

Applying Freezing Test to Quantify Cold Acclimation in *Medicago truncatula*

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ABSTRACT. Understanding cold acclimation (CA) is important for concurrently improving autumn yield and winter survival in alfalfa (*Medicago sativa* L.). *Medicago truncatula* Gaertn., an annual relative of alfalfa, could be used to determine genetic bases of CA, if the ability and conditions required for its CA are determined. The major objective of this study was to develop a laboratory screening procedure to quantify CA in *M. truncatula*. Two genotypes, Jemalong-6 and W6 5018, were grown in nonacclimation (NA) and three CA regimes (CA1, CA2, and CA3). CA was quantified by measuring freezing tolerance [LT₅₀ (the freeze temperature at which 50% injury occurred)], as estimated by ion leakage (IL) from leaf tissues. The percentage of injury and LT₅₀ were derived from freeze injury data. Cold-acclimated plants had reduced stem length, number of leaves, stem dry weight, leaf dry weight, and root dry weight compared with control. Root-to-shoot ratio was higher in cold-acclimated than in control plants. These results indicate the clear initiation of acclimation response in cold-acclimated plants. Average LT₅₀ temperatures were -3, -8, -7.8, and ≥ -12.5 °C in NA, CA1, CA2, and CA3 regimes, respectively. While 80% injury was induced by -7 °C in NA plants, -20 °C only induced an average of 52% injury in CA3 plants. While the percentage of injury was lower in Jemalong-6 than in W6 5018 for all CA regimes, CA3 regime was most effective in distinguishing CA ability of the two genotypes. Our results demonstrated the capability of *M. truncatula* to cold acclimate under the controlled CA regimes and the possible use of IL as a rapid laboratory method to quantify CA.

Increased autumn growth is one means of improving yield in alfalfa. However, an inverse relationship between biomass and winterhardiness often complicates the simultaneous improvement of both traits (Brummer, 2004). Alfalfa is known to acclimate as temperatures fall and photoperiod decreases during autumn (McKenzie et al., 1988), and significant advances in understanding the physiological changes that alfalfa undergoes during cold acclimation have been made (Castonguay et al., 2006). However, alfalfa is a tetrasomic tetraploid, complicating genetic analysis, and outcrossing with inbreeding depression, which prevents the development of inbred lines. Both aspects of alfalfa hinder investigations on the genetic basis of autumn acclimation (also called “fall dormancy”), cold tolerance, and winter survival.

Medicago truncatula (barrel medic) is an annual legume, closely related to alfalfa, grown as a winter forage and green manure crop in Mediterranean climatic zones around the world. In the north-central United States, annual medics can produce high yields of quality forage when grown as short season annual crops for autumn harvest (Zhu et al., 1996). Unlike alfalfa, *M. truncatula* is diploid and self-pollinated, characteristics that make it more tractable for genetic analysis than alfalfa. Although *M. truncatula* does not survive winter conditions in the northern United States, it often encounters and survives

frost when cultivated, and therefore, it may offer some clues about cold tolerance in alfalfa.

Numerous biochemical/physiological changes occur during cold acclimation, including increases in cold-stress proteins (Arora and Wisniewski, 1994; Arora et al., 1997; Marian et al., 2004), the accumulation of cryoprotectants such as proline (Wanner and Junttila, 1999) and sugar (Haagenson et al., 2003; Koster and Lynch, 1992; Patton et al., 2007), and increases in the unsaturated-to-saturated fatty acid ratio and in phospholipids in the plasma membrane (Palta et al., 1993). A key function of cold acclimation is to stabilize membranes against freezing-induced cellular dehydration (Thomashow, 1999). Ion leakage (IL) resulting from decreased membrane integrity of freeze-damaged tissues can be easily measured in the laboratory to determine freezing tolerance of diverse species (Arora and Palta, 1991; Nunes and Smith, 2003; Webb et al., 1994; Welling et al., 2002), including alfalfa (Sulc et al., 1991). This method is simple, repeatable, and rapid, ensures freezing stress on intact tissues, and has been used to predict field performance (Teutonico et al., 1993).

Medicago truncatula cold acclimation has been assessed in several experiments, with contradictory results. The cultivar Paraggio, grown at 10 °C, appeared to acclimate to the cool temperature by exhibiting a 2-fold increase in the quantum yield of photosystem II electron transport (Antolín et al., 2005). In contrast, plants exposed to cool conditions (10 °C day/5 °C night) exhibited poor regrowth capacity compared with

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nonacclimated plants when it was assessed by measuring dry matter production after freezing test. The cold-acclimated plants had decreased sucrose phosphate activity in leaves and with no accumulation of starch in roots, suggesting ineffective cold acclimation process in the species (Hekneby et al., 2006). Brandsæter et al. (2000) evaluated 12 winter legume species hardened at 2 °C for 2 weeks to investigate their potential as overwintering cover crops and reported the poorest frost survival in *M. truncatula*. The lack of consensus among these studies on the ability of *M. truncatula* to exhibit cold acclimation possibly results from the different freezing test procedures used or the different genotypes used in the studies.

