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ORIGINAL PAPER

Dehydrin variants associated with superior freezing tolerance in alfalfa (*Medicago sativa* L.)

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Abstract A cDNA (*msa*CIG) encoding a cold-inducible Y_2K_4 dehydrin in alfalfa (*Medicago sativa* spp. *sativa*) was shown to share extensive homology with sequences from other species and subspecies of *Medicago*. Differences were mainly the result of the occurrence of large indels, amino acids substitutions/deletions and sequence duplications. Using a combination of a bulk segregant analysis and RFLP hybridization, we uncovered an *msa*CIG polymorphism that increases in frequency in response to recurrent selection for superior freezing tolerance. Progenies from crosses between genotypes with (D+) or without (D−) the polymorphic dehydrin significantly differed in their tolerance to subfreezing temperatures. Based on the *msa*CIG sequence, we looked for intragenic variations that could be associated to the polymorphism detected on Southern blots. Amplifications with primers targeting the 3′ half side of *msa*CIG revealed fragment size variations between pools of genotypes with (+) or without (−) the polymorphism. Three major groups of amplicons of ≈370 nt (G1), 330 nt (G2), and 290 nt (G3) were distinguished. The G2 group was more intensively amplified in pools of genotypes with the polymorphic dehydrin and was associated to a superior freezing tolerance phenotype. Sequences analysis revealed that size variation in the 3′ half was attributable to the variable occurrence of large indels. Single amino acid substitutions and/or deletions caused major differences in the prediction of the secondary structure of the polypeptides. The identification of dehydrin variants associated to superior freezing tolerance paves the way to the development of functional markers and the fixation of favorable alleles in various genetic backgrounds.

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Introduction

Alfalfa (*Medicago sativa* spp. *sativa*) belongs to the species complex (*M. sativa* L.) that includes diploid and tetraploid interfertile subspecies (Muller et al. 2006). Although this perennial species is widely distributed in temperate zones of the world, its reliability in northern climates is compromised by a lack of winter hardiness (Castonguay et al. 2006). Breeding for greater winter hardiness through conventional approaches has not been very effective and only limited progress has been achieved in recent years (Volenec et al. 2002). Lack of progress is attributable to a number of factors including the quantitative nature of winter hardiness inheritance, lack of precise screening methodologies and insufficient knowledge of the molecular and genetic bases of adaptation.

Alfalfa is a cross-pollinated perennial species with an autotetraploid ($2n = 4x = 32$) genome (Jones and Bingham 1995). Cultivars are highly heterogeneous synthetic populations developed by random mating of parental genotypes selected for desirable agronomic characteristics. Populations

selectively improved for their tolerance to freezing (TF populations) were recently developed using a recurrent selection protocol entirely performed under controlled conditions (Castonguay et al. 2009). Several cycles of recurrent phenotypic selection have been performed within different genetic backgrounds and resulted in a significant increase in freezing tolerance and survival to harsh winter conditions. Although selection for freezing tolerance under environmentally controlled conditions is more predictable than field selection, the process remains lengthy and requires several cycles of selection to achieve the desired level of tolerance.

Breeding programs can greatly benefit from the identification of molecular markers for genes that have a significant impact on traits of interest and from their subsequent integration into marker-assisted selection (MAS) approaches (Morgante 2006; Weising et al. 2005; Xu and Crouch 2008). Bulk segregant analysis (BSA) of pools of DNA is a simple and very effective approach to identify differences in genes frequency between groups of plants with contrasting phenotypes (Michelmore et al. 1991). Considering that selection was solely targeted toward the improvement of freezing tolerance, BSA of TF populations offers a unique opportunity to uncover variation in genomic DNA closely linked to loci controlling freezing tolerance.

Dehydrins constitute the group 2 (or D11 family) of the late embryogenesis abundant (LEA) family of proteins that are induced by a variety of environmental stresses including dehydration, low temperature, salinity and wounding (Kosova et al. 2007). These highly hydrophilic proteins remain stable under denaturating conditions and are defined by the presence of one or more lysine-rich motifs (the K segment; Close 1996). Dehydrins can possess other typical features including a consensus Y-segment (DEYGNP) near the N terminus region, a serine-rich tract (the S-segment) that can be modified by phosphorylation, and less conserved stretches rich in polar amino acids (the Φ segments). Five distinct groups of dehydrins can be distinguished based on the presence and number (*n*) of the signature motifs: Y_nSK_n, SK_n, K_n, Y_nK_n and K_nS (Rorat 2006). In *Medicago* spp., both the Y_nK_n, K_n and SK_n types have been identified (Ivashuta et al. 2002; Pennycooke et al. 2008; Wolfraim and Dhindsa 1993; Wolfraim et al. 1993). A number of genetic studies have linked the presence of dehydrins to superior adaptation to low temperature stress. Allelic variation of a dehydrin gene was found to co-segregate with the chilling tolerance of cowpea (*Vigna unguiculata* L. Walp.) seedlings (Ismail et al. 1999). A close relationship between the accumulation of dehydrins and freezing tolerance was observed among *Rhododendron* species (Marian et al. 2004) and zoysiagrass (*Zoysia* spp.) genotypes (Patton et al. 2007). Additionally, the expression of dehydrins in transgenic plants has improved the freezing tolerance of a number of species including strawberry (*Fragaria* × *ananassa* Duch.; Houde et al. 2004), *Arabidopsis* (Brini et al. 2007) and cucumber (*Cucumis sativus* L.; Yin et al. 2006).

Although dehydrins have been previously isolated from alfalfa, no information is currently available regarding their adaptive value for tolerance to low subfreezing temperatures in this species. Genetic variation of dehydrins has yet to be explored in alfalfa and a potential association between the occurrence of allelic variants and superior freezing tolerance needs to be investigated. Therefore, our objective was to assess the relationship between variation in dehydrin sequences and differences in freezing tolerance among alfalfa TF populations. Dehydrin variants associated with freezing tolerance could provide robust markers for the fixation of favorable alleles in different alfalfa genetic backgrounds. This research is part of a long-term effort to exploit the inherent genetic diversity within the alfalfa gene pool through the identification of novel alleles and genes tightly linked to superior freezing tolerance.

