

# 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 15 *M. truncatula* accessions, the progeny of a F6 recombinant inbred line 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 2 weeks after regrowth and foliar electrolyte leakage (EL) 2 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, 3–6 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.

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## 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 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 and 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 and FT have been reported in various species and FT QTL with coincidental positions with WH QTL have been identified (barley: Francia et al. 2004; wheat: Baga et al. 2007; oat: Wooten et al. 2009; ryegrass: Xiong et al. 2007; pea: Dumont et al. 2009).

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

*Medicago truncatula* Gaertn (*M. truncatula*) is a model legume that shows a high level of synteny with pea (Choi et al. 2004b; Aubert et al. 2006) and has a genome whose sequence is under completion (Young et al. 2005, 2011). Despite that its origin and cropping regions are restricted to Mediterranean climates, freezing tolerance variability has already been reported in *M. truncatula* (Hekneby et al. 2001; Brandsæter et al. 2002; Pennycooke et al. 2008). However, information is still lacking about the localization of the genome regions controlling this trait. Identifying such genetic factors for FT in *M. truncatula* should therefore enhance the understanding of FT in legumes and facilitate applications toward cultivated species.

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

## Materials and methods

### Plant materials

#### *Collection lines, parental lines and RILs*

A set of 15 *M. truncatula* accessions was tested for freezing tolerance. They included (a) 11 inbred lines, among which 8 constitute the *M. truncatula* core collection 8 (CC8) that matches broad geographic origins (Ronfort et al. 2006), (b) the reference line Jemalong A17 and (c) three additional accessions chosen for particular characteristics (Table 1): DZA220-H is a population belonging to the tricycle subspecies of *M. truncatula*, ESP096-A is an inbred line which was collected from a high altitude site in the south of Spain and F34042-D is a population which was collected from one of the highest latitude sites in the north of Montpellier (France). Two inbred line accessions—DZA045-5 (used as male), originating from Algeria and F83005-5 (used as female), originating from Salernes, France—were crossed to generate a segregating population of 178 F6-derived RILs (namely LR3) produced by single seed descent. DZA045-5 was not tested among the collection lines but is closely related to DZA045-6. Both lines were derived from the same population and no molecular difference was found between them (Prosperi, unpublished results). The frost tolerance level of DZA045-5 can thus be inferred from that of DZA045-6. All the plant material was provided by INRA of Mauguio (Montpellier, France: <http://www1.montpellier.inra.fr/BRC-MTR/>).

**Table 1** Fifteen *M. truncatula* accessions analyzed for freezing tolerance with their agro-ecological descriptions and freezing damage scores after 2 weeks under rewarming conditions (FD2W)

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	36.55N	3.18E	200	600–900	No frost	4	a
L000736	DZA045-6	Inbred line	Algeria	36.92N	7.74E	100	600–900	No frost	3.7	a b
L000422	ESP096-A	Inbred line	Spain	36.98N	3.14W	1,200	600–900	Light	3.5	a b
L000174	SA28064	Inbred line	Cyprus	34.78N	33.17E	550	–	–	3.4	b
L000542	DZA233-4	Inbred line	Algeria	35.85N	4.95E	1,370	400–600	Light (–2.7 °C)	3.2	b c
L000163	SA22322	Inbred line	Syria	35.02N	37.10E	500	–	–	3.2	b c
L000530	F83005-5	Inbred line	France	43.56N	6.13E	260	–	Light to average	3.2	b c
L000734	DZA315-16	Inbred line	Algeria	34.72N	0.16E	1,070	300–400	No frost	3	b c
L000738	A17 (Jemalong)	Inbred line	Australia	–	–	–	–	–	2.8	c d
L000544	ESP105-L	Inbred line	Spain	38.08N	3.81W	350	400–600	No frost	2.5	d e
L000541	DZA220-H	Population	Algeria	34.51N	3.46E	1,050	150–300	Light (–0.7 °C)	2.4	d e
L000651	Salses 71B	Inbred line	France	42.80N	2.93E	5	–	Light	2.3	d e f
L000460	F20026	Inbred line	France	42.92N	9.36E	20	600–900	Light	2	e f
L000551	F34042-D	Population	France	43.70N	3.77E	150	600–900	Light to average	1.9	e f
L000549	F11005-E	Inbred line	France	43.10N	3.09W	10	–	Light to average	1.8	f

