM) which fits a  
 230 multiple QTL model to increase precision in the QTL detection. This was especially  
 231 important in cases where multiple LOD peaks were detected with CIM. We used the  
 232 CIM QTL peaks as starting positions for the MIM and progressively refined the model  
 233 using the  $BIC-M2 = 2\ln(n)$  criterion. QTL positions that did not remain significant  
 234 when fitted with others were then dropped from the model. QTL effects and their  
 235 percentage of variance were estimated with the final model fitted in MIM.  
 236 For each QTL, the inner and outer limits of the confidence interval corresponded to  
 237 LOD value decreases by 1 and 2 units respectively, relatively to the LOD value of the  
 238 most likely position. Mapchart 2.2 (Voorrips 2002) was used to draw the QTL on the  
 239 linkage map.  
 240 QTL by QTL additive epistatic interactions were first detected for the significant QTL  
 241 using MIM in Windows QTL Cartographer 2.5. To detect all pairwise additive  
 242 epistatic interactions without prior knowledge of QTL positions, we used the program  
 243 Epistacy (Holland 1998), which relies on linear models and least squares statistics  
 244 from SAS system. Epistacy was run for all traits with an initial P-value filter of 0.001.  
 245 All interactions involving markers within 50 cM distance were removed to avoid  
 246 linkage effects. To control for multiple comparisons, we used a final P-value cutoff of  
 247  $3.57E-4$  obtained by dividing an initial P-value of 0.01 by a factor of  $g(g-1)/2$  where  
 248  $g$  is the number of linkage groups, as suggested by Holland (1998).

249

## 250 **Results**

251 *Characterization of frost tolerance within 15 M. truncatula accessions*

252 Freezing visual damage was scored two weeks after freezing treatment and the fifteen  
253 *M. truncatula* accessions ranged from 1.8 (F11005-E) to 4 (DZA012-J) for FD (Table  
254 1). Accessions originating from higher latitudes were globally the most tolerant (*f*  
255 group according to the SNK test for comparison of means, Table 1), which may reflects  
256 the adaptation tendency of accessions having evolved in geographical areas  
257 characterized by more frequent, even if not very intense, frost events. F83005-5 was  
258 an exception within the group of high latitude accessions, exhibiting a medium level  
259 of frost tolerance (FD = 3). Comparatively, DZA045-6 was one of the most sensitive  
260 accessions (*a* group according to the SNK test for comparison of means, Table 1).

261

262 *Quantitative genetics*

263 All traits studied showed a normal Gaussian distribution within the RIL population  
264 (Fig. 1), except EL for which the distribution of the RILs was shifted towards the mean  
265 value of DZA045-5, the sensitive parental line. Box-Cox transformation did not  
266 improve significantly the distribution. The frequency distributions also showed that,  
267 in many cases, there was limited difference between both parents of the RILs.  
268 Nevertheless, transgressive individuals were found in the population and related QTL  
269 were detected.

270 Significant genetic variation was detected for all traits and the coefficient of genetic  
271 variation varied between 3.23 for FD8D and 35.14 for RDW (Table 3). Heritability  
272 values ranged from 0.43 to 0.89 with the highest values for CCI (0.61), FD2W (0.72)  
273 and EL (0.89). Furthermore, the high heritability of FD2W also indicated that this trait  
274 is appropriate to select for freezing tolerant lines.

275 Phenotypic correlations (Table 4) were not significant between FD or EL on the one  
276 hand and other physiological traits (NOL, LA, CCI, SDW, RDW) on the other hand.  
277 Genotypic correlations (Table 4) were low but consistently negative, with higher  
278 values of NOL, LA, CCI, SDW and RDW being associated with lower values of FD  
279 and EL.

280

#### 281 *Genetic linkage map and QTL mapping*

282 The genetic linkage map of the LR3 population comprised a total of 182 markers,  
283 including 111 SSR, 57 AFLP, 12 RAPD and 2 EST-CAPS, distributed over 8 linkage  
284 groups (LG, Fig. 2). The linkage map covered 1225 cM Kosambi (1 marker every 6.7  
285 cM, on average). Thirty-five SSR markers were anchored to sequenced BACs and 29  
286 were common to the integrated genetic-physical map  
287 (<http://www.medicago.org/genome/map.php>, Univ. Minn. 2006; Mun et al. 2006).  
288 Order conservation of these 29 common markers between the present genetic map and  
289 the reference *M. truncatula* genetic and physical maps was very high. Few genomic  
290 regions remained poorly covered by the genetic linkage map, with the largest interval  
291 between two markers in the middle region on LG8.

292 The QTL detected by the CIM and refined by MIM are presented in Fig. 2 and Table  
293 5. We detected in total 29 QTL with 1 to 5 QTL per trait. Most of them lay on three  
294 linkage groups (LG1, LG4 and LG6). Those regions can roughly be delimited between  
295 markers MTIC125 and MTIC064 (37 cM) on LG1, between L07.600 and MTIC037  
296 (12 cM) on LG4 and between E39M61.511 and E12M49.265 (8 cM) on LG6. Two  
297 linkage groups (LG3 and LG8) had no QTL and only one QTL was observed on  
298 linkage groups LG2, LG5 and LG7.

299

300 *QTL for freezing damage*

301 QTL for freezing damage with overlapping confidence intervals were detected for  
302 FD8D and FD2W on LG1, LG4 and LG6. Those traits had also the most explanatory  
303 QTL, with a proportion of phenotypic variance  $R^2$  of almost 40% on LG6. FD1D had  
304 only one QTL on LG6 ( $R^2 = 12.4\%$ ) which did not overlap with any other FD QTL.  
305 All the FD QTL had a negative value of the additive effect for F83005-5, indicating  
306 that the F83005-5 alleles contributed to increase frost tolerance and are thus favorable  
307 alleles for this trait. Taking into account the results from analysis of variance,  
308 heritability and correlations, we found that the best period to assess the variability of  
309 FT within the RIL population was after two weeks of rewarming, rather than during  
310 the frost period itself. The QTL detection and additive effects (much higher for FD2W)  
311 further confirmed this tendency.

312

313 *QTL for physiological traits*

314 Seventeen among the 19 QTL for physiological traits lay on LG1, LG4 and LG6, and  
315 6 of them overlapped with FD QTL (Fig. 2). EL QTL colocalized with the most  
316 explanatory FD QTL on LG4 and LG6, with an  $R^2$  value of 18.8% on the latter.  
317 Additive effects of EL (Table 5) showed that the F83005-5 alleles were favorable for  
318 all the positions, i.e. corresponded to lower loss of electrolytes from foliar tissues.  
319 Among the five QTL detected for CCI, one also colocalized with the FD QTL on LG6  
320 with an  $R^2$  value equal to 4.9% and a positive F83005-5 additive effect.  
321 Overlapping confidence intervals between FD and physiological traits QTL were also  
322 found on LG4, where the positions of FD8D and FD2W QTL coincided with those of  
323 QTL for EL ( $R^2=5.1$ ), RDW ( $R^2=5.4\%$ ) and NOL ( $R^2=9.2\%$ ). For these three traits,  
324 the values of the additive effects of F83005-5 were positive.

325 On LG1, overlapping confidence intervals were observed between FD8D, FD2W and  
326 NOL, with an  $R^2$  value of 8.1% for the latter and a positive additive effect of F83005-  
327 5.