In this experiment, we developed a freezing tolerance assay for *M. truncatula* that is rapid and reproducible to test the hypotheses that cellular injury decreases as the acclimation temperature to which plants are exposed decreases. We evaluated two *M. truncatula* genotypes under four acclimation regimes to determine their maximum freezing tolerance under controlled conditions. Growth and development were measured concurrently to understand the physiology of cold acclimation in *M. truncatula*.

Materials and Methods

PLANT MATERIAL. Two *M. truncatula* genotypes, Jemalong-6 (derived from the Australian cultivar Jemalong) and W6 5018 (a wild accession from Italy), were used for this study. Seeds of Jemalong-6 were obtained from Institut National de la Recherche Agronomique (INRA), Mauguio, France, and W6 5018 from the U.S. Department of Agriculture National Plant Germplasm System (NPGS), Western Plant Introduction Center, Pullman, WA. Jemalong-6 is one of the parents of a mapping population that has been analyzed for morphogenetic traits and QTL analysis in *M. truncatula* (Julier et al., 2007). Our previous study showed that W6 5018 had an erect plant type, was late flowering, and produced high biomass, while Jemalong-6 was early flowering, produced decumbent shoots, and produced low biomass under simulated autumn conditions (unpublished results). We used these two phenotypically contrasting genotypes to assess genotypic differences in cold acclimation. In alfalfa, cultivars with short, prostrate growth in autumn tend to be winterhardy, while those with erect shoots and rapid growth are not (Sheaffer et al., 1992).

GROWTH CONDITIONS. Seeds were scarified using medium grain sandpaper 1 d before seeding into individual plastic pots (capacity of ≈2500 cm³) filled with Sunshine Professional growing mix (SB300 Universal Mix; Sun Gro Horticulture, Bellevue, WA). The pots were placed in a greenhouse at 22 °C day/18 °C night temperatures with a 16-h photoperiod (Moreau et al., 2006). A total of 260 pots, 130 of each genotype, were seeded with five seeds per pot. Pots were thinned to one seedling after 2 weeks. After all plants developed at least five leaves (≈4 weeks), they were transferred to growth chambers and allocated to one of three temperature regimes.

Plants of each genotype were divided into three groups; 60 in nonacclimation (NA), 35 in cold acclimation regime 1 (CA1), and 35 in cold acclimation regime 2 (CA2). Genotypes

were assigned to positions on growth chamber benches using a completely random experimental design. The temperatures, photoperiod, and photosynthetic photon flux (*PPF*) applied to each growth regime, together with the duration plants were exposed to those conditions, are presented in Table 1. After plants in the CA2 treatment were sampled for freezing tolerance, they were transferred to another chamber for the cold acclimation 3 (CA3) regime, where they were further exposed to a still lower temperature of 3.5 °C day/−1 °C night for 1 week at 180 μmol·m⁻²·s⁻¹ *PPF* and a 16-h photoperiod and were sampled for freezing tolerance of the CA3 regime. In CA3, plants were uniformly sprayed with an ice water mix to initiate ice-nucleation, three times in alternate days, during the treatment time. Growth chamber temperatures were monitored using a HOBO logger (Onset, Pocasset, MA). Light intensity was routinely monitored by a quantum sensor (LI-185; LICOR, Lincoln, NE) throughout the experiment and the height of lights from the top of the plant canopy were adjusted to maintain constant intensity. Plants in all chambers were fertilized with Peters Excel (15N–2.2P–12.5K; Scotts, Marysville, OH) diluted 100-fold with distilled water. Each plant received ≈250 mL of nutrient solution every 2 weeks and was lightly watered every day.

DETERMINATION OF FREEZING TOLERANCE. IL was used to assess freezing tolerance (FT) and cold acclimation in *M. truncatula* by exposing tissue to a laboratory-controlled freeze-thaw protocol. IL and injury assessment were patterned after Peng et al. (2007). FT of nonacclimated plants was measured three times (week 1, week 2, and week 3) after the treatments began to determine developmental influences on the tolerance, if any. FT of plants in the CA regimes was measured at the end of the treatment period, after 4 weeks for CA1, 5 weeks for CA2, and 6 weeks for CA3 (Table 1). The freezing test temperatures were as follows: −1, −2, −3, −4, −5, −6, and −7 °C for NA; −3, −5, −6, −7, −8, −9, −10, −11, −13, and −15 °C for CA1; −5, −7, −9, −10, −11, −12, −13, −15, and 17 °C for CA2; −4, −6, −8, −10, −12, −14, −16, −18, and −20 °C for CA3. For each test temperature, two leaves of similar age (the first fully expanded leaf from the tip) were sampled from each of three replicate plants for a total of six replications and were placed in test tubes. Six unfrozen samples of each genotype were placed on ice, as unfrozen controls. Treatment samples were placed in a glycol bath at −1 °C for 1 h, after which freezing was initiated

Table 1. Air temperature, photoperiod, photosynthetic photon flux (*PPF*), and growth duration of *Medicago truncatula* genotypes, Jemalong-6 and W6 5018, at nonacclimation (NA = 3 weeks) and cold acclimation (CA1 = 4 weeks; CA2 = 5 weeks; CA3 = 6 weeks) growth regimes. CA2 plants were transferred to CA3 regime for 1 week at lower temperature (3.5 °C day/−1 °C night).