Core collection information is provided according to the website: <http://www.montpellier.inra.fr/BRC-MTR/>

## Culture conditions

### Germination

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

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

Plantlets were placed in home-made polystyrene blocks, of 100 or 50 holes each, to protect roots from freezing injury

based on previous observations of their greater sensitivity to frost than shoots (Chen et al. 1983). Each hole (diameter 41 mm; height 80 mm) was half filled with perlite, to promote the development of roots, topped with a well-moistened (water-saturated for up to 3 h) Jiffy-7 pellet (diameter 41 mm; height when fully moistened 40 mm) made of sphagnum peat (<http://www.jiffygroup.com/jiffy/catalogue>). Lime and a special fertilizer with low ammonium content were pre-added to the peat to stimulate growth. One germinated seed was inserted in each pellet and was lightly watered to allow a firm anchoring in the substrate. During the establishment phase, the seedlings were frequently watered with deionised water to avoid desiccation of the roots. To prevent a premature yellowing of the leaves, about 10 ml fertilizer was added in the pellets. This was performed about once a week using the commercial liquid fertilizer NPK (in the ratio 6:3:6) supplemented with micronutrients (Substral<sup>®</sup>, SCOTT'S,

according to European Molecular Biology Organization practical course available at <http://www.isv.cnrs-gif.fr/embo01/manuels/index.html>.

The different experimental phases, i.e. establishment, cold acclimation, freezing and rewarming, were conducted as presented in Table 2. The cold acclimation phase was carried out following two steps as inspired by Thapa et al. (2008) to prevent plants from a cold shock upon transfer from 20 °C/14 °C day/night to 8 °C/2 °C. Experiments were performed in a growth chamber (DAGARD; size: 3.2 × 3.2 × 2.4 m; type: MA 100, class M1) lighted by a combination of two types of PHILIPS lamps: HPI-T400W (metal iodide) and SON-TP400W (high-pressure sodium), with a mean photosynthetic active radiation (PAR) of 450 μmol m<sup>-2</sup> s<sup>-1</sup>. Plant material was grown in controlled conditions during three series of experiments (and 3 individual experiments in each series), the first one including the collection lines and the second and third ones comprising the parental lines DZA045-5 and F83005-5, and their recombinant inbred progenies (LR3). For each of the three experiments dedicated to the evaluation of visual freezing damages of the collection lines, each line was represented by 50 individuals allocated to 5 blocks. Three experiments were also carried out to evaluate visual freezing damages on the parental and recombinant lines, in which each line was represented by 15 individuals allocated to 3 blocks. Lastly, for each of the three experiments conducted to observe physiological traits on the parental and recombinant lines, each genotype was represented by 15 individuals allocated to 3 blocks.

#### Visual freezing damage scoring

The levels of freezing damage (FD) were recorded twice during the freezing phase, after 1 and 8 days of freezing (FD1D and FD8D, respectively), and once after 2 weeks under rewarming conditions (FD2W). Plants were individually given a score from 0 (no damage) to 5 (dead). The plants were scored 1 for frost burn limited to the leaf edges, 2 for frost burn on the majority of the leaf surface, 3 for frost burn on upper third of the stem, 4 for frost burn on

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

upper three-quarters of the stem, as described for pea by Lejeune-Hénaut et al. (2008).

#### Growth characterization

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

#### Chlorophyll content assay

A non-destructive measurement of the chlorophyll content was realized at the end of the acclimation phase with a hand-held chlorophyll content meter CCM-200 (Opti-Sciences, Hudson, NH, USA) which provides an index (chlorophyll content index, CCI) estimating the leaf chlorophyll content (Richardson et al. 2002; Biber 2007). It was performed on five leaves per plant and three times on each leaf.

#### Electrolyte leakage assay

This assay was performed on foliar organs that underwent the freezing period, excluding newly expanded leaves, if any. Three trifoliolate leaves of each plant sampled after 2 weeks of the rewarming phase were detached and placed in a 50-ml tube containing 25 ml of ultra-pure water. The tubes were kept at room temperature for about 20 h; they were shaken before measuring the conductivity of the solution (C1) with an ION check 30 conductivity meter (Radiometer Analytica). The tubes were then frozen for a minimum of 1 day at –80 °C and thawed over night at room temperature. Thereafter they were shaken and the total potential conductivity (C2) was measured. The mean electrolyte leakage (EL) was calculated as a percentage of the total leakage from frozen samples (C1/C2 × 100).

