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Title:

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

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Abstract

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

Key words

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

Introduction

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

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

Considered as a valuable basis for marker assisted selection, mapping of Quantitative Trait Loci (QTL) has also been helpful to gain insight into the genetic determinism of both winter hardiness and freezing tolerance for grain legume species. Under field conditions, QTL affecting WH have been detected for lentil (Kahraman et al. 2004) and pea (Lejeune-Hénaut et al. 2008), and also for other important crops such as cereals (barley: Pan et al. 1994; Francia et al. 2004; wheat: Börner et al. 2002; oat: Wooten et al. 2009), forage grasses (ryegrass: Xiong et al. 2007; alfalfa: Brouwer et 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 $\mu mol.m^{-2}.s^{-1}$. 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

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

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 ε_{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 σ_G^2 represents the genetic variance, σ_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 ≤ 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 (α=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 = 2ln(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

424 study are a first insight in the genetic determinism of frost tolerance in the model
425 legume *M. truncatula* which could help to identify candidate genes underlying QTL
426 and benefit to closely related cultivated species like pea or faba bean, provided that
427 syntenic QTL are identified. As an example, Tayeh et al. (2013) have recently shown
428 that the major frost tolerance QTL identified on *M. truncatula* linkage group 6 in the
429 present study was syntenic to the frost tolerance QTL identified on pea linkage group
430 6 by Lejeune-Hénaut et al. (2008) and Dumont et al. (2009). Based on the existing
431 variability of this trait within the *M. truncatula* species (Table 1), it would be worth to
432 study a segregating population from a cross showing a wider phenotypic variation for
433 the freezing tolerance traits, for instance between F20026 or F11005-E and DZA045-
434 5, in order to further confirm and/or to identify additional QTL that affect freezing
435 tolerance.
436
437 QTL for physiological traits such as electrolyte leakage (EL), number of leaves (NOL),
438 leaf area (LA), chlorophyll content index (CCI) or root dry weight (RDW) colocalized
439 with QTL for FD in genetic regions that can roughly be delimited between markers
440 MTIC125 and MTIC146 (24 cM) on LG1, between L07.600 and MTIC037 (25.1 cM)
441 on LG4 and between E39M61.511 and E12M49.265 (7.7 cM) on LG6. The fact that
442 genotypic correlations between FD or EL on the one hand and other physiological
443 traits on the other hand were negative, suggests that higher values of NL, LA, CCI,
444 SDW and RDW are associated with lower values of FD and EL, i.e. higher freezing
445 tolerance. This is consistent with the signs of the additive effects observed at the
446 positions where QTL for freezing damage traits (FD or EL) colocalized with QTL for
447 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

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558

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TABLES

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

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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.

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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

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808 **Table 3** Means, genetic variances (Vg) and their coefficients of variation (CVg expressed as √Vg/trait mean), broad-sense heritabilities (H^2) 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^2	STD Error H^2
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

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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.

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Trait	FD1D	FD8D	FD2W	EL	NOL	LA	CCI	SDW	RDW
FD1D		0.457	0.522	0.440	-0.087	-0.023	-0.203	-0.110	-0.088
		0.103	0.070	0.072	0.116	0.121	0.106	0.093	0.095
FD8D	0.457		0.992	0.708	-0.279	-0.276	-0.491	-0.223	-0.248
	<.0001		0.039	0.071	0.137	0.141	0.119	0.108	0.111
FD2W	0.472	0.830		0.681	-0.122	-0.052	-0.336	-0.079	-0.132
	<.0001	<.0001		0.047	0.108	0.113	0.094	0.087	0.089
EL	0.392	0.562	0.625		-0.081	-0.071	-0.293	-0.082	-0.068
	<.0001	<.0001	<.0001		0,103	0.106	0.091	0.083	0.085
NOL	-0.071	-0.153	-0.107	-0.057		0.757	0.454	0.696	0.561
	0.344	0.042	0.157	0.452		0.061	0.111	0.075	0.091
LA	-0.060	-0.140	-0.062	-0.057	0.793		0.727	0.912	0.687
	0.426	0.062	0.411	0.452	<.0001		0.091	0.054	0.106
CCI	-0.224	-0.296	-0.300	-0.256	0.376	0.533		0.658	0.510
	0.003	<.0001	<.0001	0.001	<.0001	<.0001		0.081	0.090
SDW	-0.104	-0.152	-0.093	-0.073	0.579	0.708	0.538		0,736
	0.167	0.043	0.215	0.334	<.0001	<.0001	<.0001		0,044
RDW	-0.098	-0.180	-0.158	-0.112	0.389	0.482	0.407	0.689	
	0.196	0.016	0.036	0.138	<.0001	<.0001	<.0001	<.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^2 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^2	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