Materials and methods

Plant materials

Genotypes of alfalfa (*Medicago sativa* spp. *sativa*), cultivar Apica (ATF0) (Michaud et al. 1983), and populations (ATF2, ATF4, ATF5 and ATF6) derived from this cultivar through recurrent cycles of selection for superior freezing tolerance (Castonguay et al. 2009) were seeded individually in Ray Leach Cone-tainers™ (SC-10 Super Cell, Stuwe & Sons Inc.) filled with a mixture of (10:3, v:v) of top soil/peat moss (Pro-mix BX, Premier Peat Moss, Rivière-du-Loup, QC, Canada) supplemented with a controlled release fertilizer [N: 17% (w:w); P: 7.31% (w:w); K: 14.1% (w:w); 250 g/35 L; Muticote 4, Haifa Chemicals Ltd, Haifa Bay, Israel]. Plants were grown for 6 weeks in an environmentally controlled chamber set to: photoperiod 16 h, day-time temperature 22°C, night-time temperature 17°C. Artificial lighting was provided by a mixture of high pressure sodium and metal halide 400 W lamps (PL light systems, Beamsville, ON, Canada) with photosynthetic photon flux density of 600–800 µmol photons m^{-2} s^{-1}. Plants were kept well watered and fertilized twice a week with a 1 g l^{-1} of a commercial fertilizer (20–20–20 plus micronutrients, Plant-Prod, Brampton, ON, Canada). Micronutrients percent composition of the fertilizer was 1.0 mg l^{-1} Mn, 0.5 mg l^{-1} Cu, 0.2 mg l^{-1} B and 0.005 mg l^{-1} Mo.

Genotypes were selected within ATF0 on the basis of the absence (−) or presence (+) of a dehydrin polymorphism and were inter crossed ($n = 10$ in each group) to generate D− and D+ populations that differ with regard to the presence of that dehydrin variant. Crosses were hand-made

using greenhouse grown plants maintained under a ≈25/15°C (day/night) temperature regime and 16 h photoperiod provided by a combination of natural irradiance supplemented by mercury vapor 1,000 W lamps (H36GW-1000/DX; Osram Sylvania, Mississauga, ON, Canada). After maturation, seeds were harvested from individual plants and identical amounts from each genotype within each group were combined to constitute a pooled sample.

Evaluation of freezing tolerance

Freezing tolerance was assessed using a procedure previously described (Castonguay et al. 1995). Fifteen plants were grown in 14-cm pots at the beginning of September 2006 under the conditions described previously. After 5–6 weeks of growth, plants were transferred to an unheated greenhouse located at a site near Quebec City, Canada (latitude 46°47′15″, longitude 71°12′00″, altitude ≈45 m asl) for their acclimation to natural hardening conditions. The unheated greenhouse was continuously ventilated during the day to keep the inside temperature close to that of the outside. When the inside air temperature remained permanently below freezing, plants were covered with a layer of Astro-Foil™ reflective insulator (Innovative Energy Inc., Lowell, IN) to simulate snow cover. Air temperature outside and inside the greenhouse and soil temperature in pots were monitored at 30-min intervals and recorded from the end of October 2006 to mid-March 2007 using stand alone data loggers (RD-temp). Plants were assessed twice (6 and 21 February 2007) for their freezing tolerance. Freezing tests were performed in a programmable walk-in freezer following a 24 h equilibration period at −2°C. Temperatures were lowered by 2°C during a 30-min period followed by a 90-min plateau at each test temperature. Plants were tested between −20 and −32°C and at the end of each temperature plateau, five pots (≈75 plants) were withdrawn from the freezer and thawed at 2°C for 24 h. Plants were then transferred to initial growth conditions for regrowth assessment. After 3 weeks, survival counts were made and used to calculate the lethal temperature for 50% of a population (LT_{50}) using the Probit curve fit of the Statistical Analysis System (2006) software. The probit model included "TSOL", "population" and their interaction as independent variables and *Y*, a discrete plant survival value (1 dead and 0 alive), as the dependent variable. After verification of absence of interactions between probit curve responses, pair comparisons of treatment intercepts (b0) were made to assess statistical differences between treatments.

Candidate gene sequence data

A cDNA clone (*msa*CIG) with sequence features typical of dehydrin genes has been isolated (EU652338; Serge Laberge, unpublished) from cold-acclimated crowns of alfalfa (*M.sativa* spp. *sativa* cv. Apica) using differential screening of a cDNA library and subsequently sequenced as described by Castonguay et al. (1994).

Northern blot analysis

Total RNA was extracted as described by De Vries et al. (1988) from a 1 g pooled sample of 50 crowns that was ground in liquid N_2. Samples from the ATF0 and ATF5 populations were collected in October 2003 before plants were transferred from environmentally controlled conditions (20/17°C, 16 h photoperiod) and in November 2003 and January 2004 after plants were acclimated to naturally declining temperatures in the unheated greenhouse. RNA blot hybridization was performed as described in Castonguay et al. (1994) using a *msa*CIG probe radiolabeled with [α-^{32}P]dCTP (Amersham Canada, Oakville, ON, Canada). Fluorograms were exposed to Kodak O-Mat XAr films for autoradiography for 4 h at −80°C.

DNA extraction and Southern blot analysis

Genomic DNA was extracted from individual and pooled samples from ≈45 genotypes from each of the cultivar Apica (ATF0) and populations (ATF2, ATF4, ATF5 and ATF6) derived from this cultivar through recurrent cycles of selection for superior freezing tolerance. Total genomic DNA was extracted using the CTAB procedure of Rogers and Bendich (1988) and subsequently used for Southern hybridization and PCR amplification. For Southern blot hybridization, 10 µg of DNA was digested with *Dra*I, electrophoresed on 0.8% agarose gels, transferred onto nylon membranes (Hybond™-N^+, Amersham Pharmacia Biotech) and subjected to hybridization with *msa*CIG (25–50 ng) labeled with α-^{32}P dCTP (Amersham Canada, Oakville, ON, Canada), using the oligolabeling procedure. Hybridizations were performed overnight at 68°C using 2 × SSC, 0.5% SDS and 0.25% (w/v) low-fat milk powder. Blots were then exposed with intensifying screens, to Kodak O-Mat XAr films for autoradiography for up to 1 week.

Primer design, fixed primers

The cDNA sequence of the cold-inducible *msa*CIG gene was used to design primers (Fig. 1; Table 1) with the Oligo Explorer software, version 1.1.0 (T. Kuulasma, University of Kuopio, Kuopio, Finland). The primer size range was set from 18 to 22 nt, the melting temperature range was set from 50 to 55°C. The basic rules of primer design such as self-complementary, primer loops and maintenance of 40–60% GC content were upheld.