#### Statistical and quantitative genetic analyses

##### *Quantitative genetic analyses*

Statistical analyses were performed with SAS statistical package v8.01 procedures (SAS Institute Inc., Cary, NC, USA; 2000). For each trait, the normality of residual distributions was tested with skewness, kurtosis and Shapiro–

Wilk statistics displayed by the “univariate” procedure. For QTL mapping, we estimated RILs best linear unbiased predictors (BLUPs) for each trait from the data recorded for the three replicated experiments using the SAS “mixed” procedure.

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

$$Y_{ijk} = \mu + E_i + G_j + \text{rep}_{(i)k} + \text{GE}_{ij} + \varepsilon_{ijk}$$

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

#### Genetic linkage map

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

#### QTL and epistasis analyses

QTL analyses were performed with Windows QTL Cartographer 2.5 (Wang et al. 2012). QTL were initially detected with composite interval mapping (CIM) using model 6. For each trait, the LOD significance threshold was determined after running 1,000 permutations test ( $\alpha = 0.05$ ). Cofactors were selected with the forward and backward regression method.

QTL were subsequently refined using multiple interval mapping (MIM) which fits a multiple QTL model to increase precision in the QTL detection. This was especially important in cases where multiple LOD peaks were

detected with CIM. We used the CIM QTL peaks as starting positions for the MIM and progressively refined the model using the  $\text{BIC-M2} = 2\ln(n)$  criterion. QTL positions that did not remain significant when fitted with others were then dropped from the model. QTL effects and their percentage of variance were estimated with the final model fitted in MIM.

For each QTL, the inner and outer limits of the confidence interval were calculated as LOD value reductions by 1 and 2 units, respectively, relatively to the LOD value of the most likely position. Mapchart 2.2 (Voorrips 2002) was used to draw the QTL on the linkage map.

QTL by QTL additive epistatic interactions were first detected for the significant QTL using MIM in Windows QTL Cartographer 2.5. To detect all pairwise additive epistatic interactions without prior knowledge of QTL positions, we used the program Epistacy (Holland 1998), which relies on linear models and least squares statistics from SAS system. Epistacy was run for all traits with an initial  $P$  value filter of 0.001. All interactions involving markers within 50 cM distance were removed to avoid linkage effects. To control for multiple comparisons, we used a final  $P$  value cutoff of  $3.57\text{E}-4$  obtained by dividing an initial  $P$  value of 0.01 by a factor of  $g(g-1)/2$ , where  $g$  is the number of linkage groups, as suggested by Holland (1998).