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823 **FIGURE LEGENDS**

824 **Fig. 1** Distribution of quantitative traits in the *M. truncatula* LR3 population. Arrows indicate
825 mean values of the two parents. Traits names are as follows: FD1D to FD8D for freezing
826 damage score after 1 to 8 days under freezing conditions, FD2W for freezing damage score
827 after 2 weeks of rewarming, EL for electrolyte leakage from leaves after 2 weeks of rewarming,
828 NOL, LA, CCI, SDW and RDW for number of leaves, leaf area, chlorophyll content index,
829 shoot dry weight and root dry weight per plant at the end of the acclimation phase, respectively.
830

831 **Fig. 2** *M. truncatula* genetic map and QTL, established from a population of 178 F6 RIL,
832 namely LR3, derived from the cross between F83005-5 and DZA045-5. Markers names are
833 indicated on the right of each linkage group (LG). Markers positions are indicated in cM
834 Kosambi on the left on each LG. Markers in bold are anchored in sequenced BACs, markers in
835 italic are common to the integrated reference genetic-physical map of *M. truncatula*
836 (http://www.medicago.org/genome/map.php; Mun et al., 2006). The confidence interval of
837 QTL position was estimated as the interval between positions where the LOD value decreased
838 respectively by 1 and 2 units, relatively to that of the most likely position. Traits names are
839 described in the legend of Fig. 1.
840