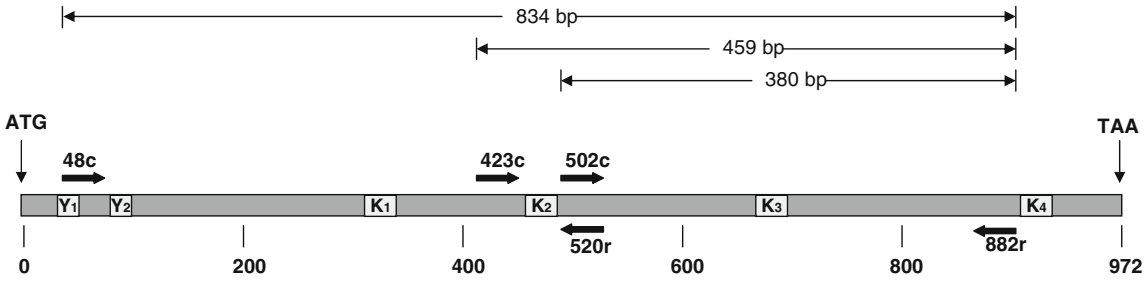

Fig. 1 Structure of the Y_2K_4 *msa*CIG open reading frame. The position and the orientation of primers are indicated by *black arrows*. Predicted length of amplicons is indicated between right and left markers. Characteristics Y and K segments are indicated

Table 1 PCR primers used for cloning, characterizing and sequencing dehydrin alleles from *Medicago sativa* cv. *Apica*

Primers	Description	Sequences (5′ → 3′)
48c	5′ Forward primer for msaCIG alleles	ATATGGAAACCCCTTGAG
423c	First intermediate forward primer for msaCIG alleles	TGGTTATGGTGATACTGG
502c	Second intermediate forward primer for msaCIG alleles	GACAAGATCAAGGAGAAGA
520r	Second intermediate reverse primer for msaCIG alleles	TCTTCTCCTTGATCTTGTC
882r	3′ Reverse primer for msaCIG alleles	GTGTTGCTCATCATGTCC
M13F	Universal M13 forward primer for sequencing in pGEM T	TGTAAAACGACGGCCAGT
M13R	Universal M13 reverse primer for sequencing in pGEM T	CAGGAAACAGCTATGAC

PCR amplifications

Each reaction was performed in a total volume of 25 µl in 0.2 ml PCR strips containing 2.5 µl of 10× PCR buffer, 1 µl each of 5 µM primers, 0.5 µl of 10 mM dNTP (Roche Diagnostics, Indianapolis, IN), 0.5 µl of 5 Prime *Taq* polymerase 5 U/µl (Inter Medico, Markham, ON, Canada) and 5 µl of 10 ng/µl genomic DNA (50 ng). The conditions for PCR were as follows: an initial denaturing step was performed at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension of 7 min at 72°C. All the reactions were performed on an Eppendorf Mastercycler ep System (VWR Canlab, Mississauga, ON, Canada).

Electrophoresis of amplified products

Upon completing PCR cycles, 20 µl of each reaction was run for 2 h at 70 V on a 2% agarose gel stained with ethidium bromide. DNA fragments were visualized using a UVP BioDoc-It system (UVP, Upland, CA). Amplicons were also separated by capillary eletrophoresis with the Experion™ system (Bio-Rad, Mississauga, ON, Canada). Capillary electrophoresis was conducted and DNA bands were analyzed with the Experion™ DNA 1K Analysis kit (Bio-Rad) according to the manufacturer's instructions.

Cloning and sequencing amplified fragments

DNA fragments were recovered from agarose gels using the QIAquick gel extraction kit (QIAGEN Inc., Mississauga, ON, Canada) according to the manufacturer's recommendations. Purified DNA was cloned into the pGEM®-T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions. Positive transformants were recovered, grown in liquid medium and plasmids purified using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Mississauga, ON, Canada). Plasmid preparations were sent for sequencing using M13 forward and reverse primers for bi-directional sequencing.

Bioinformatic analyses

BLASTn and BLASTp sequence identity searches were performed at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Chromatograms from the sequencing of the different fragments were edited and assembled using BioEdit software version 7.0.9.0 (Hall 1999). Amino acid sequences were aligned using the CLUSTALW tool included in BioEdit and refined manually. VecScreen, a online contaminant vector screening software developed by NCBI (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to remove vector sequence. The method of Chou and Fasman (1978) was used with the GCG program Peptidestructure (Genetics Computer Group, Madison, WI) to predict the secondary structure of the putative dehydrin sequences from *Medicago* spp.

Sequence data from this article have been deposited with the GenBank data library under the following names and associated accession numbers (in parentheses): *msa*CIG

(EU652338), D− (381) (GU045323), D− (375) (GU045324), TF0 (359) (GU04525), TF5 (351) (GU045326), D+ (363) (GU045327), D+ (333) (GU045328), TF5 (333a) (GU04 5329), TF5 (321) (GU045330), TF5 (333b) (GU045331), D+ (327) (GU045332), TF5 (327a) (GU045333), TF5 (327b) (GU045334), TF0 (283a) (GU045335), TF0 (283b) (GU045336), TF0 (291) (GU045337), and TF5 (291) (GU045338).

Results

*msa*CIG encodes a Y_2K_4 dehydrin

The nucleotide sequence of the full length cDNA of *msa*CIG is 972 nt long and contains one open reading frame coding for 323 amino acids. The MSACIG polypeptide has an Y_2K_4 configuration according to Close (1996) nomenclature for dehydrin. The msaCIG cDNA probe hybridizes with two groups of transcripts that are strongly induced under naturally declining temperatures in fall and winter while it remains uninduced at warm temperature (Fig. 2). Amino acid sequences from subspecies of tetraploid *M. sativa* and from the diploid *Medicago truncatula* were manually adjusted to avoid mismatch and particular care was taken to align the four conserved K segments (Fig. 3). The deduced MSACIG polypeptide shares extensive homology with other Y_2K_4 dehydrins from the *Medicago* spp. complex. The N terminus section that includes the two characteristic Y segments (DE/QYGNP) was identical among *Medicago* sequences with the sole exception of four additional amino acids in the *M. truncatula* sequence. In contrast, major differences occurred in the intervening region between the N-terminal section and the first K segment. Analysis of the sequences between the K1 and K2 segments revealed a major indel of 35 amino acids when compared to the *Ms*CAS30 sequence. This indel was mainly due to the presence in *Ms*CAS30 of a duplicated motif of 19 residues (GTTGVGHQQHGDTYGTGTG) followed by a 14-amino acid segment (GHGTTGYGATDVGH) identical to one present between the K2 and K3 segments in *Mf*CAS30 and *msa*CIG. A β-sheet structure just before the K2 segment was predicted in the *Mt*CAS31 and in the *msa*CIG sequences. The presence of seven additional amino acids immediately after the K2 segment of *M. truncatula* was also associated with the prediction of a β-sheet structure. The *Mt*CAS31and *Ms*CAS30 sequences share a similar 21–23 amino acid deletion in the intra-region between the K2 and K3 segments. A predicted β-sheet structure after the K3 segment was observed only in *Mf*CAS30 and *msa*CIG, despite a single amino acid difference between all four sequences in that region. Furthermore, a 12–14 amino acid gap is also present in the intra-region between the K3 and K4 segments of *Mt*CAS31 and *Ms*CAS30. There is a rather exceptional occurrence of one cysteine residue at the C-terminal end of the *Medicago* spp. Y_2K_4 dehydrins. We also observed major differences in the amino acid composition of the C-terminal part of *Ms*CAS30 as compared to the other *Medicago* sequences after the K4 segment. These variations led to the prediction of two β-sheet structures interrupted by an α-helix structure that appears to be unique.