328 Among the QTL for physiological traits that did not colocalize with FD QTL, a  
329 particular position was observed on LG1, where QTL for EL ( $R^2=3.7$ ), CCI ( $R^2=13.3$ ),  
330 LA ( $R^2=16.4$ ), SDW ( $R^2=6.5$ ), RDW ( $R^2=9.5$ ) overlapped.

331 Phenotypic correlations were not significant between FD or EL on the one hand and  
332 other physiological traits (NOL, LA, CCI, SDW, RDW) on the other hand. Genotypic  
333 correlations were low but consistently negative, suggesting that higher values of NOL,  
334 LA, CCI, SDW and RDW are generally associated with lower values of FD and EL,  
335 i.e. higher freezing tolerance. This is consistent with the signs of the additive effects  
336 observed at the positions where QTL for freezing damage traits (FD or EL) colocalized  
337 with physiological traits (NOL, CCI, RDW).

338

#### 339 *Epistatic interactions*

340 Running the program Epistacy enabled the identification of 88 significant results with  
341 3 to 22 interactions per trait. Keeping only one representative marker with the lowest  
342 P-value when significant interactions involved several very close markers for the same  
343 traits allowed us to further filter the results. Final results with 38 significant additive-  
344 by-additive epistatic interactions from Epistacy output are presented in Online  
345 Resource 1. We detected 3 to 6 significant interactions per trait. Only 2 interactions  
346 were detected between QTL pairs and both were related to CCI. One of them (QTL on  
347 LG6 and LG1) was also detected by MIM in Windows QTL Cartographer. For both  
348 of these CCI x CCI interactions, one or two doses of the F83005-5 allele corresponded  
349 to higher CCI genotypic means (Online Resource 1). Four interactions were detected



350 between a QTL (CCI on LG6, LA and RDW on LG4 and NOL on LG7) and a locus  
351 of the genetic background. Finally 32 interactions were detected between loci of the  
352 genetic background. Partial  $R^2$  values varied between 6.3% and 12.7%.

353 Three loci, namely MTB14 on LG3, MTIC059 and G17.1300 on LG5, were involved  
354 in 6, 5 and 4 epistatic interactions, respectively, suggesting pleiotropic effects on  
355 FD1D, FD8D, FD2W, EL and RDW for MTB14, FD1D, FD8D, FD2W and EL for  
356 MTIC059 and on FD2W, EL, NOL and RDW for G17.1300. MTIC059 and MTB14  
357 were involved four times in the same epistatic interaction. On FD traits and EL, one  
358 or two DZA045-5 alleles at interacting loci implying one or two of these markers were  
359 associated with higher values of frost damages and foliar electrolyte leakage.

360

## 361 **Discussion**

### 362 *Genetic variability for freezing tolerance*

363 The results obtained for 15 accessions of diverse geographical origins (Table 1)  
364 pointed out a relatively large genetic variability in the *M. truncatula* tolerance to  
365 subfreezing temperatures after a cold acclimation phase. These results demonstrated a  
366 global relationship between the geographical origin of the accessions and their freezing  
367 tolerance. Most of the accessions originating from lower latitudes (Algeria, Cyprus,  
368 Syria) were found to be more sensitive than those from France. However, no clear  
369 relationship was found between freezing tolerance and the altitude of the area of origin,  
370 which is illustrated by the fact that both high and low collection sites were represented  
371 for sensitive lines from the same latitude (DZA.233-4 and DZA045-6, respectively).  
372 The influence of the geographical origin of plants on their level of freezing tolerance  
373 has been documented in various species. It is indeed frequently reported, within a  
374 given species, that ecotypes from northern locations, which generally experiment

375 lower winter temperatures, present superior levels of freezing tolerance. This statement  
376 is generally verified for herbaceous species (Finne et al. 2000; Hamilton et al. 2002;  
377 Nakayama et al. 1997; Rukavina et al. 2007; Leinonen et al. 2011), although it can be  
378 disturbed by the presence of snow which probably reduces the selection pressure  
379 exerted by low temperatures (Dionne et al. 2010). The altitude effect has also  
380 sometimes been evidenced (Annicchiarico et al. 2001; Loik and Redar 2003), the high  
381 altitudinal ecotypes being more tolerant to frost. A similar relationship between  
382 altitudinal provenance and freezing damage was also observed in four other *Medicago*  
383 species (Yahia and Fyad-Lameche 2003). Screening of a collection of *M. truncatula*  
384 including accessions from a broader range of altitude may be required to confirm our  
385 preliminary observations on 15 accessions.

386

#### 387 *Genetic linkage map in M. truncatula*

388 The linkage map of LR3 covered 1225 cM Kosambi, which is higher than the size of  
389 genetic maps previously established from A17 x A20 F2 populations (513 cM in Choi  
390 et al. 2004a; 566 cM in Mun et al. 2006). But this is consistent with genetic map lengths  
391 previously reported for Jemalong-6 x DZA.315-16 F2 or F6-RIL populations (1225  
392 cM and 1097 cM in Thoquet et al. (2002) and Huguet (2004), respectively) as well as  
393 for the LR3 RIL population by Gorton et al. (2012). Several of the SSR markers were  
394 located in sequenced BACs or common with the genetic-physical map of the species.  
395 Those markers are likely to facilitate the exploration of interesting regions detected by  
396 QTL mapping.

397

#### 398 *QTL for freezing tolerance and related physiological traits in M. truncatula*

399 Results obtained for QTL mapping in the LR3 population showed that freezing  
400 tolerance in *M. truncatula* is controlled by a limited number of QTL mainly  
401 colocalizing in three regions on LG1, LG4 and LG6. Even if it can partly be due to the  
402 limited contrast between parents (Table 1), this observation is also consistent with the  
403 relatively low number of QTL generally found for traits reflecting FT or WH in legume  
404 species. In alfalfa, Brouwer et al. (2000) identified seven QTL for winter injury, among  
405 which only two were consistent among the two years of experimentation. In lentil,  
406 Eujayl et al. (1999) found that the frost tolerance trait, evaluated in field conditions,  
407 was inherited as a 1:1 ratio of tolerant to sensitive in a population of RILs, suggesting  
408 monogenic inheritance. In a different genetic background of lentil, Kahraman et al.  
409 (2004) detected one to three QTL of winter survival in different field environments,  
410 with one QTL being common to all environments. In faba bean, three and two QTL  
411 were found for frost tolerance in controlled conditions for unhardened and hardened  
412 plants respectively (Arbaoui et al. 2008). In pea, Liesenfeld et al. (1986) estimated that  
413 as few as three additive genes or tight linkage groups may condition winter hardiness  
414 in the progenies they experimented and Lejeune-Hénaut et al. (2008), studying winter  
415 freezing damages in a population of RILs, detected six QTL among which three were  
416 identified for all their field experiments. These three consistent QTL coincided with  
417 three (out of four) QTL for freezing damages assessed for the same population under  
418 controlled conditions (Dumont et al. 2009). In other plant families, QTL analysis for  
419 frost tolerance also suggested that a limited number of regions of the genome were  
420 involved (review on Triticeae: Cattivelli et al. 2002; oat: Wooten et al. 2009 ; ryegrass:  
421 Xiong et al. 2007 ; rapeseed: Kole et al. 2002; Asghari et al. 2008). When such QTL  
422 positions are consistent among environmental conditions and genetic backgrounds  
423 they constitute valuable targets for marker assisted selection. The results of the present

study are a first insight in the genetic determinism of frost tolerance in the model legume *M. truncatula* which could help to identify candidate genes underlying QTL and benefit to closely related cultivated species like pea or faba bean, provided that syntenic QTL are identified. As an example, Tayeh et al. (2013) have recently shown that the major frost tolerance QTL identified on *M. truncatula* linkage group 6 in the present study was syntenic to the frost tolerance QTL identified on pea linkage group 6 by Lejeune-Hénaut et al. (2008) and Dumont et al. (2009). Based on the existing variability of this trait within the *M. truncatula* species (Table 1), it would be worth to study a segregating population from a cross showing a wider phenotypic variation for the freezing tolerance traits, for instance between F20026 or F11005-E and DZA045-5, in order to further confirm and/or to identify additional QTL that affect freezing tolerance.