Growth regimes	Temperature [°C (day/night)]	Photoperiod (h)	<i>PPF</i> (μmol·m ⁻² ·s ⁻¹)	Duration (weeks)
Nonacclimation (NA)	22/18	16	400	3
Cold acclimation 1 (CA1)	18/10	16	400	2
	10/5	16	400	2
Cold acclimation 2 (CA2)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
Cold acclimation 3 (CA3)	18/10	16	400	1
	10/5	16	400	2
	7/4	16	180	2
	3.5/−1	16	180	1

by ice-nucleation produced by dropping a small ice crystal into each tube and kept for 1 h before further lowering the temperature.

The temperature in glycol bath was lowered at 1 °C/40 min and the frozen samples were removed at each test temperature and thawed in ice overnight. On the following day, the samples were removed from ice and kept at 4 °C for 1.5 h and at 22 °C (room temperature) for another 1 h. Twenty milliliters of deionized water was added to each sample, which were then vacuum infiltrated four times (1 min each) and placed on a gyratory platform shaker (Innova-2300; New Brunswick Scientific, Edison, NJ) at 250 rpm for 1.5 h. An initial IL measurement was then taken with a conductivity meter (model 3100; YSI, Yellow Springs, OH). Samples were autoclaved at 121 °C for 20 min with a slow exhaust cycle of 0.45 kg·min⁻¹ and after being brought to room temperature, the total IL was measured. Initial leakage was expressed as percentage of the final IL value, and percentage of leakage for each treatment was converted to percentage of injury as

$$\text{Percentage of injury} = \left[\frac{\% \text{ IL (t)} - \% \text{ IL (c)}}{100 - \% \text{ IL (c)}} \right] \times 100$$

where % IL (t) and % IL (c) are measurements of percentage of IL from the respective freeze-treatment temperature and the unfrozen control, respectively. LT₅₀, a measure of FT, was derived for both the genotypes in all growth regimes by determining the freeze test temperature at which 50% injury (midpoint of maximum and minimum percentage of injury) occurred (Fig. 1), as explained in Lim et al. (1998).

GROWTH EVALUATIONS. The number of leaves and the length of the primary stem were measured on four randomly selected plants from each treatment (NA, CA1, and CA2) at weekly intervals for the first 3 weeks after treatments began. Plants were sampled for dry weights at FT sampling time of each growth regime. Roots, stems, and leaves of each plant were

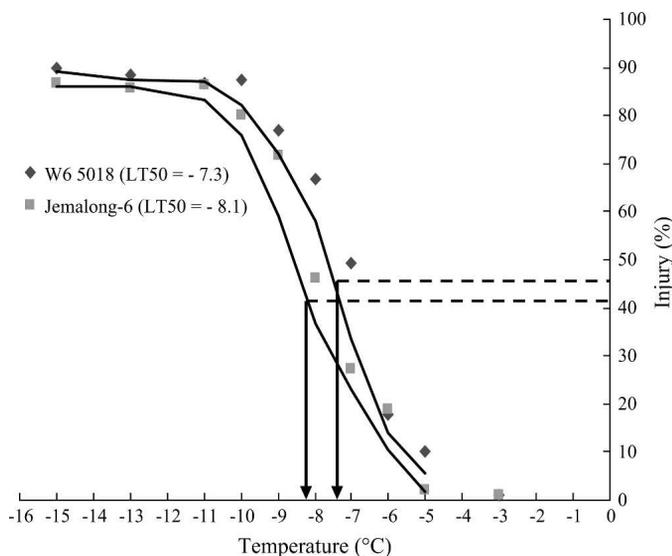


Fig. 1. Freezing tolerance [LT₅₀ (temperature at which 50% injury occurred)] of *Medicago truncatula* genotypes W6 5018 and Jemalong-6 at cold acclimation (CA1) growth regime. The dashed lines show 50% injury of a particular genotype and the bold arrows show the respective LT₅₀ temperature. LT₅₀ was derived as [(maximum % injury - minimum % injury) / 2] + minimum % injury. Injury was measured at each test temperature after 4 weeks of CA1 growth regime.

separated and dried for 3 d at 60 °C to estimate the dry weight (Hoy et al., 2002). Leaf area (LA) was measured using a leaf area meter (LI-3100; LI-COR). Specific leaf area [SLA (leaf area per leaf dry weight)] and root-to-shoot ratio (RS) were also determined on each sample.