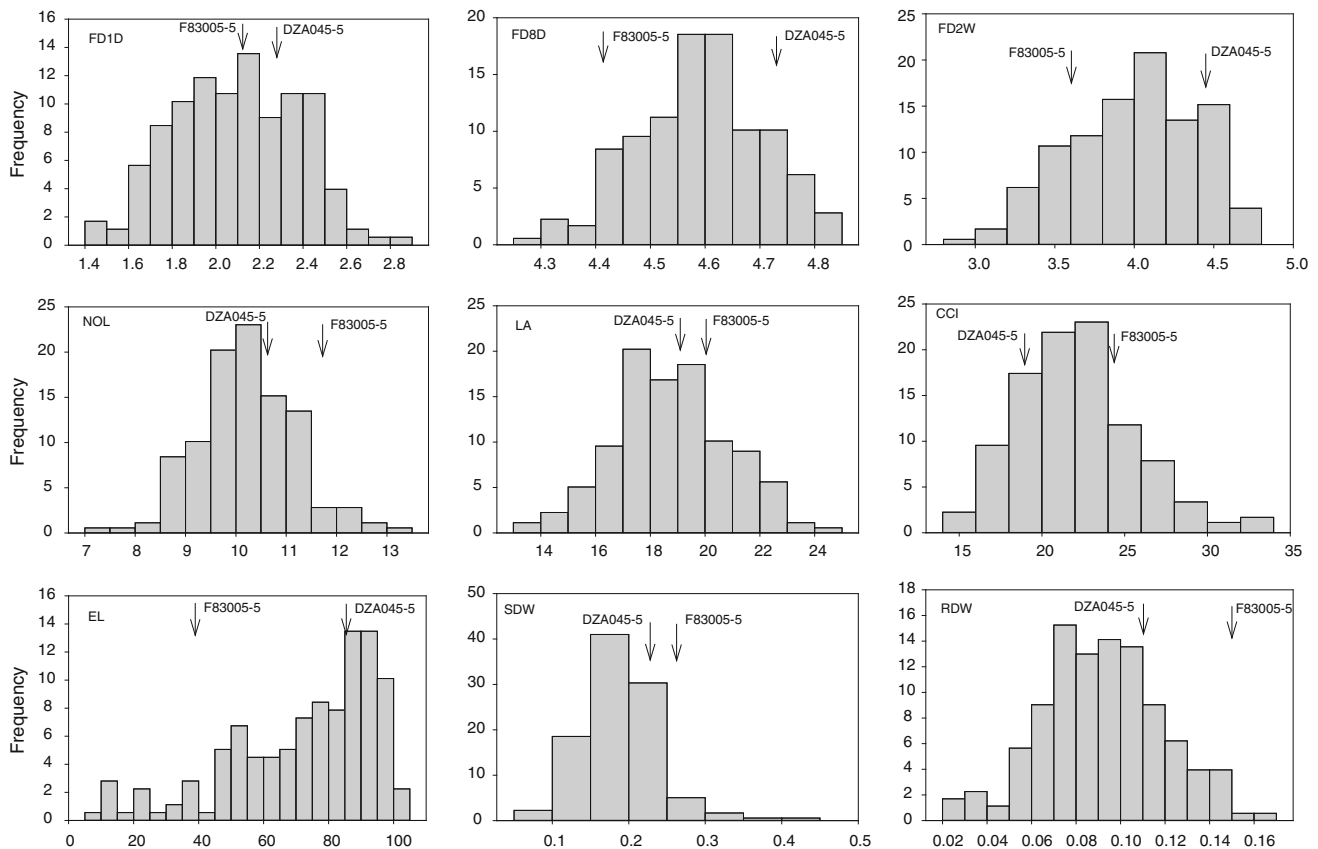
## Results

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

Visual freezing damage was scored 2 weeks after freezing treatment and the 15 *M. truncatula* accessions ranged from 1.8 (F11005-E) to 4 (DZA012-J) for FD (Table 1). Accessions originating from higher latitudes were globally the most tolerant ( $f$  group according to the SNK test for comparison of means, Table 1), which may reflect the adaptation tendency of accessions having evolved in geographical areas characterized by more frequent, even if not very intense, frost events. F83005-5 was an exception within the group of high latitude accessions, exhibiting a medium level of frost tolerance (FD = 3). Comparatively, DZA045-6 was one of the most sensitive accessions ( $a$  group according to the SNK test for comparison of means, Table 1).

### Quantitative genetics

All traits studied showed a normal Gaussian distribution within the RIL population (Fig. 1), except EL for which the distribution of the RILs was shifted toward the mean value



**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 1–8 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

of DZA045-5, the sensitive parental line. Box–Cox transformation did not improve significantly the distribution. The frequency distributions also showed that, in many cases, there was limited difference between both parents of the RILs. Nevertheless, transgressive individuals were found in the population and related QTL were detected.

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

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

## Genetic linkage map and QTL mapping

The genetic linkage map of the LR3 population comprised a total of 182 markers, including 111 SSR, 57 AFLP, 12 RAPD and 2 EST-CAPS, distributed over 8 linkage groups (LG, Fig. 2). The linkage map covered 1,225 cM Kosambi (1 marker every 6.7 cM, on average). Thirty-five SSR markers were anchored to sequenced BACs and 29 were common to the integrated genetic-physical map (<http://www.medicago.org/genome/map.php>, Univ. Minn. 2006; Mun et al. 2006). Order conservation of these 29 common markers between the present genetic map and the reference *M. truncatula* genetic and physical maps was very high. Few genomic regions remained poorly covered by the genetic linkage map, with the largest interval between two markers in the middle region of LG8.