QTL for physiological traits such as electrolyte leakage (EL), number of leaves (NOL), leaf area (LA), chlorophyll content index (CCI) or root dry weight (RDW) colocalized with QTL for FD in genetic regions that can roughly be delimited between markers MTIC125 and MTIC146 (24 cM) on LG1, between L07.600 and MTIC037 (25.1 cM) on LG4 and between E39M61.511 and E12M49.265 (7.7 cM) on LG6. The fact that genotypic correlations between FD or EL on the one hand and other physiological traits on the other hand were negative, suggests that higher values of NL, LA, CCI, SDW and RDW are associated with lower values of FD and EL, i.e. higher freezing tolerance. This is consistent with the signs of the additive effects observed at the positions where QTL for freezing damage traits (FD or EL) colocalized with QTL for physiological traits (NOL, CCI, RDW).

448 Leakage of ions, evaluated by the conductivity test, reflects freezing damages to cell  
449 membranes and its post freezing measure is generally used as an indirect indicator of  
450 freezing injury (Dexter et al. 1930; Levitt 1980). QTL for EL have been reported to  
451 colocalize with QTL for FD in pea (Dumont et al. 2009) and with QTL for winter  
452 injury or winter survival in alfalfa (Brouwer et al. 2000) and ryegrass (Xiong et al.  
453 2007) respectively. Even if the loss of cell membrane permeability may be reversible  
454 depending on the degree of injury (Palta and Li 1980), it has been shown on *Medicago*  
455 *sativa* that a cellular rupture actually results from a -8°C freezing treatment (Sulc et al.  
456 1991a). On the same species, EL measurements on roots progressively increased with  
457 decreasing freezing temperatures, except for the most sensitive genotypes which tend  
458 to plateau from -6°C to -10°C (Sulc et al. 1991b). For *M. truncatula*, data presented  
459 on LR3 resulted in a coefficient of genotypic correlation of 68% between EL and  
460 FD2W (Table 4), which is partly reflected by the colocalization of QTL for both traits  
461 on LG4 and LG6. The relative ranking of the parents of LR3 for EL, i.e. 85% after 2  
462 weeks of rewarming for the freezing sensitive DZA045-5 against 39% for the freezing  
463 tolerant F83005-5, as well as the sign of the additive effects of EL QTL indicate that  
464 F83005-5 is probably able to limit irreversible freezing damages on cell membranes  
465 and that this ability is partly transmissible to its progeny.

466 QTL mapping for NOL and CCI suggests that frost tolerance is improved when the  
467 photosynthetic apparatus presents sufficient size and efficiency at the end of the  
468 acclimation phase, as shown by the colocalizations between FD QTL and NOL on  
469 LG1 and LG4 and between FD QTL and CCI on LG6. In pea, Lejeune-Hénaut et al.  
470 (2010) have already observed that pea seedlings must have a minimum number of  
471 leaves (2 or 3) to resist frost. Dumont et al. (2009) showed that pea plantlets  
472 characterized by more numerous branches at the end of the acclimation phase in

473 controlled conditions were more tolerant to frost, with QTL for the corresponding trait  
474 coinciding with two of the most consistent QTL for frost tolerance. Ashgari et al.  
475 (2008) found a single minor effect QTL for winter survival of rapeseed which  
476 coincided with a QTL for the number of leaves at the rosette stage. For both traits, the  
477 favorable alleles were brought by the tolerant parent. In *M. truncatula*, QTL for the  
478 number of leaves and aerial fresh weight under salt stress were detected on the linkage  
479 group 6 within the population Jemalong A17 x F83005-5 (Arraouadi et al. 2011), at a  
480 position that would deserve to be more precisely compared to the LR3 genetic map,  
481 thanks to common markers which are currently lacking.

482 Coincidental QTL mapping for RDW and FD on LG4 suggests that superior allocation  
483 of dry matter to the root system may indirectly contribute to superior freezing tolerance  
484 in *M. truncatula*. Alternatively, this may reflect a superior potential for cold  
485 acclimation which allows the accumulation of C and N metabolites that could help  
486 sustain a more vigorous regrowth after freezing. A higher partitioning of dry matter in  
487 roots than in shoots under low temperatures (LT) has already been reported in *M.*  
488 *truncatula*. Thapa et al. (2008) observed that growing under LT regimes,  
489 comparatively to control conditions, resulted in a global reduction of dry matter for  
490 Jemalong 6 and W6 5018, together with an increase of the root:shoot ratio for both  
491 genotypes. The growth of Jemalong 6 under LT was however less reduced than for  
492 W6 5018 and this genotype especially proved to be able to produce the same amount  
493 of root dry weight (RDW) under LT than under control conditions. The authors related  
494 this behaviour to the higher frost tolerance exhibited by Jemalong 6 after a LT period,  
495 highlighting its greater cold acclimation ability. In pea, Lejeune-Hénaut et al. (2010)  
496 showed that dry matter production was higher under LT conditions than under control  
497 for Champagne and Terese, and that a higher root:shoot ratio was also observed under

498 LT, particularly for the frost tolerant genotype Champagne. Similar effects of LT, i.e.  
499 higher DW production and higher root:shoot ratio were also reported in other species  
500 like wheat (Equiza et al. 1997) with higher values observed for the most frost tolerant  
501 genotypes.

502 It seems finally that F83005-5 is able to produce more leaves, to maintain its  
503 photosynthetic ability to a higher level and to allocate more dry matter to roots during  
504 acclimation to low non-freezing temperatures. The plant capacity to acclimate and  
505 maintain growth at low above freezing temperatures would allow superior tolerance to  
506 freezing and a more vigorous regrowth upon return to warmer temperatures. To which  
507 extent the ability to acclimate and maintain growth at low temperatures is involved in  
508 the development of superior cryoprotection, which is suggested by limited damage on  
509 cell membranes, has not been tested here.

510 A fine mapping approach is necessary, at least for the most explanatory frost tolerance  
511 QTL to determinate whether the coincident positions of the QTL are due to single loci  
512 with pleiotropic effect or to genetic linkage.

513

#### 514 *Epistatic interactions*

515 Epistatic interactions among genes can be important for phenotypic expression or  
516 genotypic variation in populations. In *M. truncatula*, such interactions have already  
517 been reported, within the LR3 population, for traits related to resistance to  
518 *Aphanomyces euteiches* (Hamon et al. 2010). In this study, we detected 38 epistatic  
519 interactions for FD traits or potentially related physiological traits. Most of these  
520 interactions (32) were detected between markers of the genetic background, a higher  
521 number than for resistance to *Aphanomyces euteiches*. For this trait, Hamon et al.  
522 (2010) found only 4 interactions between genomic regions that did not have an additive

523 effect on resistance to *Aphanomyces euteiches*, among 12 identified in total. In  
524 particular in the present study, no epistatic interaction was directly associated to FD or  
525 EL QTL, but 19 significant epistatic interactions were identified for these traits  
526 between markers of the genetic background and cannot be neglected. Attention should  
527 be especially paid to loci such as MTB14 on LG3, MTIC059 and G17.1300 on LG5,  
528 which are involved in 8 among 19 interactions and for which one or two DZA045-5  
529 alleles at interacting loci were associated with higher values of frost damages and foliar  
530 electrolyte leakage.