STATISTICAL ANALYSIS. The experimental design was a completely randomized design with genotypes and temperature treatments as fixed factors. Statistical analyses were conducted using the GLM procedure of SAS (version 8; SAS Institute, Cary, NC). Differences between the treatment means were separated by Fisher's protected least significance (LSD) test at 0.05 and 0.01 *P* levels. Regressions were performed separately for each genotype at different growth regimes using freeze test temperatures and PI data.

Results

DEVELOPMENT OF FREEZING TOLERANCE. The two *M. truncatula* genotypes maintained at NA and CA regimes varied for leaf PI with test temperatures (Fig. 2, A–D). For NA plants, no effect of sampling time (week 1, week 2, or week 3) or genotype × sampling time interaction was evident, therefore only one sampling time (week 2) was used to compare the results with CA regimes (Fig. 2A). Nonacclimated tissues did not suffer any injury when frozen at –1 °C (data not shown), had the lowest injury at –2 °C, and suffered incrementally more pronounced injury with decreasing test temperatures (Fig. 2A). Nonacclimated plants of the two genotypes differed in injury only at –3 °C when W6 5018 showed 10% injury compared with 23% for Jemalong-6. The maximum injury recorded for nonacclimated tissues was about 90% at –7 °C.

Leaves from CA1 plants did not show the incidence of freeze injury until –3 °C (Fig. 2B). However, freezing injury increased steadily from –5 to –10 °C, after which there was no further increase in injury in either genotype. A similar trend was observed at CA2 (Fig. 2C). The temperatures at which genotypes differed varied among cold treatments, with CA1 [–5, –7, and –8 °C (Fig. 2B)] and with CA2 [–5, –9, and –10 °C (Fig. 2C)]. In contrast to nonacclimated samples, Jemalong-6 had higher freezing tolerance than W6 5018, as it suffered lower freeze injury at test temperatures in CA1 and CA2 at which the genotypes differed (Fig. 1, B and C). Interestingly, the differences in PI between the two genotypes were apparent at almost every test temperature in CA3 (Fig. 2D). In addition, the difference between genotypes in PI widened in CA3 with decreasing test temperatures, reaching 65% in W65018, but only 39% in Jemalong-6 at the lowest test temperature (–20 °C). Although the injury increased steadily with decreasing test temperatures at CA3 regime, it did not reach a plateau for either genotype.

FT (LT₅₀) for genotype and its interaction with growth regimes was not significant. FT of the *M. truncatula* genotypes varied with growth regime (Table 2). Within the NA growth durations (week 1, week 2, and week 3), FT of the genotypes was similar, with LT₅₀ about –3.0 °C. However, LT₅₀ temperatures of all the CA regimes were lower than the nonacclimated plants. FT of plants grown at CA1 and CA2 regimes did not differ, as evident by similar average LT₅₀, –7.6 and –7.7 °C, respectively, across genotypes. When the CA2 plants were exposed to a lower temperature (3.5 °C day/–1 °C night) for an additional week (CA3 regime), the FT of the plants were increased by almost ≥ –4 °C. Whereas two genotypes exhibited

