

**Analysis of dehydrin sequences from alfalfa populations  
recurrently selected for freezing tolerance**

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We recently developed alfalfa populations that show a significant increase in tolerance to freezing and survival to harsh winter conditions (TF populations) using a recurrent selection protocol entirely performed under environmentally-controlled conditions. Although this approach is more predictable than field selection, the breeding process could be further accelerated by the identification of molecular markers closely associated to superior freezing tolerance. Dehydrins are ubiquitously found in plants that undergo a dehydration-related stress. These highly hydrophilic proteins are thought to play key adaptive roles with regard to tolerance to freezing-induced cell desiccation. A cDNA clone (*msaCIG*; Serge Laberge unpublished) with sequence features typical of dehydrins has been previously isolated from cold-acclimated alfalfa (*M. sativa* cv. Apica).

RFLP analysis of pooled DNA samples ( $\approx 45$  genotypes) from each of the cultivar Apica (ATF0) and ATF populations recurrently selected for improved freezing tolerance within that initial background were hybridized with a *msaCIG* probe. Even though several bands equally hybridized with *msaCIG* in all TF populations, a polymorphic fragment that markedly intensified in response to selection was clearly detected. Progenies from crosses of ATF0 genotypes with (D+) or without (D-) the polymorphic dehydrin significantly differed in their freezing tolerance. Improvement of freezing tolerance in D+ as compared to ATF0 accounted for more than 25% of the increase achieved after five cycles of recurrent selection in ATF5. This result indicated a major impact of this polymorphic dehydrin on freezing tolerance. Based on the modular nature of dehydrins, we looked for intragenic variations associated to the polymorphism detected on Southern blots. Genomic DNA from genotypes of ATF0 and ATF5 was pooled on the basis of the presence (+) or the absence (-) of the *msaCIG* polymorphism. Amplifications with primers targeting the 5' half side of *msaCIG* did not differ between pools of negative (-) and positive (+) genotypes. Contrastingly, there were significant variations in the size of amplified fragments in the 3' half side of *msaCIG*. Sequence analysis revealed the presence of *msaCIG* homologs with major insertions/deletions (indel) that did not affect the open reading frame. Whereas a fragment with two large indels decreased in frequency in pools of genotypes that show the *msaGIG* polymorphism on Southern blots, another fragment with a single 60 bp indel markedly increased in intensity in the same group of genotypes. Amplification with pooled DNA samples from ATF0 and ATF5 confirmed an increased frequency of the *msaCIG* sequence variant with the single 60 bp indel in response to selection for superior freezing tolerance.

The identification of dehydrin sequence variants closely associated to superior freezing tolerance paves the way to the development of functional markers and the fixation of favourable alleles in various genetic backgrounds.