531

#### 532 *Perspectives for fine mapping*

533 Three main LG were highlighted by the present results of QTL mapping. Among them,  
534 LG6 was the most interesting for it gathered several QTL of correlated traits among  
535 which were the most explanatory for frost damages. On LG6, the FD QTL had their  
536 peak LOD values at 122.2 cM, on the marker MTIC153. The FD2W QTL presented  
537 moreover a short confidence interval of 2.5 cM and 3.8 cM with LOD-1 and LOD-2  
538 boundaries respectively, which is a favorable point for further fine mapping and  
539 identification of candidate genes.

540 *M. truncatula* LG6 is a particular LG as it appeared to be the shortest one, according  
541 to the physical map data (<http://www.medicago.org/genome/map.php>) and the most  
542 heterochromatic (Kulikova *et al.*, 2001). This latter characteristic may not facilitate the  
543 final assembly of the physical map. Particularly, the microsatellite marker MTIC153,  
544 which has been developed from an EST accession (AL369679, Julier *et al.* 2003), is  
545 not integrated into the 3.5 *M. truncatula* physical map. It would be further interesting  
546 to add physically anchored markers in the QTL interval and to refine QTL mapping in  
547 large segregating populations in order to propose a list of candidate genes.



548

## 549 **Acknowledgements**

550 We are very grateful to M. Delalande from INRA center of Mauguio (Montpellier,  
551 France) for kindly providing seeds of the *M. truncatula* LR3 population. We are also  
552 grateful to F. Depta, J-F. Hu, B. Decaux, R. Devaux, M. Boilleau, O. Jaminon, A-S.  
553 Niquet, K. Lourgant, A. Ketele, G. Deniot and I. Le Goff for either their technical  
554 support for devices used or great help for periodical sampling. This work was  
555 supported by the Picardie region and by the UNIP (Union Nationale  
556 Interprofessionnelle des Plantes Riches en Protéines). The development of the F83005-  
557 5 x DZA045-5 genetic map was supported within the EU-FP6 GLIP project.

558

## 559 **References**

- 560 Annicchiarico P, Collins RP, Fornasier F, Rhodes I (2001) Variation in cold tolerance  
561 and spring growth among Italian white clover populations. *Euphytica* 122  
562 (2):407-416. doi:10.1023/a:1012918100232
- 563 Arbaoui M, Link W, Satovic Z, Torres AM (2008) Quantitative trait loci of frost  
564 tolerance and physiologically related trait in faba bean (*Vicia faba* L.).  
565 *Euphytica* 164 (1):93-104. doi:10.1007/s10681-008-9654-0
- 566 Arraouadi S, Chardon F, Huguet T, Aouani ME, Badri M (2011) QTLs mapping of  
567 morphological traits related to salt tolerance in *Medicago truncatula*. *Acta*  
568 *Physiologiae Plantarum* 33 (3):917-926. doi:10.1007/s11738-010-0621-8
- 569 Asghari A, Mohammadi SA, Moghaddam M, Shokuhian AA (2008) Identification of  
570 SSR and RAPD markers associated with QTLs of winter survival and related  
571 traits in *Brassica napus* L. *Afr J Biotechnol* 7 (7):897-903
- 572 Aubert G, Morin J, Jacquin F, Loridon K, Quillet M, Petit A, Rameau C, Lejeune-  
573 Hénaut I, Huguet T, Burstin J (2006) Functional mapping in pea, as an aid to

574 the candidate gene selection and for investigating synteny with the model  
 575 legume *Medicago truncatula*. Theoretical and Applied Genetics 112 (6):1024-  
 576 1041. doi:doi:10.1007/s00122-005-0205-y  
 577 Baga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB, Chibbar RN (2007)  
 578 Identification of quantitative trait loci and associated candidate genes for low-  
 579 temperature tolerance in cold-hardy winter wheat. Funct Integr Genomics 7  
 580 (1):53-68. doi:10.1007/s10142-006-0030-7  
 581 Biber PD (2007) Evaluating a Chlorophyll Content Meter on Three Coastal Wetland  
 582 Plant Species. Journal of Agricultural, Food and Environmental Sciences 1  
 583 (2):1-11  
 584 Börner A, Schumann E, Fürste A, Cöster C, Leithold B, Röder M, Weber W (2002)  
 585 Mapping of quantitative trait loci determining agronomic important characters  
 586 in hexaploid wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics  
 587 105 (6):921-936. doi:10.1007/s00122-002-0994-1  
 588 Brandsæter LO, Olsmo A, Tronsmo AM, Fykse H (2002) Freezing Resistance of  
 589 Winter Annual and Biennial Legumes at Different Developmental Stages.  
 590 Crop Science 42 (2):437-443. doi:10.2135/cropsci2002.4370  
 591 Brouwer DJ, Duke SH, Osborn TC (2000) Mapping genetic factors associated with  
 592 winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. Crop  
 593 Science 40 (5):1387-1396  
 594 Calenge F, Saliba-Colombani V, Mahieu S, Loudet O, Daniel-Vedele F, Krapp A  
 595 (2006) Natural Variation for Carbohydrate Content in Arabidopsis. Interaction  
 596 with Complex Traits Dissected by Quantitative Genetics. Plant Physiology 141  
 597 (4):1630-1643. doi:10.1104/pp.106.082396

598 Cattivelli L, Baldi P, Crosatti C, Di Fonzo N, Faccioli P, Grossi M, Mastrangelo AM,  
 599 Pecchioni N, Stanca AM (2002) Chromosome regions and stress-related  
 600 sequences involved in resistance to abiotic stress in Triticeae. *Plant Molecular*  
 601 *Biology* 48 (5):649-665

602 Chen TH-H, Gusta LV, Fowler DB (1983) Freezing Injury and Root Development in  
 603 Winter Cereals. *Plant Physiology* 73 (3):773-777. doi:10.1104/pp.73.3.773

604 Choi H-K, Kim D, Uhm T, Limpens E, Lim H, Mun J-H, Kalo P, Penmetsa RV, Seres  
 605 A, Kulikova O, Roe BA, Bisseling T, Kiss GB, Cook DR (2004a) A Sequence-  
 606 Based Genetic Map of *Medicago truncatula* and Comparison of Marker  
 607 Colinearity with *M. sativa*. *Genetics* 166 (3):1463-1502.  
 608 doi:10.1534/genetics.166.3.1463

609 Choi H-K, Mun J-H, Kim D-J, Zhu H, Baek J-M, Mudge J, Roe B, Ellis N, Doyle J,  
 610 Kiss GB, Young ND, Cook DR (2004b) Estimating genome conservation  
 611 between crop and model legume species. *PNAS* 101 (43):15289-15294.  
 612 doi:10.1073/pnas.0402251101

613 Dexter ST, Tottingham WE, Graber LF (1930) Preliminary results in measuring the  
 614 hardness of plants. *Plant Physiol* 5:215-223. doi:doi:10.1104/pp.5.2.215

615 Dionne J, Rochefort S, Huff DR, Desjardins Y, Bertrand A, Castonguay Y (2010)  
 616 Variability for Freezing Tolerance among 42 Ecotypes of Green-Type Annual  
 617 Bluegrass. *Crop Science* 50 (1):321-336. doi:10.2135/cropsci2008.12.0712

618 Dumont E, Fontaine V, Vuylstekker C, Sellier H, Bodele S, Voedts N, Devaux R, Frise  
 619 M, Avia K, Hilbert JL, Bahrman N, Hanocq E, Lejeune-Henaut I, Delbreil B  
 620 (2009) Association of sugar content QTL and PQL with physiological traits  
 621 relevant to frost damage resistance in pea under field and controlled conditions.