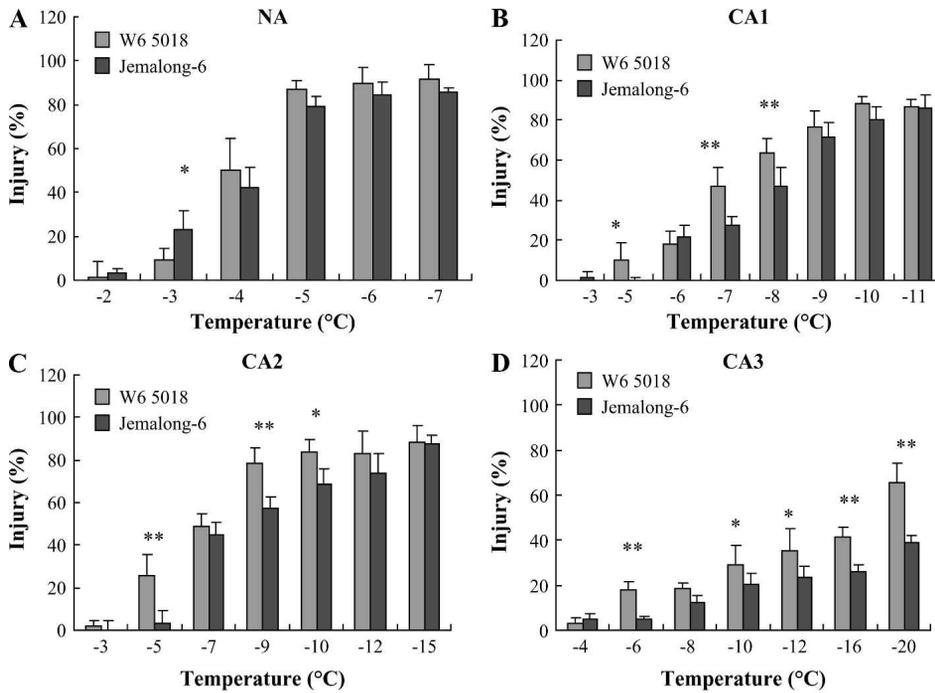


Fig. 2. Percentage of injury measured at different freeze test temperatures in leaves of *Medicago truncatula* genotypes W6 5018 and Jemalong-6 at (A) nonacclimation (NA), (B) cold acclimation 1 (CA1), (C) cold acclimation 2 (CA2), and (D) cold acclimation 3 (CA3) regimes. Measurements in CA1 (18 °C day/10 °C night for 2 weeks followed by 10 °C day/5 °C night for 2 weeks), CA2 (18 °C day/10 °C night for 1 week followed by 10 °C day/5 °C night for 2 weeks and 7 °C day/4 °C night for 2 weeks), and CA3 (CA2 regime followed by 3.5 °C day/–1 °C night for 1 week) were taken after 4, 5, and 6 weeks, respectively, after the treatments. Measurement in NA (22 °C day/18 °C night) was taken at the first week under the treatment. The vertical bars represent \pm SD of six replications. Significant differences between the two genotypes at 0.05 and 0.001 confidence levels at a particular test temperature are marked with * and **, respectively.

Table 2. Freezing tolerance [LT₅₀ (temperature at which 50% injury occurred)] of *Medicago truncatula* genotypes, W6 5018, and Jemalong-6, as influenced by nonacclimation [NA (22 °C day/18 °C night)] at a weekly interval (week 1, week 2, week 3) and cold acclimation (CA1, CA2, and CA3) regimes. Measurements in CA1 (18 °C day/10 °C night for 2 weeks followed by 10 °C day/5 °C night for 2 weeks), CA2 (18 °C day/10 °C night for 1 week followed by 10 °C day/5 °C night for 2 weeks and 7 °C day/4 °C night for 2 weeks), and CA3 (CA2 regime followed by 3.5 °C day/–1 °C night for 1 week) were taken after 4, 5, and 6 weeks, respectively, after the treatments. Mean LT₅₀ values \pm SD are shown for three replicate plants. LT₅₀ was derived as [(maximum % injury – minimum % injury) / 2] + minimum % injury.

Growth regime	LT ₅₀ [°C (mean \pm SD)]	
	W65018	Jemalong-6
NA		
Week 1	–3.4 \pm 0.49 a ^z	–3.1 \pm 0.36 a
Week 2	–2.9 \pm 0.23 a	–2.9 \pm 0.40 a
Week 3	–3.4 \pm 0.15 a	–3.6 \pm 0.10 a
CA		
CA1	–7.3 \pm 0.53 b	–8.1 \pm 0.43 b
CA2	–7.5 \pm 0.61 b	–7.9 \pm 0.25 b
CA3	\geq –13.3 \pm 2.43 c	\geq –12.3 \pm 2.10 c

^zValues within columns with different letters are significantly different based on LSD at $P = 0.01$.

difference in their cold acclimation ability (Fig. 2, B–D), cultivar difference in LT₅₀ was not evident in nonacclimated or in cold-acclimated leaf tissues. Consequently, LT₅₀ values differentiated FT between nonacclimated and cold-acclimated

plants but was not an effective measure of discriminating the tolerance between the two *M. truncatula* genotypes before or after cold acclimation.

GROWTH EVALUATIONS. The number of leaves per plant and stem length varied with growth regimes (Fig. 3, A and B). Stem length was reduced drastically in CA1 and CA2 compared with NA regimes at every week (Fig. 3A) during the first 3 weeks after treatment regimes began, but to a smaller extent between the CA1 and CA2 regimes. Similar results were also observed for the number of leaves per stem (Fig. 3B). In general, W6 5018 produced longer stems than Jemalong-6 under NA and CA2 (week 2), while this difference between the genotypes narrowed in the CA1 regime. Jemalong-6 developed average of one leaf more than W6 5018 in CA1, while they had similar number of leaves in the CA2 growth regime (Fig. 3B).