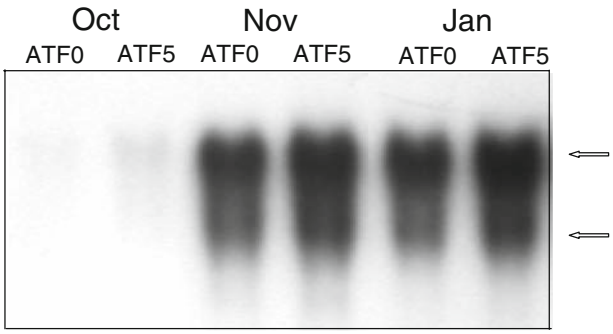

Fig. 2 Northern blot analysis of *msa*CIG transcript accumulation in crowns of plants from populations ATF0 and ATF5. Plants were acclimated to natural conditions in an unheated greenhouse during the 2003–2004 overwintering season. Samples were collected in October (*Oct*) from plants grown indoor at 20/17°C and after acclimation to naturally declining temperatures in November (*Nov*) and January (*Jan*). The daily mean temperature for the months of November and January in Quebec City are, respectively, of −0.7 and −12.8°C. *Arrows* indicate two groups of transcripts that hybridize with the *msa*CIG probe

The 15-amino acid K1 segment (DNRGVV/MDKIKEKIPG) was highly conserved in all *Medicago* spp. sequences. The presence of a glutamic acid (E) residue at the beginning of the K2 segment led to the prediction of an extended α-helix structure in all *Medicago* sequences except for *Mt*CAS31 where an aspartic acid (D) residue is present. Removing the additional G residue before the K2 segment of *Mt*CAS31 and substituting the D residue for an E residue allowed us to retrieve the predicted extended α-helix structure (data not shown). A duplicated GH motif immediately before the K3 segment of the *Ms*CAS30 sequence causes noticeable changes in the predicted secondary structure resulting in an extended α-helix segment. This extended α-helix for K3 is reminiscent of the K2 segment predicted for all sequences except for that from *Medicago truncatula* (*Mt*CAS31). Based on the alignment, it appears that an asparagine (N) residue is missing in the K3 segment of *Mt*CAS31 and *Ms*CAS30 when compared to the K1 and K2 segments of the other sequences. The simulated inclusion of a missing N residue in *Mt*CAS31 led to the prediction of an extended α-helix structure (data not shown). Thus, point mutations leading to an amino acid substitution and/or addition/deletion of amino acids markedly affected the prediction of the secondary structure of K segments of dehydrins with potential consequences on their functions.

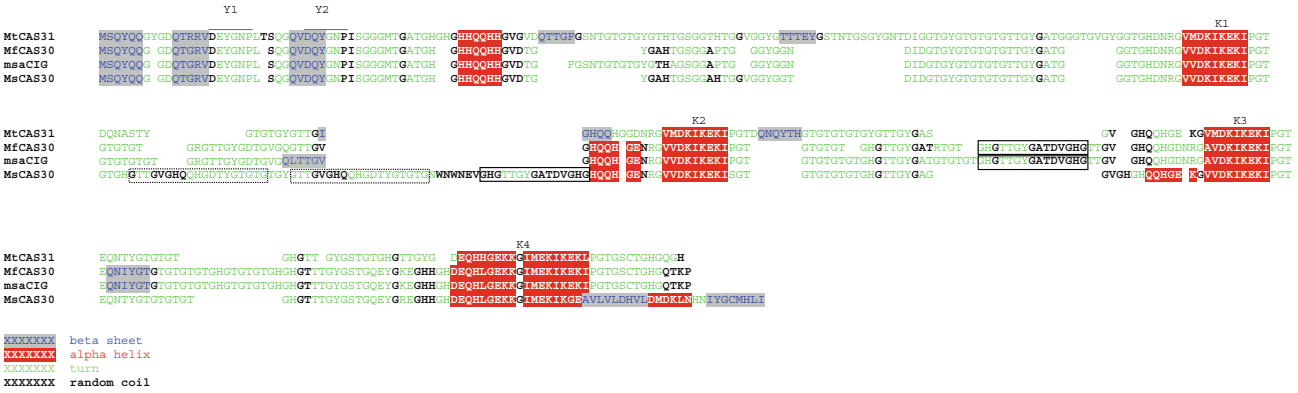

Fig. 3 Alignments of *Medicago* dehydrin polypeptide sequences: *msa*CIG (*Medicago sativa* spp. *sativa*; GenBank accession EU652338, this study), *Mt*CAS31 (*Medicago truncatula*; GenBank accession EU139871, Pennycooke et al. 2008), *Mf*CAS30 (*Medicago sativa* spp. *falcata*; GenBank accession EU149865, Pennycooke et al. 2008) and *Ms*CAS30 (*Medicago sativa* spp. *media*; GenBank accession AF411554, Ivashuta et al. 2002). Characteristics Y and K segments and predicted secondary structures are indicated. Duplicated motifs are enclosed in *dashed boxes*. Conserved motifs are enclosed in *solid boxes*

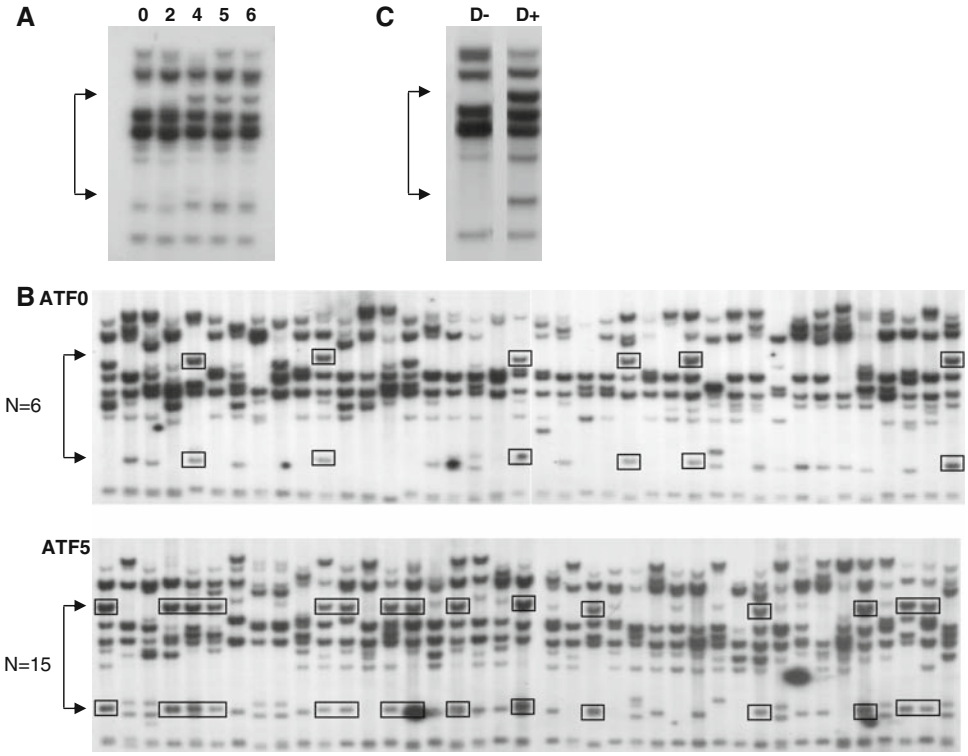

Fig. 4 Southern blot hybridization of *msa*CIG using *Dra*I-digested DNA from **a** pooled samples from populations selectively improved for their tolerance to freezing within *Medicago sativa* spp. *sativa* cv. Apica (ATF0 to ATF6). **b** Forty individual genotypes within each of the ATF0 and ATF5 populations. **c** Pooled DNA samples from populations D− and D+ obtained by inter-crossing ATF0 genotypes without (−) or with (+) the polymorphic dehydrin. The polymorphic dehydrin that increases in frequency in response to recurrent selection is indicated by the *arrows*

The K4 segment was highly conserved among all species with a high level of amino acid identity.