622           Theoretical and Applied Genetics 118 (8):1561-1571. doi:10.1007/s00122-  
 623           009-1004-7  
 624   Equiza MA, Mirave JP, Tognetti JA (1997) Differential inhibition of shoot vs. root  
 625           growth at low temperature and its relationship with carbohydrate accumulation  
 626           in different wheat cultivars. Annals of Botany 80 (5):657-663.  
 627           doi:10.1006/anbo.1997.0503  
 628   Eujayl I, Erskine W, Baum M, Pehu E (1999) Inheritance and Linkage Analysis of  
 629           Frost Injury in Lentil. Crop Science 39 (3):639-642.  
 630           doi:10.2135/cropsci1999.0011183X003900020004x  
 631   Finne MA, Rognli OA, Schjelderup I (2000) Genetic variation in a Norwegian  
 632           germplasm collection of white clover (*Trifolium repens* L.)-1. Population  
 633           differences in agronomic characteristics. Euphytica 112 (1):33-44  
 634   Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS,  
 635           Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature  
 636           tolerance in a 'Nure' (winter) x 'Tremois' (spring) barley map. Theoretical and  
 637           Applied Genetics 108 (4):670-680. doi:10.1007/s00122-003-1468-9  
 638   Gorton AJ, Heath KD, Pilet-Nayel M-L, Baranger A, Stinchcombe JR (2012) Mapping  
 639           the Genetic Basis of Symbiotic Variation in Legume-Rhizobium Interactions  
 640           in *Medicago truncatula*. G3: Genes|Genomes|Genetics 2 (11):1291-1303.  
 641           doi:10.1534/g3.112.003269  
 642   Hamilton NRS, Skot L, Chorlton KH, Thomas ID, Mizen S (2002) Molecular  
 643           genecology of temperature response in *Lolium perenne*: 1. preliminary analysis  
 644           to reduce false positives. Mol Ecol 11 (9):1855-1863. doi:10.1046/j.1365-  
 645           294X.2002.01567.x

646 Hamon C, Baranger A, Miteul H, Lecointe R, Le Goff I, Deniot G, Onfroy C, Moussart  
 647 A, Prosperi JM, Tivoli B, Delourme R, Pilet-Nayel ML (2010) A complex  
 648 genetic network involving a broad-spectrum locus and strain-specific loci  
 649 controls resistance to different pathotypes of *Aphanomyces euteiches* in  
 650 *Medicago truncatula*. Theoretical and Applied Genetics 120 (5):955-970.  
 651 doi:10.1007/s00122-009-1224-x

652 Hayes PM, Blake T, Chen THH, Tragoonrung S, Chen F, Pan A, Liu B (1993)  
 653 Quantitative Trait Loci on Barley (*Hordeum-Vulgare* L) Chromosome-7  
 654 Associated with Components of Winterhardiness. Genome 36 (1):66-71

655 Hekneby M, Antolin MC, Sanchez-Diaz M (2001) Cold response of annual  
 656 Mediterranean pasture legumes. Options Méditerranéennes Série A,  
 657 Séminaires Méditerranéens (45):157-161

658 Holland J (1998) Computer note. EPISTACY: A SAS program for detecting two-locus  
 659 epistatic interactions using genetic marker information. Journal Of Heredity 89  
 660 (4):374-375. doi:10.1093/jhered/89.4.374

661 Holland JB (2006) Estimating Genotypic Correlations and Their Standard Errors  
 662 Using Multivariate Restricted Maximum Likelihood Estimation with SAS Proc  
 663 MIXED. Crop Science 46 (2):642-654. doi:10.2135/cropsci2005.0191

664 Holland JB, Nyquist WE, Cervantes-Martínez CT (2003) Estimating and interpreting  
 665 heritability for plant breeding: an update Plant Breeding Reviews 22, 9-111  
 666 22:9-111

667 Huguet T (2004) Genetic map of the *Medicago truncatula* LR4 population  
 668 [http://medicago.toulouse.inra.fr/Mt/GeneticMAP/LR4\\_MAP.html](http://medicago.toulouse.inra.fr/Mt/GeneticMAP/LR4_MAP.html) LIPM,  
 669 Toulouse, France

670 Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998)  
 671 *Arabidopsis* CBF1 Overexpression Induces COR Genes and Enhances  
 672 Freezing Tolerance. *Science* 280 (5360):104-106.  
 673 doi:10.1126/science.280.5360.104  
 674 Julier B, Flajoulot S, Barre P, Cardinet G, Santoni S, Huguet T, Huyghe C (2003)  
 675 Construction of two genetic linkage maps in cultivated tetraploid alfalfa  
 676 (*Medicago sativa*) using microsatellite and AFLP markers. *BMC Plant Biology*  
 677 3 (1):9. doi:10.1186/1471-2229-3-9  
 678 Kahraman A, Kusmenoglu I, Aydin N, Aydogan A, Erskine W, Muehlbauer FJ (2004)  
 679 QTL mapping of winter hardiness genes in lentil. *Crop Science* 44 (1):13-22  
 680 Kole C, Thormann CE, Karlsson BH, Palta JP, Gaffney P, Yandell B, Osborn TC  
 681 (2002) Comparative mapping of loci controlling winter survival and related  
 682 traits in oilseed *Brassica rapa* and *B. napus*. *Molecular Breeding* 9 (3):201-  
 683 210. doi:10.1023/a:1019759512347  
 684 Leinonen PH, Remington DL, Savolainen O (2011) Local adaptation, phenotypic  
 685 differentiation, and hybrid fitness in diverged natural populations of  
 686 *Arabidopsis lyrata*. *Evolution* 65 (1):90-107. doi:10.1111/j.1558-  
 687 5646.2010.01119.x  
 688 Lejeune-Hénaut I, Delbreil B, Devaux R, Guillioni L (2010) Cold temperatures and the  
 689 functioning of the canopy in pea. In: Quae (ed) *Physiology of the pea crop*.  
 690 Science Publishers, Enfield, USA, pp 272: 168-180  
 691 Lejeune-Hénaut I, Hanocq E, Béthencourt L, Fontaine V, Delbreil B, Morin J, Petit A,  
 692 Devaux R, Boilleau M, Stempniak JJ, Thomas M, Lainé AL, Foucher F,  
 693 Baranger A, Burstin J, Rameau C, Giauffret C (2008) The flowering locus *Hr*  
 694 colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum*

695 L. Theoretical and Applied Genetics 116 (8):1105-1116. doi:10.1007/s00122-  
 696 008-0739-x

697 Levitt J (1980) Chilling, freezing and high temperature stress, vol 1. Responses of  
 698 plants to environmental stresses. Academic Press,

699 Liesenfeld DR, Auld DL, Murray GA, Swensen JB (1986) Transmittance of  
 700 winterhardiness in segregated populations of peas. Crop Science 26:49-54

701 Lincoln SE, Daly MJ, Lander ES (1992) Constructing genetic maps with  
 702 MAPMAKER/EXP version 3.0. Technical Report, 3rd edn. Whitehouse  
 703 Institute, Cambridge, Mass.,