Dry weights of the two genotypes did not differ at the first week of NA regime, although W6 5018 had higher leaf area compared with Jemalong-6 (Table 3). The difference in dry weights between the two

genotypes was apparent at week 2 and week 3, and at both times, W6 5018 had higher leaf dry weight (LDW), stem dry weight (SDW), and root dry weight (RDW) compared with Jemalong-6. SLA and RS remained the same for both the genotypes at all NA growth durations. However, both the genotypes produced similar dry weights, LA, RS, and SLA at CA1 and CA2 regimes. W6 5018 produced higher SDW and LA compared with Jemalong-6 at CA3 regime, but both the genotypes had similar LDW, RDW, and RS. Comparing the nonacclimated plants to cold-acclimated plants, it was clear that CA treatments reduced the growth of the plants as evident by reduced dry weights in both genotypes. Plants grown for 6 weeks in the CA3 regime produced much lower dry weights than week 3 plants. CA3 plants had comparable LDW and SDW, but higher RDW and RS compared with week 2 NA plants. However, the dry weights of CA1 plants with only 4 weeks of growth were comparable to 3 weeks old NA plants. These results clearly indicate that temperature had larger effect in dry weights than the growth duration at any CA growth regime. However, both the genotypes had comparable LA but reduced SLA at each cold acclimation regime.

Discussion

An IL test performed on leaf tissue after a freeze-thaw event was efficient for determining FT and acclimation ability differences in the two genotypes of *M. truncatula*. Cell membranes are critical sites of freezing injury in plants. Numerous factors are involved in membrane damage at

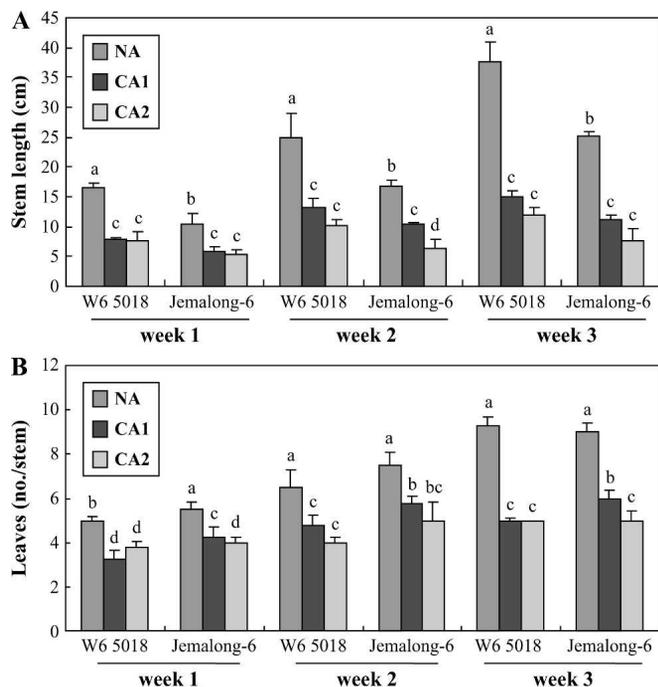


Fig. 3. Effect of growth regimes [nonacclimation (NA), cold acclimation 1 (CA1), and cold acclimation 2 (CA2)] on stem length (A) and number of leaves on the first primary stem (B) of *Medicago truncatula* genotypes W65018 and Jemalong-6 during the first 3 weeks (week 1, week 2, and week 3). Means with the same letters in each week were not significantly different based on LSD at $P < 0.05$. The vertical bars represent \pm SD based on mean of four replications.

freezing temperatures, such as cellular dehydration (lamellar-to-hexagonal-II phase transitions), expansion-induced lysis, and damage caused by reactive oxygen species, protein denaturation, etc. (Thomashow, 1999). Thus, protection of the cell membrane damage from such factors is important to improving FT. Cold acclimation induces metabolic changes in

tissues to maintain membrane fluidity and to protect the membrane against freeze-dehydration (Graham and Patterson, 1982; Leborgne et al., 1992; Perras and Sarhan, 1989)

There was a substantial reduction in membrane injury in cold-acclimated plants compared with nonacclimated, indicating that CA regimes were effective. In previous literature (Antolín et al., 2005; Hekneby et al., 2006), the 10 °C day/5 °C night temperature regime was used to assess CA and frost tolerance in *M. truncatula*. Acclimation temperatures used in our studies were comparable to those studies (CA1), but in addition, we included lower temperatures (7 °C day/4 °C night) in the CA2 regime. However, PI and FT (LT_{50}) measured in CA1 and CA2 plants were similar. This result suggests that the extended growth at lower temperature did not contribute to the hardiness level of the plants. Sakai and Larcher (1987) modeled the seasonal CA process in woody plants from temperate zones in two stages. The first stage proceeds at 10 to 20 °C in the fall and involves accumulation of organic substances and attributes to a relatively small (–10 °C) level of hardiness in plants. Similarly, CA1 and CA2 plants had increased FT in our studies within the range of temperatures.