Identification of a polymorphism associated with freezing tolerance

*Dra*I-digested DNA from ≈45 genotypes from each of the cultivar Apica (ATF0) and populations recurrently selected for superior tolerance to freezing (ATF populations) within that initial background were hybridized with *msa*CIG (Fig. 4a). Several bands that strongly hybridized with *msa*CIG were detected equally in all ATF populations regardless of selection pressure. A polymorphism that progressively intensified with the number of selection cycles was however clearly noticeable after four cycles of recurrent selection as indicated by the joined arrows. Subsequent assessment of genotypic variability of the dehydrin polymorphism detected with pools of DNA among 40 genotypes randomly selected within each of ATF0 and ATF5 revealed a marked increase in the frequency of this polymorphic band in response to selection for superior freezing tolerance (Fig. 4b). Whereas six genotypes were found to have that particular hybridization signal in ATF0, the number of positive genotypes more than doubled in ATF5.

Genotypes selected within ATF0 on the basis of the absence (D−) or presence (D+) of the observed polymorphism were intercrossed ($n = 10$ in each group) to generate the populations D− and D+ that differ with regard to the presence of the dehydrin variant. Subsequent hybridization of pooled samples of *Dra*I-digested DNA from these two populations confirmed a striking contrast for the dehydrin polymorphism between the D− and D+ populations (Fig. 4c). In order to assess the association between the dehydrin polymorphism and cold adaptation, progenies of the D− and D+ crosses that differ with regard to the presence of the polymorphic dehydrin (Fig. 5a) were subsequently tested for their freezing tolerance and compared to ATF0 and ATF5. The D+ population, preferentially enriched for the polymorphic dehydrin, was significantly more tolerant to freezing than the D− and ATF0 populations (Fig. 5b). Although the D+ progeny was significantly less tolerant to freezing than ATF5, the observed improvement nevertheless reached nearly 30% of the increase observed after five cycles of recurrent selection.

Analysis of intragenic size variation in *msa*CIG variants

Evidence for an *msa*CIG variant associated with the improvement of freezing tolerance in alfalfa prompted us to look for the sequence variation underlying the observed polymorphism. Two primer sets (48c-520r and 502c-882r) were used to search for length polymorphisms (Table 1; Fig. 1). Genomic DNA from genotypes of ATF0 or ATF5 was pooled ($n = 15$) on the basis of the presence (+) or the absence (−) of the polymorphic fragment. Whereas amplification of the 5′ half of the dehydrin sequences did not reveal any noticeable size variation among both groups of genotypes (Fig. 6a), the second set of primers showed a clear length polymorphism (Fig. 6b). Three major bands were identified on agarose gels. Either with the ATF0 or the ATF5 genotypes, DNA fragments identified by the dark arrow showed a stronger signal in pools of positive (+) than in pools of negative (−) genotypes. Conversely, fragments identified by the open arrow followed a complete opposite pattern with a stronger signal in genotypes that lack the polymorphic dehydrin. These observations were confirmed by capillary electrophoresis (Fig. 6c) which allowed us to allocate the amplicons to three groups based on their size. The average length of the fragments estimated with the Experion™ software yielded values of 370, 330 and 290 nucleotides for the first (G1), the second (G2) and the third group (G3), respectively. The mean length of the amplicons within the G1 group was near the predicted 380 bp deduced from the *msa*CIG sequence (Fig. 1). PCR amplification with bulk samples from the D− and D+ populations yielded results similar to those obtained with pools of genotypes (Fig. 6d). In both populations, G1 amplicons remained

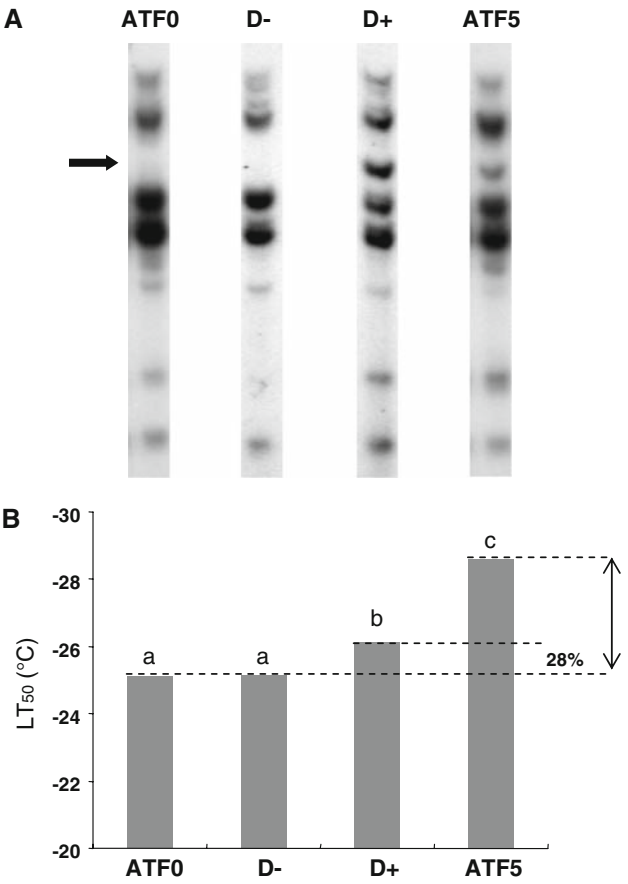

Fig. 5 **a** Southern blot hybridization of *msa*CIG using *Dra*I-digested DNA from a population recurrently selected for superior freezing tolerance (ATF5) and its initial background (ATF0) and from populations generated by inter-crossing genotypes selected within ATF0 on the basis of the absence (D−) or presence (D+) of the polymorphic dehydrin. The position of polymorphic *Dra*I fragment on Southern blots is indicated by the *black arrow* (**b**). Freezing tolerance expressed as the temperature at which 50% of the plants are killed (LT_{50}) for the four populations described above. LT_{50} values without the *same letters* are significantly different at $\alpha \leq 0.05$. The percentage value indicates the increase in freezing tolerance in D+ plants as compared to what was achieved in ATF5 after five cycles of recurrent selection for freezing tolerance

stable. Although G3 fragments were detected in both populations, the intensity of the signal decreased in the D+ population along with the intensification of the G2 fragments. Amplifications with bulked DNA samples from ATF0 and ATF5 confirmed the marked intensification of the G2 fragments in the ATF5 population with a superior freezing tolerance phenotype (Fig. 6e).