The QTL detected by CIM and refined by MIM are presented in Fig. 2 and Table 5. We detected in total 29 QTL with 1–5 QTL per trait. Most of them lay on three linkage groups (LG1, LG4 and LG6). Those regions can roughly be delimited between markers MTIC125 and

MTIC064 (37 cM) on LG1, between L07.600 and MTIC037 (12 cM) on LG4 and between E39M61.511 and E12M49.265 (8 cM) on LG6. Two linkage groups (LG3 and LG8) had no QTL and only one QTL was observed on linkage groups LG2, LG5 and LG7.

#### QTL for freezing damage

QTL for freezing damage with overlapping confidence intervals were detected for FD8D and FD2W on LG1, LG4

**Table 3** Means, genetic variances ( $V_g$ ) and their coefficients of variation ( $CV_g$  expressed as  $\sqrt{V_g}/\text{trait mean}$ ), broad-sense heritabilities ( $H^2$ ) their standard errors for traits mapped in the *M. truncatula* LR3 population

Trait	Mean	$V_g$	$CV_g$	$H^2$	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

Traits names are described in the legend of Fig. 1

**Table 4** Genotypic and phenotypic correlations between each pair of the different traits analyzed in the *M. truncatula* LR3 population: genotypic correlations (above the diagonal) are on the top lines and

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	

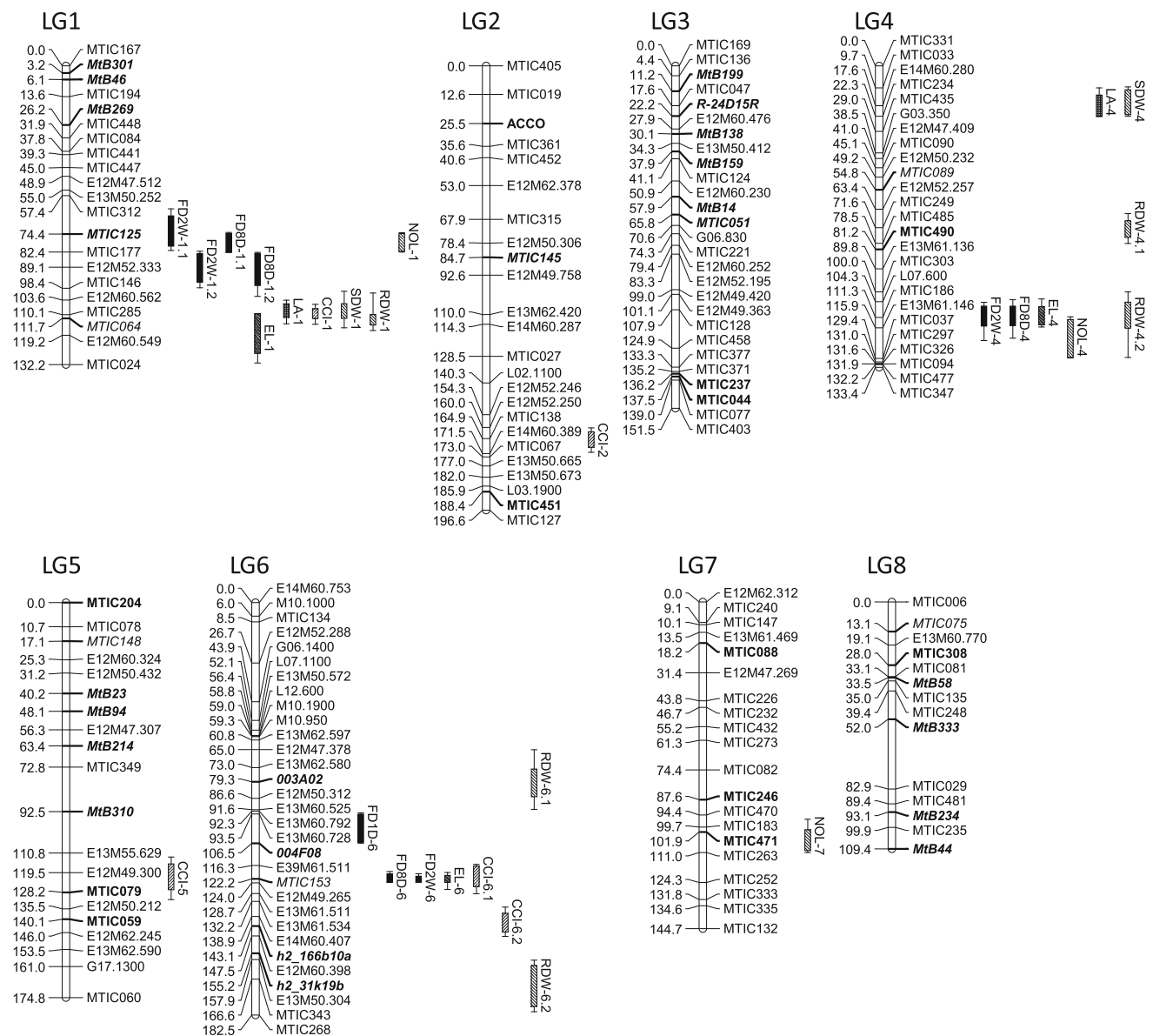
Traits names are described in the legend of Fig. 1