Our results indicate that *M. truncatula* has a considerable ability to cold acclimate at subzero temperatures. Plants acclimated in the CA3 regime (3.5 °C day/–1 °C night) for 1 week exhibited the lowest injury compared with CA1 and CA2 at any test temperature. The significantly lower percentage of injury observed in CA3 plants indicates that the subzero temperature exposure during CA3 was largely effective in inducing higher CA in the plants. In addition, we demonstrated a significant decrease of LT_{50} temperature in both genotypes, from about –3 °C in nonacclimated to \geq –13 °C in cold-acclimated (CA3) plants. Freezing at subzero temperatures is the second phase hardening (subzero hardening) wherein cold-acclimated plants enhance FT further than that achieved during hardening with above-freezing temperatures. Second phase hardening has been documented in alfalfa (Castonguay et al., 1993), as well as other species, including wheat (*Triticum*

Table 3. Leaf area (LA), specific leaf area (SLA), root-to-shoot ratio (RS), leaf dry weight (LDW), stem dry weight (SDW), and root dry weight (RDW) of *Medicago truncatula* genotypes, W6 5018, and Jemalong-6 at nonacclimation (NA; week 1, week 2, and week 3) and cold acclimation (CA1, CA2, and CA3) regimes. Measurements in CA1 (18 °C day/10 °C night for 2 weeks followed by 10 °C day/5 °C night for 2 weeks), CA2 (18 °C day/10 °C night for 1 week followed by 10 °C day/5 °C night for 2 weeks and 7 °C day/4 °C night for 2 weeks), and CA3 (CA2 regime followed by 3.5 °C day/–1 °C night for 1 week) were taken after 4, 5, and 6 weeks, respectively, after the treatments. Measurements in NA (22 °C day/18 °C night) were taken during the first 3 weeks under the treatment.

Regime	Genotype	LA (cm ²)	SLA (cm ² .g ⁻¹)	RS (ratio)	LDW (g/plant)	SDW (g/plant)	RDW (g/plant)
NA							
Week 1	W6 5018	108 a ^z	178 a	1.08 a	0.61 a	0.42 a	1.19 a
	Jemalong-6	73 b	173 a	1.20 a	0.42 a	0.33 a	0.89 a
Week 2	W6 5018	194 a	154 a	0.94 a	1.28 a	0.99 a	2.19 a
	Jemalong-6	163 a	186 a	0.89 a	0.91 b	0.79 a	1.48 b
Week 3	W6 5018	281 a	105 a	0.82 a	2.7 a	3.31 a	4.91 a
	Jemalong-6	194 b	128 a	0.89 a	1.5 b	1.51 b	2.72 b
CA							
CA1	W6 5018	116 a	74 a	0.63 a	1.67 a	1.27 a	1.98 a
	Jemalong-6	139 a	83 a	0.68 a	1.68 a	1.54 a	2.16 a
CA2	W6 5018	82 a	83 a	0.98 a	1.01 a	0.95 a	1.99 a
	Jemalong-6	73 a	88 a	1.39 a	0.83 a	0.83 a	2.31 a
CA3	W6 5018	108 a	108 a	1.32 a	1.00 a	1.13 a	3.04 a
	Jemalong-6	93 b	122 a	1.84 a	0.76 a	0.77 b	2.70 a

^zMean values of genotype (n = 4) for response at a particular regime. Values within a regime with different letters are significantly different based on LSD at $P < 0.05$.

aestivum L.) (Herman et al., 2006), rye (*Secale cereale* L.) (Oline, 1984), barley (*Hordeum vulgare* L.) (Livingston, 1996), and oat (*Avena sativa* L.) (Livingston, 1996). Plants withstand dehydration and the physical effects of ice formation in the intercellular spaces during the second phase of CA by changing cell constituents and structures (Saxe et al., 2001; Weiser, 1970). Distinct changes in translatable mRNA in alfalfa cultivars acclimated at subzero temperatures are associated with achieving maximal hardiness during snow cover in winter (Castonguay et al., 1993).

The ability to acclimate at subzero temperatures is important for winter survival and spring growth for *M. truncatula*. In the present study, plants in CA2 developed a smaller but significant FT, while those in CA3 (subzero temperature) acquired still greater FT. These results imply that the events in induction of CA in *M. truncatula* may also be explained by the biphasic process, as in woody plants, and may indicate its potential for winter survival. By contrast, Brandsæter et al. (2000) reported poor frost resistance and survival in *M. truncatula* acclimated for 2 weeks at 2 °C. However, in their study, plants had already reached the flowering stage before they were frozen, and this could be the reason for poor survival. The most effective temperature in inducing CA depends on species, tissues, and developmental stages of the plant. A temperature slightly above 0 °C and slight below zero (−3 °C) are the two essential steps for developing full frost hardiness (Sakai and Larcher, 1987). Our CA3 regime included both the levels of temperature and the plants developed the two distinct levels of CA capabilities. However, neither genotype reached a plateau for percentage of injury in the CA3 regime. Further studies using the treatment temperatures colder than −20 °C in the freeze test may provide the maximum cold acclimation ability in this species under artificially controlled CA regimes.