Analysis and alignment of *msa*CIG size variants

The 423c and 882r primer pair combination (Table 1; Fig 1) was used to amplify pooled DNA samples from ATF0, ATF5, D− and D+ populations. A non-exhaustive sampling identified 16 Y_2K_4 variants homologous to *msa*CIG within

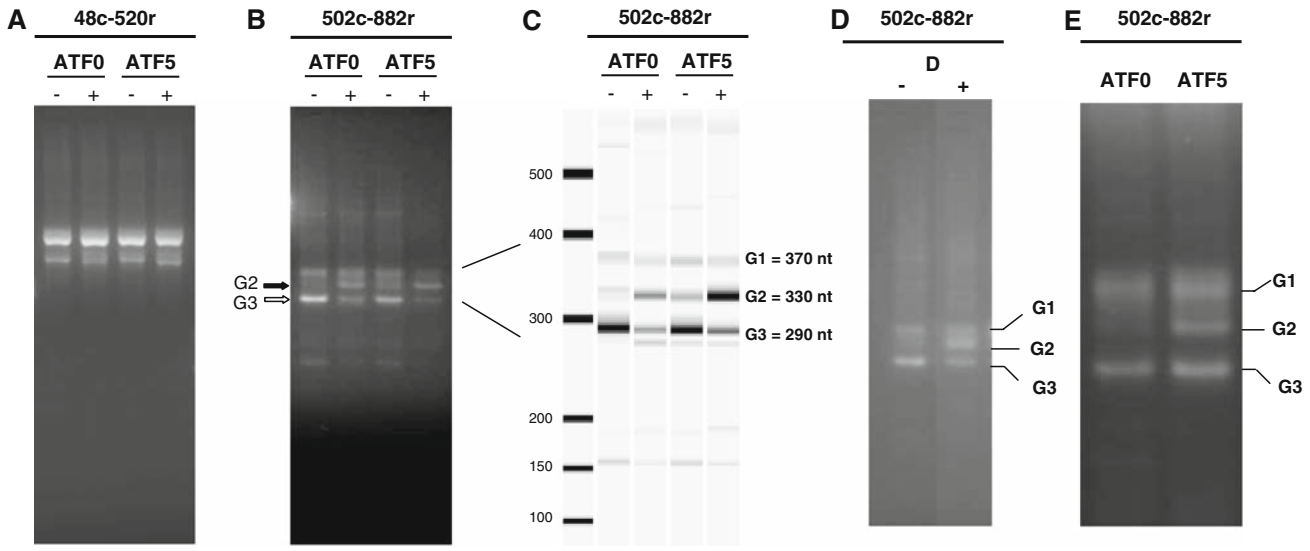

Fig. 6 Amplification of the 5′ half and the 3′ half sides of *msa*CIG using pooled DNA from ATF0 genotypes selected on the basis of the absence (−) or the presence (+) of the polymorphic *Dra*I fragment. Fifteen genotypes were pooled in each group. The two sets of primers (48c-520r in **a** and 502c-882r in **b**) are described in Table 1. Amplicons were separated either on 2% agarose gels (**a**, **b**) or by capillary electrophoresis (**c**). Amplicons were grouped into three categories (G1, G2 and G3) based on their average size (**c**); length in nucleotides (nt) is indicated. Amplification of the C-terminal region (502c-882r) was also performed with pooled samples of progenies from the D− and D+ populations (**d**) and from populations ATF0 and ATF5 (**e**). Fragments identified by the *dark arrow* in **b** are more highly amplified in genotypes with (+) the dehydrin polymorphic between ATF populations. Conversely, fragments identified by the *open arrow* in **b** are more highly amplified in genotypes without (−) the dehydrin polymorphic between ATF populations

the Apica genetic background (Fig. 7). These variants differ mainly by the presence of indels, amino acids substitutions and amino acid addition/deletion that have, in some instances, a major impact on the prediction of the secondary structure. Sequence analyses of amplicons confirmed the existence of three major groups that correspond to the fragment size estimates obtained by agarose and capillary gel electrophoresis (Fig. 6b, c). Comparison of variants with the *msa*CIG sequence within the G1 and G2 groups revealed the absence of a predicted β-sheet structure before the K2 segment as a result of non-conservative substitutions. The three groups had a conserved K2 segment yielding a predicted extended α-helix structure that was also observed in all *Medicago* sequences with the exception of the non-winterhardy *M. truncatula* (Fig. 3). The G1 and G2 groups differed mainly by the presence between the K2 and the K3 segments of a large indel (16–18 amino acids). In these two groups, we also observed the variable presence of a smaller indel (2–6 amino acids) after the K3 segment. Two major sub-groups were apparent within G2. One subgroup had sequences almost identical to *msa*CIG whereas the second was characterized by a few substitutions before the K3 segment and the lack of a GT motif deletion after the K3 segment. The G3 group was characterized by the presence of a large indel of 23 amino acids not found in the G1 and G2 groups. This large indel was also observed in *Mt*CAS31 and *Ms*CAS30 sequences (Fig. 3). It is noteworthy that the EQNTY motif that follows the K2 segment in sequences of the G3 group is conspicuously absent in the sequences from the G1 and G2 groups and from previously documented *Medicago* sequences. Conversely, the presence of this motif after the K3 segment is confirmed in all the sequences regardless of the size group. However, a single amino acid substitution of threonine (T) to isoleucine (I) within this motif in the G1 and G2 sequences allowed the prediction of β-sheet structure. A histidine (H) residue insertion occurred just before the EQNTY motif present after the K3 segment in the sequences from the G3 group. It is noteworthy that the K3 segment of the sequences of the G3 group is almost identical to the K3 segment present in the *Ms*CAS30 and *Mt*CAS31. The duplication of $(GH)_n$ motif before the K3 was associated with the prediction of an extended α-helix, another feature unique to the G3 variants and to the *Ms*CAS30 sequence. The deletion of an asparagine (N) residue just before the K3 segment again appears to have important implications for the predicted conformation of the secondary structure. A major deletion of 14-aa residues after the K3 segment was another important characteristic that differentiates the G3 group from the two other groups. This deletion is also present in *Mt*Cas31 and *Ms*Cas30 sequences (Fig. 3).