841 Figure 1

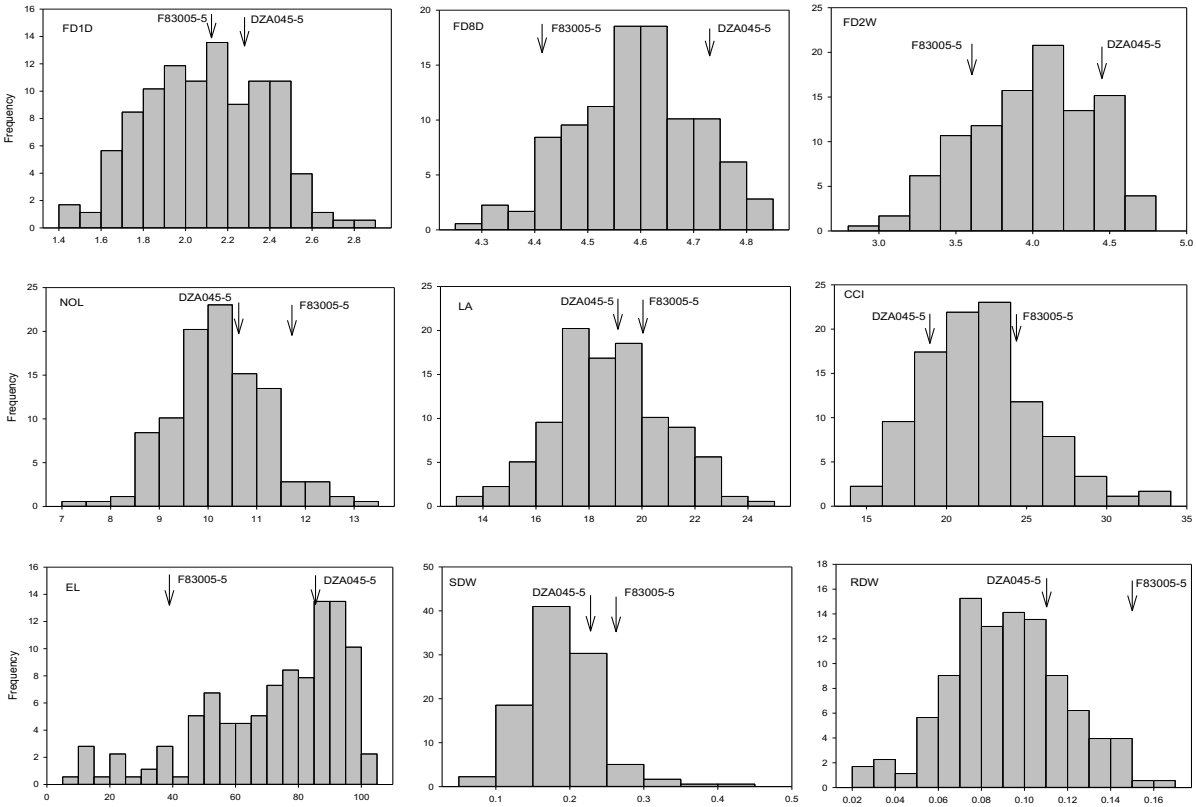

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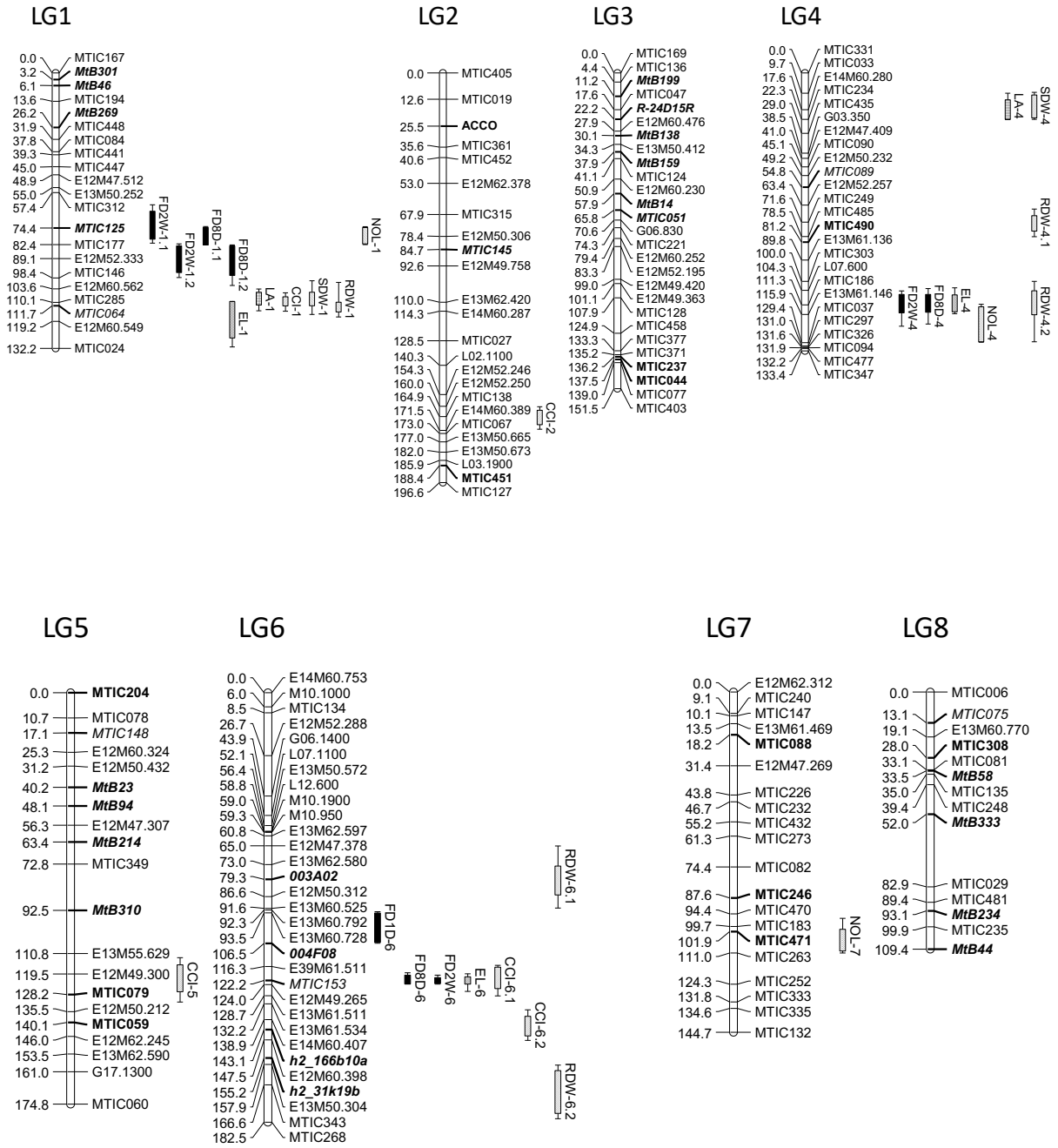
Figure 2