In comparative analyses, freezing injury in W6 5018 initiated and reached the maximum earlier than Jemalong-6 in all CA regimes. Webb et al. (1994) demonstrated genotypic differences in leaf injury, rate of increase in freezing tolerance, and achievement of maximal LT₅₀ in spring oat, winter oat, and winter rye acclimated at 2 °C for 4 weeks, which is consistent with our results. Although the freezing tolerance of the two genotypes did not vary in cold acclimation regimes used in this study, the percentage of injury in Jemalong-6 was significantly lower than W6 5018 at most of the test temperatures in CA3 (Table 2). In addition, the initial and maximum freeze injury recorded in Jemalong-6 were at lower freeze test temperatures compared with W6 5018. These results clearly indicate that Jemalong-6 had superior cold acclimation ability than W6 5018 under the CA regimes used in the study. Castonguay et al. (1993) showed differential cold-inducible gene products in contrasting winter-hardy alfalfa cultivars and suggested a close association between gene expression and FT within cultivars. Genotypic differences in CA abilities observed in this study implies that there may be differences in genes or expression of genes involved in regulatory and sensing mechanisms in controlling the cold acclimation process in *M. truncatula*.

Exposure to different growth regimes resulted in differential growth responses in *M. truncatula*. Reduced number of leaves and stem length were observed in cold-acclimated plants within 1 week of initiation of CA regimes (Fig. 3). Low temperature reduces photosynthetic carbon fixation and sucrose synthesis, which reduces growth and phloem export of sucrose in plants (Strand et al., 1999). At low temperatures, reduction in

photosynthesis is considered as a major cause for growth cessation, as indicated by reduced chlorophyll content and photosynthetic capacities in sensitive maize genotypes (Haldimann, 1998; Leipner et al., 1999). However, overwintering cereals can grow and survive at low temperatures and have the capacity to increase or maintain high photosynthesis for increased FT (Hurry et al., 1994). They use most of the photosynthates for energy and the carbon skeleton needed for metabolic accumulation for cryoprotection and storage as adaptive responses. The observed reduced growth and development during the first 3 weeks in cold-acclimated plants in the present study, therefore, may indicate the allocation of more photosynthates for the induction of adaptive processes compromising the normal growth. Genotypes differed clearly in terms of growth and phenological development at NA while they had similar performance at CA regimes (Fig. 3 and Table 3). However, growth and development of Jemalong-6 was reduced less in CA regimes compared with its growth in NA regime than did W6 5018. These results further suggest that Jemalong-6 may have a relatively lower temperature requirement to attain maximum acclimation and may thereby explain its significantly greater cold acclimation ability at CA3 regimes.

We observed reduction in leaf dry weight from CA1 to CA3 with an increase in RDW, resulting in a higher RS ratio in both genotypes. A higher partitioning of dry matter in roots than in shoots has been reported in many species under low temperature stress (Wilson, 1988). Low shoot growth may be due to low absorption and transportation of water and nutrients by roots at low temperatures (BassiriRad et al., 1991). A higher RS ratio may also reveal a greater partitioning of carbon to the nutrient-absorbing tissues in low temperature conditions (Chapin, 1974). High SLA measured in nonacclimated plants in this study may indicate that the plants may have a high rate of CO₂ exchange per unit leaf area and thus may have achieved higher growth. High SLA has been regarded as an important trait in crops, such as sugarcane (Terauchi and Matsuoka, 2000) and winter cereal (Richards, 2000), attributing to high light interception during early growth and development. However, increase in leaf density (lower SLA) has been demonstrated as a common response to low temperature in chilling-sensitive (*Phaseolus vulgaris* L. and *Zea mays* L.) and chilling-tolerant species (*Pisum sativum* L. and *Spinacia oleracea* L.), which is consistent with our results (Wolfe, 1991). Reduced LA and SLA observed in CA regimes maybe adaptive strategies by which the cold-acclimated plants minimize leaf injury, reducing leaf expansion, and thereby reducing the surface area exposed to low temperatures.

In conclusion, the present study clearly demonstrated that *M. truncatula* has an ability to cold acclimate as evident by significant increase in FT with the exposure to specific CA regimes (accompanied by reduced growth). Quantifying FT by measuring IL in leaves proved to be a successful strategy as the difference in acclimation conditions and genotypes were well demonstrated with this approach. Because the two genotypes significantly differed in their freezing injury at various subzero test temperatures while still exhibiting similar LT₅₀ at CA regimes, we chose to present both the measurements in this study. Notably, this result implies that LT₅₀ may not be a comprehensive parameter to differentiate CA abilities among *M. truncatula* genotypes and that actual freezing injury at given test temperatures should be used in addition to LT₅₀ to evaluate this trait in *M. truncatula*. Various CA regimes (CA1, CA2, and

CA3) had differential effects on inducing cold hardening. Overall, the maximum FT was achieved under the CA3 treatment, the only regime that included subzero temperature exposure. Thus, CA3 appears to be the best regime of the three regimes tested for inducing maximal CA under controlled conditions in *M. truncatula* for physiological and biochemical studies.

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