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

#### QTL for physiological traits

Seventeen among the 19 QTL for physiological traits lay on LG1, LG4 and LG6, and 6 of them overlapped with FD QTL (Fig. 2). EL QTL colocalized with the most explanatory FD QTL on LG4 and LG6, with an  $R^2$  value of 18.8 % on the latter. Additive effects of EL (Table 5) showed that the F83005-5 alleles were favorable for all the positions, i.e., corresponded to lower loss of electrolytes from foliar tissues. Among the five QTL detected for CCI, one also colocalized with the FD QTL on LG6 with an  $R^2$  value equal to 4.9 % and a positive F83005-5 additive effect.

their standard errors on the bottom lines; phenotypic correlations (below the diagonal) are on the top lines and associated  $P$  values on the bottom lines



**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

Overlapping confidence intervals between FD and physiological traits' QTL were also found on LG4, where the positions of FD8D and FD2W QTL coincided with those of QTL for EL ( $R^2 = 5.1$ ), RDW ( $R^2 = 5.4$  %) and NOL ( $R^2 = 9.2$  %). For these three traits, the values of the additive effects of F83005-5 were positive.

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

The signs of the additive effects observed at the positions where QTL for freezing damage traits (FD or EL) colocalized with physiological traits (NOL, CCI, RDW) are consistent with the negative genotypic correlations reported between these two groups of traits in Table 4.

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



**Table 5** QTL parameters in the *M. truncatula* LR3 population

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
FD2 W	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

Traits names are described in the legend of Fig. 1. QTL are named by their trait 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 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 percentage of phenotypic variance explained by each QTL and  $a$  is the additive effect relative to the parent F83005-5

### Epistatic interactions

Running the program Epistacy enabled the identification of 88 significant results with 3–22 interactions per trait. Keeping only one representative marker with the lowest  $P$  value when significant interactions involved several very close markers for the same traits allowed us to further filter the results. Final results with 38 significant additive-by-additive epistatic interactions from Epistacy output are presented in Online Resource 1. We detected three to six significant interactions per trait. Only two interactions were detected between QTL pairs and both were related to CCI. One of them (QTL on LG6 and LG1) was also detected by MIM in Windows QTL Cartographer. For both of these

CCI  $\times$  CCI interactions, one or two doses of the F83005-5 allele corresponded to higher CCI genotypic means (Online Resource 1). Four interactions were detected between a QTL (CCI on LG6, LA and RDW on LG4 and NOL on LG7) and a locus of the genetic background. Finally 32 interactions were detected between loci of the genetic background. Partial  $R^2$  values varied between 6.3 and 12.7 %.

Three loci, namely MTB14 on LG3, MTIC059 and G17.1300 on LG5, were involved in 6, 5 and 4 epistatic interactions, respectively, suggesting pleiotropic effects on FD1D, FD8D, FD2W, EL and RDW for MTB14, on FD1D, FD8D, FD2W and EL for MTIC059 and on FD2W, EL, NOL and RDW for G17.1300. MTIC059 and MTB14

were involved four times in the same epistatic interaction. On FD traits and EL, one or two DZA045-5 alleles at interacting loci imply that one or two of these markers were associated with higher values of frost damages and foliar electrolyte leakage.