Variations in fragments size among individual genotypes

In order to analyze the relationship between specific allelic variants with the polymorphic dehydrin detected on Southern

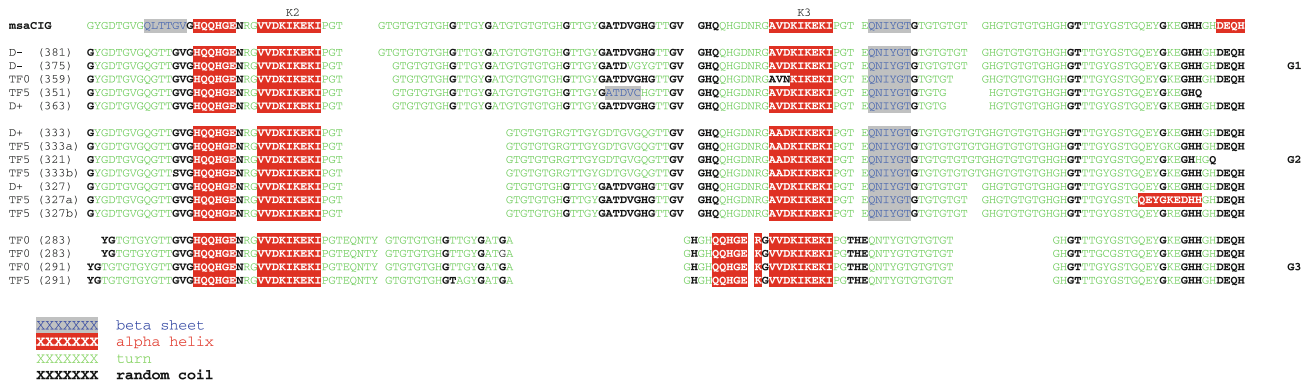

Fig. 7 Alignment of the predicted C-terminal amino acid sequence of *msa*CIG homologs from *Medicago sativa* spp. *sativa* cv. Apica. The K segments and predicted secondary structures are indicated. The C-terminal region between position 423 and 882 of *msa*CIG was amplified from ATF0, ATF5, D− and D+ populations. *Numbers* in *brackets* indicate the length in nucleotides of these sequences between position 502 and 882 of *msa*CIG and are provided for ease of comparison with the size groups described in Fig. 6. Sequences were assigned to one of three groups based on their length

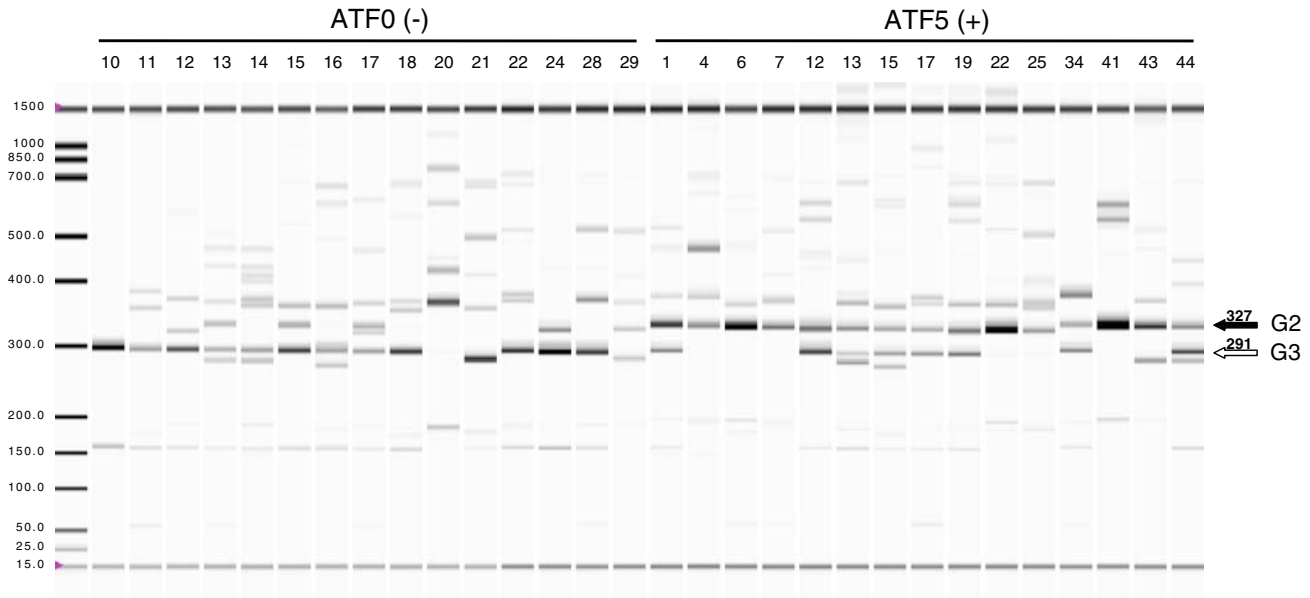

Fig. 8 Amplification of the 3′ half side of *msa*CIG variants (502c-882r) using DNA from 15 ATF0 genotypes without (−) and 15 ATF5 genotypes with (+) the polymorphic dehydrin. Amplicons were separated by capillary electrophoresis. *Dark* and *open arrows* indicate, respectively, the G2 and G1 groups of *msa*CIG variants. *Numbers* above the *arrows* indicate the average length in nucleotides of the amplicons

blots, amplicons from 15 ATF0− genotypes and 15 ATF5+ genotypes were analyzed by capillary gel electrophoresis (Fig. 8). The frequency of the G2 and G3 fragments appeared to be inversely proportional between ATF0− and ATF5+ genotypes. Whereas G3 fragments were found in mostly all genotypes of ATF0−, several ATF5+ genotypes did not have this fragment. The opposite situation was observed with G2 fragments found in low frequency in ATF0− genotypes whereas it was present in all ATF5+ genotypes with a clear intensification of the signal.

Discussion

Y_2K_4 dehydrins are commonly found in legumes

Apart from their previous identification in the genus *Medicago*, the presence of Y_nK_n dehydrins has been reported in other legumes including *Pisum sativum* (Y_2K_2) (Grosselindemann et al. 1998), *Glycine max* (Y_2K) (Momma et al. 2003), *Vigna unguiculata* (Y_2K) (Ismail et al. 1999), *Vicia monantha* (Y_2K) (accession no. AB506695.1) and *Cicer pinnatifidum* (Y_2K) (Bhattarai and Fettig 2005). This type

of dehydrin was also isolated from deciduous trees including *Prunus dulcis* (Y_2K_4) (Artlip et al. 1997), *Prunus persica* (Y_2K_9) (accession no. DQ061115), *Betula pubescens* (partial Y_3K_2 genomic clone) (Welling et al. 2004) and from the herbaceous species *Spinacia oleracea* (YK_{11}) (Neven et al. 1993) and *Cichorium intybus* (Y_2K_2) (Mingeot et al. 2009). Several of these Y_nK_n dehydrins were shown to be induced by low temperatures (Artlip et al. 1997; Bhattarai and Fettig 2005; Ismail et al. 1999; Ivashuta et al. 2002; Neven et al. 1993; Welling et al. 2004) or to have cryoprotective activity (Momma et al. 2003; Neven et al. 1993; Wisniewski et al. 1999).