LG1
0.0 MTIC167
3.2 MtB301
6.1 MtB46
13.6 MTIC194
26.2 MtB269
31.9 MTIC448
37.8 MTIC084
39.3 MTIC441
45.0 MTIC447
48.9 E12M47.512
55.0 E13M50.252
57.4 MTIC312
74.4 MTIC125
82.4 MTIC177
89.1 E12M52.333
98.4 MTIC146
103.6 E12M60.562
110.1 MTIC285
111.7 MTIC064
119.2 E12M60.549
132.2 MTIC024
FD2W-1.1
FD8D-1.1
FD2W-1.2
FD8D-1.2
EL-1
LA-1
CCI-1
SDW-1
RDW-1
NOL-1
LG2
0.0 MTIC405
12.6 MTIC019
25.5 ACCO
35.6 MTIC361
40.6 MTIC452
53.0 E12M62.378
67.9 MTIC315
78.4 E12M50.306
84.7 MTIC145
92.6 E12M49.758
110.0 E13M62.420
114.3 E14M60.287
128.5 MTIC027
140.3 L02.1100
154.3 E12M52.246
160.0 E12M52.250
164.9 MTIC138
171.5 E14M60.389
173.0 MTIC067
177.0 E13M50.665
182.0 E13M50.673
185.9 L03.1900
188.4 MTIC451
196.6 MTIC127
CCI-2
LG3
0.0 MTIC169
4.4 MTIC136
11.2 MtB199
17.6 MTIC047
22.2 R-24D15R
27.9 E12M60.476
30.1 MtB138
34.3 E13M50.412
37.9 MtB159
41.1 MTIC124
50.9 E12M60.230
57.9 MtB14
65.8 MTIC051
70.6 G06.830
74.3 MTIC221
79.4 E12M60.252
83.3 E12M52.195
99.0 E12M49.420
101.1 E12M49.363
107.9 MTIC128
124.9 MTIC458
133.3 MTIC377
135.2 MTIC371
136.2 MTIC237
137.5 MTIC044
139.0 MTIC077
151.5 MTIC403
LG4
0.0 MTIC331
9.7 MTIC033
17.6 E14M60.280
22.3 MTIC234
29.0 MTIC435
38.5 G03.350
41.0 E12M47.409
45.1 MTIC090
49.2 E12M50.232
54.8 MTIC089
63.4 E12M52.257
71.6 MTIC249
78.5 MTIC485
81.2 MTIC490
89.8 E13M61.136
100.0 MTIC303
104.3 L07.600
111.3 MTIC186
115.9 E13M61.146
129.4 MTIC037
131.0 MTIC297
131.6 MTIC326
131.9 MTIC094
132.2 MTIC477
133.4 MTIC347
FD2W-4
FD8D-4
EL-4
NOL-4
LA-4
SDW-4
RDW-4.1
RDW-4.2
LG5
0.0 MTIC204
10.7 MTIC078
17.1 MTIC148
25.3 E12M60.324
31.2 E12M50.432
40.2 MtB23
48.1 MtB94
56.3 E12M47.307
63.4 MtB214
72.8 MTIC349
92.5 MtB310
110.8 E13M55.629
119.5 E12M49.300
128.2 MTIC079
135.5 E12M50.212
140.1 MTIC059
146.0 E12M62.245
153.5 E13M62.590
161.0 G17.1300
174.8 MTIC060
CCI-5
LG6
0.0 E14M60.753
6.0 M10.1000
8.5 MTIC134
26.7 E12M52.288
43.9 G06.1400
52.1 L07.1100
56.4 E13M50.572
58.8 L12.600
59.0 M10.1900
59.3 M10.950
60.8 E13M62.597
65.0 E12M47.378
73.0 E13M62.580
79.3 003A02
86.6 E12M50.312
91.6 E13M60.525
92.3 E13M60.792
93.5 E13M60.728
106.5 004F08
116.3 E39M61.511
122.2 MTIC153
124.0 E12M49.265
128.7 E13M61.511
132.2 E13M61.534
138.9 E14M60.407
143.1 h2_166b10a
147.5 E12M60.398
155.2 h2_31k19b
157.9 E13M50.304
166.6 MTIC343
182.5 MTIC268
FD1D-6
FD8D-6
FD2W-6
EL-6
CCI-6.1
CCI-6.2
RDW-6.1
RDW-6.2
LG7
0.0 E12M62.312
9.1 MTIC240
10.1 MTIC147
13.5 E13M61.469
18.2 MTIC088
31.4 E12M47.269
43.8 MTIC226
46.7 MTIC232
55.2 MTIC432
61.3 MTIC273
74.4 MTIC082
87.6 MTIC246
94.4 MTIC470
99.7 MTIC183
101.9 MTIC471
111.0 MTIC263
124.3 MTIC252
131.8 MTIC333
134.6 MTIC335
144.7 MTIC132
NOL-7
LG8
0.0 MTIC006
13.1 MTIC075
19.1 E13M60.770
28.0 MTIC308
33.1 MTIC081
33.5 MtB58
35.0 MTIC135
39.4 MTIC248
52.0 MtB333
82.9 MTIC029
89.4 MTIC481
93.1 MtB234
99.9 MTIC235
109.4 MtB44