## Discussion

### Genetic variability for freezing tolerance

The results obtained for 15 accessions of diverse geographical origins (Table 1) pointed out a relatively large genetic variability in the *M. truncatula* tolerance to sub-freezing temperatures after a cold acclimation phase. These results demonstrated a global relationship between the geographical origin of the accessions and their freezing tolerance. Most of the accessions originating from lower latitudes (Algeria, Cyprus, Syria) were found to be more sensitive than those from France. However, no clear relationship was found between freezing tolerance and the altitude of the area of origin, which is illustrated by the fact that both high and low collection sites represented sensitive lines from the same latitudes (DZA.233-4 and DZA045-6, respectively). The influence of the geographical origin of plants on their level of freezing tolerance has been documented in various species. It is indeed frequently reported, within a given species, that ecotypes from northern locations (which generally experience lower winter temperatures) present superior levels of freezing tolerance. This statement is generally verified for herbaceous species (Finne et al. 2000; Hamilton et al. 2002; Nakayama et al. 1997; Rukavina et al. 2007; Leinonen et al. 2011), although it can be disturbed by the presence of snow which probably reduces the selection pressure exerted by low temperatures (Dionne et al. 2010). The altitude effect has also sometimes been evidenced (Annicchiarico et al. 2001; Loik and Redar 2003), the high altitudinal ecotypes being more tolerant to frost. A similar relationship between altitudinal provenance and freezing damage was also observed in four other *Medicago* species (Yahia and Fyad-Lameche 2003). Screening of a collection of *M. truncatula* including accessions from a broader range of altitude may be required to confirm our preliminary observations on 15 accessions.

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

The linkage map of LR3 covered 1,225 cM Kosambi, which is higher than the size of genetic maps previously established from A17 × A20 F2 populations (513 cM in Choi et al. 2004a; 566 cM in Mun et al. 2006). But this is consistent with genetic map lengths previously reported for Jemalong-6 × DZA.315-16 F2 or F6 RIL populations

(1,225 and 1,097 cM in Thoquet et al. 2002 and Huguet 2004, respectively) as well as for the LR3 RIL population by Gorton et al. (2012). Several of the SSR markers were located in sequenced BACs or common with the genetic-physical map of the species. Those markers are likely to facilitate the exploration of interesting regions detected by QTL mapping.

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

Results obtained for QTL mapping in the LR3 population showed that freezing tolerance in *M. truncatula* is controlled by a limited number of QTL mainly colocalizing in three regions on LG1, LG4 and LG6. Even if it can partly be due to the limited contrast between parents (Table 1), this observation is also consistent with the relatively low number of QTL generally found for traits reflecting FT or WH in legume species. In alfalfa, Brouwer et al. (2000) identified seven QTL for winter injury, among which only two were consistent among the 2 years of experimentation. In lentil, Eujayl et al. (1999) found that the frost tolerance trait, evaluated in field conditions, was inherited as a 1:1 ratio of tolerant to sensitive in a population of RILs, suggesting monogenic inheritance. In a different genetic background of lentil, Kahraman et al. (2004) detected one to three QTL of winter survival in different field environments, with one QTL being common to all environments. In faba bean, three and two QTL were found for frost tolerance in controlled conditions for unhardened and hardened plants, respectively (Arbaoui et al. 2008). In pea, Liesenfeld et al. (1986) estimated that as few as three additive genes or tight linkage groups may condition winter hardiness in the progenies they experimented, and Lejeune-Hénaut et al. (2008), studying winter freezing damages in a population of RILs, detected six QTL among which three were identified for all their field experiments. These three consistent QTL coincided with 3 (of 4) QTL for freezing damages assessed for the same population under controlled conditions (Dumont et al. 2009). In other plant families, QTL analysis for frost tolerance also suggested that a limited number of regions of the genome were involved (review on Triticeae: Cattivelli et al. 2002; oat: Wooten et al. 2009; ryegrass: Xiong et al. 2007; rapeseed: Kole et al. 2002; Asghari et al. 2008). When such QTL positions are consistent among environmental conditions and genetic backgrounds, they constitute valuable targets for marker assisted selection. The results of the present study are a first insight into the genetic determinism of frost tolerance in the model legume *M. truncatula* which could help identify candidate genes underlying QTL and benefit to closely related cultivated species like pea or faba bean, provided that syntenic QTL are identified. For 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, to further confirm and/or to identify additional QTL that affect freezing tolerance.