704 Loik ME, Redar SP (2003) Microclimate, freezing tolerance, and cold acclimation  
 705 along an elevation gradient for seedlings of the Great Basin Desert shrub,  
 706 *Artemisia tridentata*. J Arid Environ 54 (4):769-782.  
 707 doi:10.1006/jare.2002.1106

708 Moreau D, Salon C, Munier-Jolain N (2006) Using a standard framework for the  
 709 phenotypic analysis of *Medicago truncatula*: an effective method for  
 710 characterizing the plant material used for functional genomics approaches.  
 711 Plant, Cell & Environment 29 (6):1087-1098. doi:10.1111/j.1365-  
 712 3040.2005.01483.x

713 Mun J-H, Kim D-J, Choi H-K, Gish J, Debellé F, Mudge J, Denny R, Endré G, Saurat  
 714 O, Dubez A-M, Kiss GB, Roe B, Young ND, Cook DR (2006) Distribution of  
 715 Microsatellites in the Genome of *Medicago truncatula*: A Resource of Genetic  
 716 Markers That Integrate Genetic and Physical Maps. Genetics 172 (4):2541-  
 717 2555. doi:10.1534/genetics.105.054791

718 Nakayama S, Daido H, Abe J (1997) Winter hardiness and growth at low temperature  
 719 in European varieties of orchardgrass (*Dactylis glomerata* L.). Grassland  
 720 Science 43:224-230

721 Palta JP, Li PH (1980) Alterations in membrane transport properties by freezing injury  
 722 in herbaceous plants. Physiologia Plantarum 50 (2):169-175.  
 723 doi:10.1111/j.1399-3054.1980.tb04446.x

724 Pan A, Hayes PM, Chen F, Chen THH, Blake T, Wright S, Karsai I, Bedo Z (1994)  
 725 Genetic-Analysis of the Components of Winterhardiness in Barley (*Hordeum-*  
 726 *Vulgare* L). Theoretical and Applied Genetics 89 (7-8):900-910

727 Pennycooke JC, Cheng H, Stockinger EJ (2008) Comparative Genomic Sequence and  
 728 Expression Analyses of *Medicago truncatula* and Alfalfa Subspecies *falcata*  
 729 COLD-ACCLIMATION-SPECIFIC Genes. Plant Physiology 146 (3):1242-  
 730 1254. doi:10.1104/pp.107.108779

731 Richardson AD, Duigan SP, Berlyn GP (2002) An evaluation of noninvasive methods  
 732 to estimate foliar chlorophyll content. New Phytologist 153 (1):185-194.  
 733 doi:10.1046/j.0028-646X.2001.00289.x

734 Ronfort J, Bataillon T, Santoni S, Delalande M, David J, Prosperi J-M (2006)  
 735 Microsatellite diversity and broad scale geographic structure in a model  
 736 legume: building a set of nested core collection for studying naturally occurring  
 737 variation in *Medicago truncatula*. BMC Plant Biology 6 (1):28.  
 738 doi:10.1186/1471-2229-6-28

739 Rukavina H, Hughes HG, Qian Y (2007) Freezing Tolerance of 27 Saltgrass Ecotypes  
 740 from Three Cold Hardiness Zones. Hortscience 42 (1):157-160



741 Stoddard F, Balko C, Erskine W, Khan H, Link W, Sarker A (2006) Screening  
 742 techniques and sources of resistance to abiotic stresses in cool-season food  
 743 legumes. *Euphytica* 147 (1):167-186. doi:10.1007/s10681-006-4723-8

744 Stone JM, Palta JP, Bamberg JB, Weiss LS, Harbage JF (1993) Inheritance of freezing  
 745 resistance in tuber-bearing *Solanum* species: evidence for independent genetic  
 746 control of nonacclimated freezing tolerance and cold acclimation capacity.  
 747 *Proceedings of the National Academy of Sciences* 90 (16):7869-7873

748 Sulc RM, Albrecht KA, Duke SH (1991a) Leakage of intracellular substances as an  
 749 indicator of freezing injury in alfalfa. *Crop Science* 31 (2):430-435

750 Sulc RM, Albrecht KA, Palta JP, Duke SH (1991b) Leakage of intracellular substances  
 751 from alfalfa roots at various subfreezing temperatures. *Crop Science* 31  
 752 (6):1575-1578

753 Teutonico RA, Yandell B, Satagopan JM, Ferreira ME, Palta JP, Osborn TC (1995)  
 754 Genetic-Analysis and Mapping of Genes-Controlling Freezing Tolerance in  
 755 Oilseed Brassica. *Molecular Breeding* 1 (4):329-339

756 Tayeh N, Bahrman N, Devaux R, Bluteau A, Prosperi JM, Delbreil B, Lejeune-Hénaut  
 757 I (2013) A high-density genetic map of the *Medicago truncatula* major freezing  
 758 tolerance QTL on chromosome 6 reveals colinearity with a QTL related to  
 759 freezing damage on *Pisum sativum* linkage group VI. In press in *Molecular*  
 760 *Breeding*. doi:10.1007/s11032-013-9869-1.

761 Thapa B, Arora R, Knapp AD, Brummer EC (2008) Applying freezing test to quantify  
 762 cold acclimation in *Medicago truncatula*. *Journal of the American Society for*  
 763 *Horticultural Science* 133 (5):684-691

764 Thoquet P, Gherardi M, Journet E-P, Kereszt A, Ane J-M, Prosperi J-M, Huguet T  
 765 (2002) The molecular genetic linkage map of the model legume *Medicago*

766           truncatula: an essential tool for comparative legume genomics and the isolation  
 767           of agronomically important genes. BMC Plant Biology 2 (1):1.  
 768           doi:10.1186/1471-2229-2-1  
 769 Voorrips RE (2002) MapChart: Software for the Graphical Presentation of Linkage  
 770           Maps and QTLs. Journal Of Heredity 93 (1):77-78. doi:10.1093/jhered/93.1.77  
 771 Wang S, Basten CJ, Zeng Z-B (2012) Windows QTL Cartographer 2.5 Department of  
 772           Statistics, North Carolina State University, Raleigh, NC  
 773           (<http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>)  
 774 Wooten DR, Livingston DP, Lyerly HJ, Holland JB, Jellen EN, Marshall DS, Murphy  
 775           JP (2009) Quantitative Trait Loci and Epistasis for Oat Winter-Hardiness  
 776           Component Traits. Crop Science 49 (6):1989-1998.  
 777           doi:10.2135/cropsci2008.10.0612  
 778 Xiong Y, Fei S-z, Arora R, Brummer EC, Barker R, Jung G, Warnke S (2007)  
 779           Identification of quantitative trait loci controlling winter hardiness in an  
 780           annual  $\times$  perennial ryegrass interspecific hybrid population. Molecular  
 781           Breeding 19 (2):125-136. doi:10.1007/s11032-006-9050-1  
 782 Yahia N, Fyad-Lameche FZ (2003) Evaluation of cold tolerance variability in annual  
 783           Medicago species at the seedling stage. Acta Bot Gall 150 (1):3-17  
 784 Young ND, Cannon SB, Sato S, Kim D, Cook DR, Town CD, Roe BA, Tabata S  
 785           (2005) Sequencing the Genespaces of Medicago truncatula and Lotus  
 786           japonicus. Plant Physiology 137 (4):1174-1181. doi:10.1104/pp.104.057034  
 787 Young ND, Debelle F, Oldroyd GED et al. (2011) The Medicago genome provides  
 788           insight into the evolution of rhizobial symbioses. Nature 480 (7378):520-524.  
 789           doi:10.1038/nature10625

790 Zhu B, Choi DW, Fenton R, Close TJ (2000) Expression of the barley dehydrin  
791 multigene family and the development of freezing tolerance. Molecular and  
792 General Genetics MGG 264 (1):145-153. doi:10.1007/s004380000299

793    **TABLES**

794

795 **Table 1** Fifteen *M. truncatula* accessions lines analyzed for freezing tolerance with their agro-ecological descriptions and freezing damage scores  
796 after 2 weeks under rewarming conditions (FD2W). Core collection information is provided according to the website:  
797 <http://www.montpellier.inra.fr/BRC-MTR/>.

