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BRIEF COMMUNICATION

Dehydrin and proline content in *Brassica napus* and *B. carinata* under cold stress at two irradiances

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Abstract

The accumulation of cold-induced dehydrin and proline was related to the frost tolerance (FT) in several *Brassica* species or cultivars. A dehydrin of molecular mass 47 kDa was detected in the leaves of an Ethiopian mustard (*B. carinata*) and a pair of dehydrins of similar molecular mass in the three (two winter, one spring) oilseed rape (*B. napus*) cultivars, when plants were maintained at 4 °C for one-month under two different irradiances. More dehydrin was accumulated in oilseed rape than in Ethiopian mustard under the high irradiance. A significant correlation was observed between leaf dehydrin content and FT, and no relationship between proline content and FT or between the proline and dehydrin contents. Protoplast-derived callus cells behaved differently from leaves sampled from intact plants, as they did not accumulate dehydrin and proline in response to cold stress.

Additional key words: Ethiopian mustard, frost tolerance, oilseed rape, protein marker, protoplast-derived callus.

Assessing the frost tolerance (FT) of *Brassica* species under field conditions is hindered by climatic unpredictability, since a mild winter will prevent the differentiation between tolerant and non-tolerant types. Thus, conventional plant breeding has only enjoyed limited success in improving FT (Thomashow 1990). A more reliable screening method is clearly needed. A significant research effort has been invested into investigating the efficacy of various cryoprotective compounds in winter cereals, in particular cold regulated/late embryogenesis abundant (COR/LEA) proteins, which belong to the dehydrin family. These proteins help to limit water loss from the cell (Kosová *et al*. 2007), probably because they are effective at immobilizing water (Ingram and Bartels 1996). The timing of induction, maintenance and turning off of dehydrin expression, and its resulting accumulation *in planta* during the cold acclimation process is positively correlated with FT (Sarhan *et al*. 1997, Bravo *et al*. 1999, Renault *et al*. 2005). In a comparison based on cold-stressed plants of the contrasting winter wheat cvs. Mironovskaya 808 and Bezostaya 1, Vítámvás *et al*. (2007) noted that FT was predictable on the basis of the accumulation of WCS120 dehydrins. As a result, dehydrin content has been proposed as a marker for FT (Kosová *et al*. 2007). In cell cultures of alfalfa (Parmentier-Line *et al*. 2002) and blueberry (Wolfraim *et al*. 1993), dehydrin content was enhanced by the imposition of a cold treatment. Proline, which has been associated with the general stress response (Kavi Kishor *et al*. 2005, Kumar *et al*. 2010, Li *et al*. 2010, Toka *et al*. 2010), may also be cryoprotective, since proline over-producers display an enhanced cold tolerance (Patton *et al*. 2007, McClinchey and Kott 2008, Dörffling *et al*. 2009, Pociecha *et al*. 2009, Gothandam *et al*. 2010). The transgenic embryo of hybrid larch (*Larix* × *lepto-europaea*) with increased proline content was resistant to cold, salt, and freezing stresses (Gleeson *et al*. 2005).

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Abbreviations: FT - frost tolerance; HI - higher irradiance (400 μmol m^{-2} s^{-1}); LI - lower irradiance (120 μmol m^{-2} s^{-1}); LT_{50} - lethal temperature; PD - protoplast-derived; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.
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The present research was focused on establishing the relationships between FT and the contents of both dehydrins and proline in the leaves of various *Brassica* species, subjected to cold and two different irradiances. We also determined whether these relationships also hold in protoplast-derived (PD) callus cells.

The set of plant material consisted of two winter oilseed rape (*Brassica napus* L.) cvs. Californium and Viking, one spring oilseed rape cv. Topas and doubled haploid line derived from Ethiopian mustard (*Brassica carinata* A. Braun) cv. Dodolla. All seedlings were grown under a day/night temperature of 22/20 °C, a 12-h photoperiod with irradiance of 270 μmol m^{-2} s^{-1} for 30 d, at which point they reached the four leaf stage. The plants were then divided into two groups. All plants were then exposed for one month to 4 ± 1 °C and a 12-h photoperiod, with one group being at lower irradiance of 120 μmol m^{-2} s^{-1} (LI), and the other at higher irradiance of 400 μmol m^{-2} s^{-1} (HI). Before and after the cold treatment, the uppermost fully developed leaf was harvested from each plant and used to assess proline and dehydrin contents. Protoplast-derived calli were prepared following Kaur *et al*. (2006). When the calli had reached a diameter of 5 - 8 mm, they were divided into two groups of 20 calli. The calli in one group were weighed immediately, and those in the second group were transferred to fresh medium and weighed after a one-month exposure to 4 ± 1 °C in the dark.

Frost tolerance was evaluated after the end of the cold treatment. The FT was determined by the electrolyte leakage method, as described by Prášil and Zámečník (1998), in which leaf discs are cooled gradually from -3 to -30 °C. A software package developed by Janáček and Prášil (1991) was used to calculate the temperature, which was lethal for 50 % of the plants (LT_{50} value).