QTL for physiological traits such as EL, NOL, LA, CCI or 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 were negative, suggests that higher values of NOL, 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).

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

QTL mapping for NOL and CCI suggests that frost tolerance is improved when the photosynthetic apparatus presents sufficient size and efficiency at the end of the acclimation phase, as shown by the colocalizations between FD QTL and NOL on LG1 and LG4 and between FD QTL and CCI on LG6. In pea, Lejeune-Hénaut et al. (2010) have already observed that pea seedlings must have a minimum NOL (2 or 3) to resist frost. Dumont et al. (2009) showed that pea plantlets characterized by more numerous branches at the end of the acclimation phase in controlled conditions were more tolerant to frost, with QTL for the corresponding trait coinciding with two of the most consistent QTL for frost tolerance. Asghari et al. (2008) found a single minor effect QTL for winter survival of rapeseed which coincided with a QTL for the NOL at the rosette stage. For both traits, the favorable alleles were brought by the tolerant parent. In *M. truncatula*, QTL for NOL and aerial fresh weight under salt stress were detected on the linkage group 6 within the population Jemalong A17  $\times$  F83005-5 (Arraouadi et al. 2011), at a position that would deserve to be more precisely compared to the LR3 genetic map, thanks to common markers which are currently lacking.

Coincidental QTL mapping for RDW and FD on LG4 suggests that superior allocation of dry matter to the root system may indirectly contribute to superior freezing tolerance in *M. truncatula*. Alternatively, this may reflect a superior potential for cold acclimation which allows the accumulation of C and N metabolites that could help sustain a more vigorous regrowth after freezing. A higher partitioning of dry matter in roots than in shoots under low temperatures (LT) has already been reported in *M. truncatula*. Thapa et al. (2008) observed that growing under LT regimes, comparatively to control conditions, resulted in a global reduction of dry matter for Jemalong 6 and W6 5018, together with an increase of the root:shoot ratio for both genotypes. The growth of Jemalong 6 under LT was, however, less reduced than for W6 5018 and this genotype especially proved to be able to produce the same amount of RDW under LT than under control conditions. The authors related this behavior to the higher frost tolerance exhibited by Jemalong 6 after a LT period, highlighting its greater cold acclimation ability. In pea, Lejeune-Hénaut et al. (2010) showed that dry matter production was higher under LT conditions than under control conditions for Champagne and Terese, and that a higher root:shoot ratio was also observed under LT, particularly for the frost tolerant genotype Champagne. Similar effects of LT, i.e. higher dry weight production in shoots and roots, were also reported in other species like wheat (Equiza et al. 1997) with higher values observed for the most frost tolerant genotypes.

It seems finally that F83005-5 is able to produce more leaves to maintain its photosynthetic ability to a higher

level and to allocate more dry matter to roots during acclimation to low non-freezing temperatures. The plant capacity to acclimate and maintain growth at low above-freezing temperatures would allow superior tolerance to freezing and a more vigorous regrowth upon return to warmer temperatures. To which extent the ability to acclimate and maintain growth at low temperatures is involved in the development of superior cryoprotection, which is suggested by limited damage on cell membranes, has not been tested here.

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

### Epistatic interactions

Epistatic interactions among genes can be important for phenotypic expression or genotypic variation in populations. In *M. truncatula*, such interactions have already been reported, within the LR3 population, for traits related to resistance to *Aphanomyces euteiches* (Hamon et al. 2010). In this study, we detected 38 epistatic interactions for FD traits or potentially related physiological traits. Most of these interactions (32) were detected between markers of the genetic background, a higher number than for resistance to *Aphanomyces euteiches*. For this trait, Hamon et al. (2010) found only four interactions between genomic regions that did not have an additive effect on resistance to *Aphanomyces euteiches*, among 12 identified in total. In particular in the present study, no epistatic interaction was directly associated to FD or EL QTL, but 19 significant epistatic interactions were identified for these traits between markers of the genetic background and cannot be neglected. Attention should be especially paid to loci such as MTB14 on LG3, MTIC059 and G17.1300 on LG5, which are involved in 8 among 19 interactions and for which one or two DZA045-5 alleles at interacting loci were associated with higher values of frost damages and foliar electrolyte leakage.

### Perspectives for fine mapping

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

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

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