Accession Number	Accession name	Collection	Country of origin	Latitude	Longitude	Altitude (m)	Annual rain (mm)	Occurring annual frost	FD2W	Stat. SNK grouping
L000368	DZA012-J	Inbred line	Algeria	36.55 N	3.18 E	200	600-900	No frost	4	a
L000736	DZA045-6	Inbred line	Algeria	36.92 N	7.74 E	100	600-900	No frost	3.7	a b
L000422	ESP096-A	Inbred line	Spain	36.98 N	3.14 W	1200	600-900	Light	3.5	a b
L000174	SA28064	Inbred line	Cyprus	34.78 N	33.17 E	550	-	-	3.4	b
L000542	DZA233-4	Inbred line	Algeria	35.85 N	4.95 E	1370	400-600	Light (-2.7°C)	3.2	b c
L000163	SA22322	Inbred line	Syria	35.02 N	37.10 E	500	-	-	3.2	b c
L000530	F83005-5	Inbred line	France	43.56 N	6.13 E	260	-	Light to average	3.2	b c
L000734	DZA315-16	Inbred line	Algeria	34.72 N	0.16 E	1070	300-400	No frost	3	b c
L000738	A17 (Jemalong)	Inbred line	Australia	-	-	-	-	-	2.8	c d
L000544	ESP105-L	Inbred line	Spain	38.08 N	3.81 W	350	400-600	No frost	2.5	d e
L000541	DZA220-H	Population	Algeria	34.51 N	3.46 E	1050	150-300	Light (-0.7°C)	2.4	d e
L000651	Salses 71B	Inbred line	France	42.80 N	2.93 E	5	-	Light	2.3	d e f
L000460	F20026	Inbred line	France	42.92 N	9.36 E	20	600-900	Light	2	e f
L000551	F34042-D	Population	France	43.70 N	3.77 E	150	600-900	Light to average	1.9	e f
L000549	F11005-E	Inbred line	France	43.10 N	3.09 W	10	-	Light to average	1.8	f

798

799

800 **Table 2** Air temperature, photoperiod and growth duration of the *M. truncatula* plant material under establishment, cold acclimation, freezing and  
801 rewarming growth regimes.

Growth regimes	Temperature (°C day/night)	Photoperiod (hours)	Duration (days)
Establishment	20/14	14	15
Cold acclimation	12/12	14	8
	8/2	14	14
Freezing	4/-6	10	8
Rewarming	16/6	14	15

808 **Table 3** Means, genetic variances (Vg) and their coefficients of variation (CVg expressed as  $\sqrt{Vg}$ /trait mean), broad-sense heritabilities (H<sup>2</sup>) their  
809 standard errors for traits mapped in the *M. truncatula* LR3 population. Traits names are described in the legend of Fig. 1.

Trait	Mean	Vg	CVg	H <sup>2</sup>	STD Error H <sup>2</sup>
FD1D	2.09	0.15	18.47	0.49	0.10
FD8D	4.59	0.02	3.23	0.59	0.06
FD2W	3.99	0.35	14.74	0.72	0.05
EL	72.23	542.86	32.26	0.89	0.01
NOL	10.21	2.10	14.19	0.51	0.07
LA	18.79	12.12	18.53	0.45	0.08
CCI	22.12	24.45	22.35	0.61	0.05
SDW	0.19	0.003	28.83	0.57	0.08
RDW	0.09	0.001	35.14	0.43	0.11

810

811

812

813

814 **Table 4** Genotypic and phenotypic correlations between each pair of the different traits analyzed in the *M. truncatula* LR3 population: genotypic  
815 correlations (above the diagonal) are on the top lines and their standard errors on the bottom lines; phenotypic correlations (below the diagonal)  
816 are on the top lines and associated *P*-values on the bottom lines. Traits names are described in the legend of Fig. 1.

Trait	FD1D	FD8D	FD2W	EL	NOL	LA	CCI	SDW	RDW
FD1D		0.457 0.103	0.522 0.070	0.440 0.072	-0.087 0.116	-0.023 0.121	-0.203 0.106	-0.110 0.093	-0.088 0.095
FD8D	0.457 <.0001		0.992 0.039	0.708 0.071	-0.279 0.137	-0.276 0.141	-0.491 0.119	-0.223 0.108	-0.248 0.111
FD2W	0.472 <.0001	0.830 <.0001		0.681 0.047	-0.122 0.108	-0.052 0.113	-0.336 0.094	-0.079 0.087	-0.132 0.089
EL	0.392 <.0001	0.562 <.0001	0.625 <.0001		-0.081 0.103	-0.071 0.106	-0.293 0.091	-0.082 0.083	-0.068 0.085
NOL	-0.071 0.344	-0.153 0.042	-0.107 0.157	-0.057 0.452		0.757 0.061	0.454 0.111	0.696 0.075	0.561 0.091
LA	-0.060 0.426	-0.140 0.062	-0.062 0.411	-0.057 0.452	0.793 <.0001		0.727 0.091	0.912 0.054	0.687 0.106
CCI	-0.224 0.003	-0.296 <.0001	-0.300 <.0001	-0.256 0.001	0.376 <.0001	0.533 <.0001		0.658 0.081	0.510 0.090
SDW	-0.104 0.167	-0.152 0.043	-0.093 0.215	-0.073 0.334	0.579 <.0001	0.708 <.0001	0.538 <.0001		0.736 0.044
RDW	-0.098 0.196	-0.180 0.016	-0.158 0.036	-0.112 0.138	0.389 <.0001	0.482 <.0001	0.407 <.0001	0.689 <.0001	



818 **Table 5** QTL parameters in the *M. truncatula* LR3 population. Traits names are described in the legend of Fig. 1. QTL are named by their trait  
819 abbreviation followed by the LG number and a rating number when more than one QTL was detected for the same trait on the same LG. The inner  
820 and outer limits of the QTL confidence intervals correspond to LOD-2 and LOD-1 decreases at each side of the QTL peaks. R<sup>2</sup> represents the  
821 percentage of phenotypic variance explained by each QTL and a is the additive effect relative to the parent F83005-5.