Y_2K_4 dehydrins from *Medicago* spp. show variations in their sequences

Comparative analysis revealed that *msa*CIG was highly homologous to other Y_2K_4 dehydrins from *Medicago* (Ivashuta et al. 2002; Pennycooke et al. 2008). The deduced amino acid sequence of *msa*CIG shows that the Y and K segments characteristics of dehydrins, closely match the consensus sequences for these motifs (Close 1996) with only a few conservative substitutions. Y segments are thought to be important for dehydrin interactions since they have been found to be homologous to the nucleotide-binding site of chaperones from plants and bacteria (Close 1996). Prediction of the secondary structure of *msa*CIG confirmed expectations that the K segments form amphipathic class A2 α-helices as previously reported in the literature (Campbell and Close 1997; Dure III 1993; Velten and Oliver 2001). Amphipathic helices could be involved in hydrophobic interactions with partially denatured proteins (Campbell and Close 1997) or membrane phospholipids (Koag et al. 2003). Xiao and Nassuth (2006) suggested that dehydrins with reported cryoprotective activity should contain at least three K segments. However, the presence of four K segments in the non-winterhardy *M. truncatula* (Thapa et al. 2008) suggests that the number of K segments may not be sufficient to confer superior cold tolerance. Pennycooke et al. (2008) obtained evidence that differences in the number of cis-acting elements in the upstream region of Y_2K_4 dehydrins from *Medicago* spp. combined with differences in gene copy number may be a factor contributing to differences in low temperature tolerance.

In contrast, the regions between the K segments were found to be highly variable among Y_2K_4 dehydrins from *Medicago* due to the frequent occurrence of large and small indels and/or single amino acid substitutions. These regions contain a large proportion of polar amino acids and Gly residues. Similar to the observations of Natali et al. (2003) in *Helianthus annuus* L., we documented several indels in *M. sativa* dehydrin variants that did not cause frameshifts. It has been reported by Ismail et al. (1999) that a deletion in a Gly-rich region was associated with a strong reduction in chilling tolerance in *Vigna unguiculata* seedlings. A duplication of a 24 amino acid motif in the polar Φ region before the first K segment has been associated with allelic variation at a dehydrin locus in *Pisum sativum* (Grosselindemann et al. 1998). The same motif was also present in one copy in a *Vicia monantha* dehydrin (accession no. AB506695.1). Interestingly, a stretch of 13 amino acids (P/TEYGSTNTGSGYG) at the beginning of that motif and two β-sheet forming stretches of five amino acids each were found in the region that preceded the first K segment uniquely in the *M. truncatula* sequence. These sequences might have been deleted from specific species lineages or inserted into the *M. truncatula* sequence subsequent to divergence from a common *Medicago* ortholog. The presence of the unusual small sequence NWNWNE between the K1 and K2 segments of the *Ms*CAS30 sequence near duplicated motifs may indicate a retro transposition event since tryptophan (W) residues are not typically found in dehydrins. It has been reported by Grosselindemann et al. (1998), that the presence or duplication of 21 amino acids residues between the K1 and K2 segments was largely responsible for size variations between three dehydrin genes in *Pisum sativum.*

As reported by Bassett et al. (2006), Peng et al. (2008) and Garcia-Bañuelos et al. (2009), we noted the rather exceptional occurence of one cysteine residue at the C-terminal end of the *Medicago* spp. Y_2K_4 dehydrins. The presence of a cysteine residue after the K4 segment was also found in dehydrins from *Pisum sativum* (Grosselindemann et al. 1998) and *Vicia monantha* (accession no. AB506695.1) and appears to be unique to a subset of dehydrins found in legumes. As suggested by Bassett et al. (2006), cysteine residues might be involved in intra- or intermolecular bridges/interactions necessary for dehydrin function.

Relationship between dehydrin variants and freezing tolerance

Our results illustrate that BSA in combination with genetic material that show clearly contrasted phenotypes is a simple but powerful approach to uncover DNA variants that were subject to selection. This approach has been successfully used for the identification of DNA markers linked to disease (Caixeta et al. 2003; Varshney et al. 2004) and drought resistance (Quarrie et al. 1999). Takeda and Matsuoka (2008) recently demonstrated that genes that were the target of selection can be identified through nucleotide sequence diversity observed between the initial background material and those lines derived from successive cycles of improvement.

Freezing tolerance of alfalfa is a complex trait under quantitative genetic control. This is supported by the

observation of extensive changes in gene expression and the identification of several cold-regulated genes in alfalfa (Castonguay et al. 2006). It is, however, noteworthy that a single MAS cycle within ATF0 based on the *msa*CIG polymorphism identified by RFLP yielded nearly 30% of the increase in freezing tolerance observed after five cycles of recurrent selection. This significant increase in freezing tolerance in populations preferentially enriched in the polymorphic dehydrin is indicative that this variant is associated with a quantitative trait locus (QTL) with a major impact on freezing tolerance. Dehydrin genes have been located within QTL intervals controlling winter hardiness (Campbell and Close 1997; Zhu et al. 2000). It is also noteworthy that the complete lack of the polymorphic dehydrin in the D− population did not reduce its capacity to withstand subfreezing temperatures below that observed with ATF0. Evidence gathered in the current study and in previous reports certainly warrants further investigations on a possible cause-and-effect relationship between specific dehydrin forms and superior freezing tolerance in alfalfa.

Based on the occurrence of duplicated segments (Grosselindemann et al. 1998) and the presence of indels (Ismail et al. 1999) in legume dehydrins, we looked for intragenic size variations potentially associated to the polymorphism uncovered on Southern blots. We observed a unique relationship between the presence of a specific indel in the region between the K2 and the K3 segments in the C-terminal region of a group of sequences of intermediary size (G2) and the polymorphism revealed on Southern blots. An indel in the same region was observed in another group of variant (G3) but was not associated to the polymorphism uncovered by Southern hybridization. This suggests that mutations occurring in the highly polar, hydrophilic Φ segments do not occur at random particularly since these regions were reported to interact with hydrophobic domains of macromolecules to help prevent their aggregation (Soulages et al. 2003).

In conclusion, our study illustrates that phenotypic recurrent selection within panmictic populations of allogamous species generates genetic material that can be used to identify genes that have a high likelihood of being associated with the selected traits. Using a BSA of alfalfa TF populations, we identified an Y_2K_4 dehydrin variant closely linked with superior freezing tolerance. Whether this polymorphic dehydrin is an allelic form of *msa*CIG, a paralog resulting from gene duplication or an ortholog acquired from an ancestor is a complex issue that will require further genetic studies. Another outstanding question is whether the observed variations in the C-terminal coding region are causally responsible for the phenotypic effect. This point will be addressed in future works. However, the information obtained in the current study paves the way to the development of a functional marker for the targeted introgression of genes with adaptive value in various genetic backgrounds of alfalfa.

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