Trait	LG	QTL name	Peak (cM)	LOD	Confidence interval (cM) (LOD-2)-(LOD-1)--(LOD-1)-(LOD-2)	R <sup>2</sup>	a	Nearest marker
FD1D	6	FD1D-6	101.5	3.0	93.0-93.6--106-106.4	12.4	-0.117	E13M60.728
FD8D	1	FD8D-1.1	77.4	3.2	73.7-74.2--82.5-82.5	4.7	-0.029	MTIC125
	1	FD8D-1.2	92.2	3.8	82.5-83.0--97.2-102.0	4.0	-0.029	E12M52.333
	4	FD8D-4	111.3	6.2	103.5-106.3--114.8-120.5	10.9	-0.035	MTIC186
	6	FD8D-6	123.3	23.4	119.0-120.0--123.7-123.8	39.3	-0.075	MTIC153
FD2W	1	FD2W-1.1	72.4	5.2	63.3-66.4--79.8-81.7	8.1	-0.132	MTIC125
	1	FD2W-1.2	91.2	4.1	82.0-83.0--95.8-98.2	6.3	-0.041	E12M52.333
	4	FD2W-4	112.3	7.3	104.6-106.3--115.1-121.5	12.0	-0.192	MTIC186
	6	FD2W-6	122.3	23.0	120.0-121.0--123.5-123.8	38.4	-0.356	MTIC153
EL	1	EL-1	116.7	3.0	109.7-109.7--127.2-131.5	3.7	-5.210	MTIC064
	4	EL-4	111.3	4.2	103.1-106.5--114.6-115.5	5.1	-5.000	MTIC186
	6	EL-6	122.3	9.3	119.4-120.7--123.8-126.9	18.8	-10.320	MTIC153
NOL	1	NOL-1	77.4	3.0	73.8-74.2--82.0-82.4	8.1	0.210	MTIC125
	4	NOL-4	119.9	2.9	111.0-112.2--129.0-129.2	9.2	0.286	E13M61.146
	7	NOL-7	104.0	3.3	96.3-100.9--110.2-111.0	12.2	0.374	MTIC471
LA	1	LA-1	108.6	6.5	103.7-105.2--111.4-114.1	16.4	0.823	MTIC285
	4	LA-4	19.6	3.1	9.7-12.9--22.3-22.4	7.7	-0.769	E14M60.280
CCI	1	CCI-1	110.1	6.2	105.5-107.4--111.8-114.3	13.3	1.294	MTIC285
	2	CCI-2	164.9	4.4	160.2-161.9--168.8-171.0	6.6	-0.907	MTIC138
	5	CCI-5	120.5	4.7	112.6-115.6--127.5-131.4	9.6	1.131	E12M49.300
	6	CCI-6.1	121.4	2.7	115.8-116.6--125.7-128.8	4.9	0.828	MTIC153
	6	CCI-6.2	141.9	4.1	134.7-137.5--145.8-147.7	9.4	1.086	E14M60.407

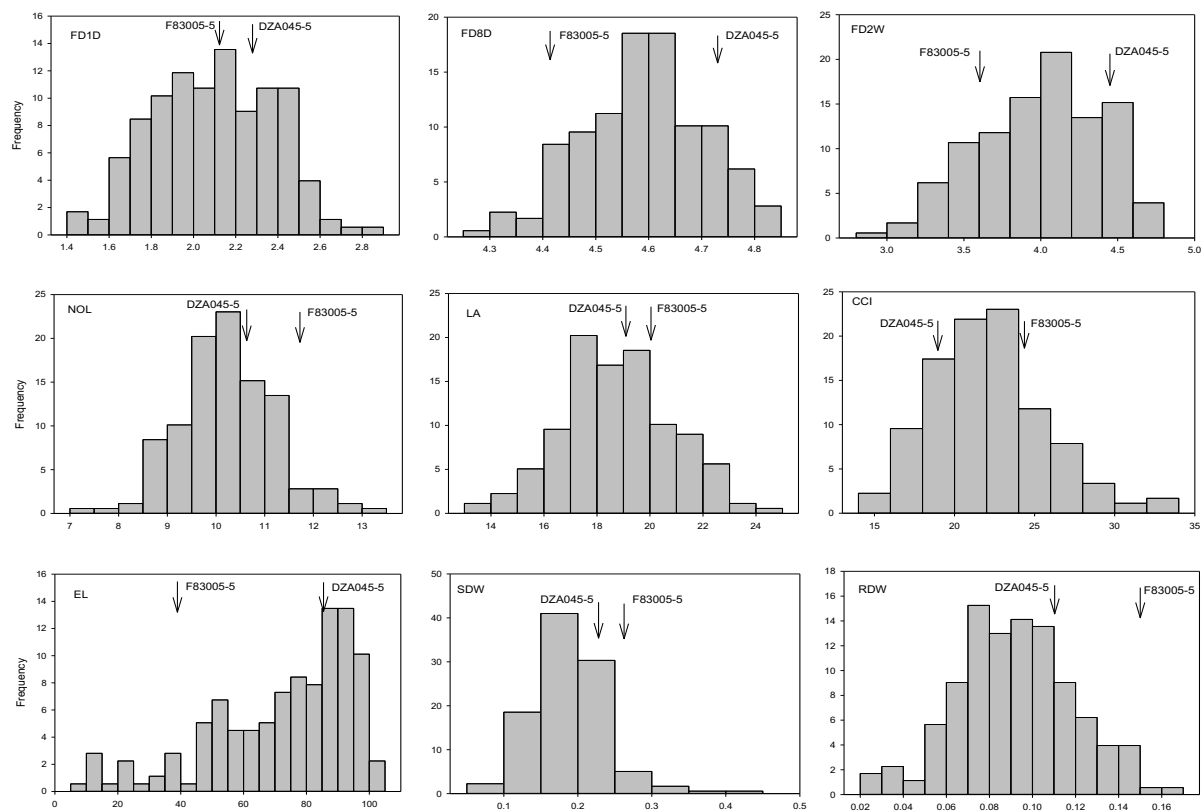
SDW	1	SDW-1	110.0	3.1	99.7-105.2--111.7-115.9	6.5	0.013	MTIC285
	4	SDW-4	17.6	3.3	9.3-10.6--21.6-22.3	7.0	-0.014	E14M60.280
RDW	1	RDW-1	111.7	5.0	100.5-110.0--114.7-117.1	9.5	0.009	MTIC064
	4	RDW-4.1	71.6	3.2	65.3-68.5--75.9-78.6	6.0	-0.007	MTIC249
	4	RDW-4.2	109.3	3.2	100.0-104.5—116.0-129.0	5.4	0.008	MTIC186
	6	RDW-6.1	79.0	3.3	65.1-73.6--85.9-91.5	3.4	0.008	E13M62.580
	6	RDW-6.2	169.6	3.8	158.2-160.5--178.7-181.0	8.2	-0.010	MTIC343

822

## FIGURE LEGENDS

**Fig. 1** Distribution of quantitative traits in the *M. truncatula* LR3 population. Arrows indicate mean values of the two parents. Traits names are as follows: FD1D to FD8D for freezing damage score after 1 to 8 days under freezing conditions, FD2W for freezing damage score after 2 weeks of rewarming, EL for electrolyte leakage from leaves after 2 weeks of rewarming, NOL, LA, CCI, SDW and RDW for number of leaves, leaf area, chlorophyll content index, shoot dry weight and root dry weight per plant at the end of the acclimation phase, respectively.

**Fig. 2** *M. truncatula* genetic map and QTL, established from a population of 178 F6 RIL, namely LR3, derived from the cross between F83005-5 and DZA045-5. Markers names are indicated on the right of each linkage group (LG). Markers positions are indicated in cM Kosambi on the left on each LG. Markers in bold are anchored in sequenced BACs, markers in italic are common to the integrated reference genetic-physical map of *M. truncatula* (<http://www.medicago.org/genome/map.php>; Mun et al., 2006). The confidence interval of QTL position was estimated as the interval between positions where the LOD value decreased respectively by 1 and 2 units, relatively to that of the most likely position. Traits names are described in the legend of Fig. 1.



843 Figure 2