Proline content was determined by spectrophotometric method according to Jiménez *et al*. (2006). The reaction was monitored at 520 nm, using a 1 mM proline solution as a standard (*Sigma-Aldrich*, St. Louis, MI, USA). Proteins solubilised by boiling were extracted according to Vítámvás and Prášil (2008). Prior to the analysis, pelleted proteins extracted from 60 mg fresh tissue were dissolved in 50 mm^3 of sample buffer, from which 5 mm^3 was taken as a sample for 10 % SDS-PAGE separation (Laemmli 1970). Thermo-stable proteins were detected by silver staining and dehydrins by Western blot analysis, according to Vítámvás and Prášil (2008). The molecular mass of the dehydrins was estimated from the gel migration of molecular mass standards (*Bio-Rad,* Hercules, CA, USA), and their quantity was expressed in the form of a relative densitometric intensity [%], by means of the software package *Quantity One 4.6.7* (*Bio-Rad*). Individual readings were corrected according to measured differences between duplicate internal standards. Three independent biological replicates and at least two technical replicates were performed. The *Statistica v 7.0* for *Windows* software package (*StatSoft*, Tulsa, OK, USA) was used to calculate correlations, simple linear regressions and multi-factorial analyses of variance. Fisher's least significant difference (LSD) was applied to discriminate between treatment means.

Dehydrins of molecular mass ~47 kDa were detected in the leaves of all the cold treated plants (Fig. 1). Two similar-sized proteins were present in all three oilseed rape cultivars, while the Ethiopian mustard only produced one dehydrin. Each of the main effects (cold treatment, irradiance, genotype) and the interaction between genotype and irradiance were significant ($P < 0.05$). The most effective accumulator of dehydrin was cv. Topas under HI (11.7 %), followed by cv. Californium/HI (9.0), cv. Viking/HI (8.4), cv. Californium/LI (7.3), cv. Viking/LI (6.8), cv. Topas/LI (4.7), cv. Dodolla/LI (3.3) and

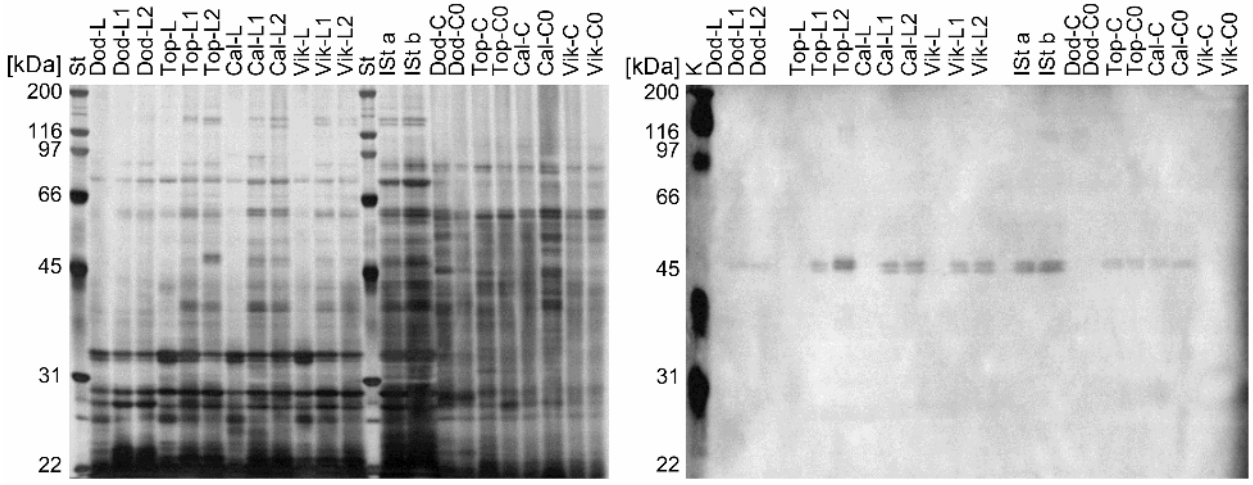

Fig. 1. SDS-PAGE profile (*left*) and Western blot probed with an anti-dehydrin antibody (*right*) derived from extracts of leaf and protoplast-derived callus. St - molecular mass standards; K - kaleidoscope broad range pre-stained standards (*Bio-Rad*); ISt a and ISt b - internal standards (b is a 1:1 dilution of a; a is an extract from cv. Californium plants held for 60 d at 4 °C under HI); Dod - cv. Dodolla; Top - cv. Topas; Cal - cv. Californium; Vik - cv. Viking; L - leaf extract; C - callus extract; 0 - cold treatment in the dark; 1 - cold treatment under LI; 2 - cold treatment under HI; unnumbered, no cold treatment.

cv. Dodolla/HI (2.6) (Fig. 2*A*).

Both irradiance and genotype had a significant effect on FT, but there was no significant genotype × irradiance interaction. The highest FT was achieved by cv. Topas/HI (LT_{50} -12.6 °C), followed by cv. Californium/HI (-11.4 °C), cv. Viking/HI (-10.7 °C), cv. Californium/LI (-10.2 °C), cv. Viking/LI (-10.0 °C), cv. Topas/LI (-9.8 °C), cv. Dodolla/LI (-4.9 °C) and cv. Dodolla/HI (-4.5 °C) (Fig. 2*B*).

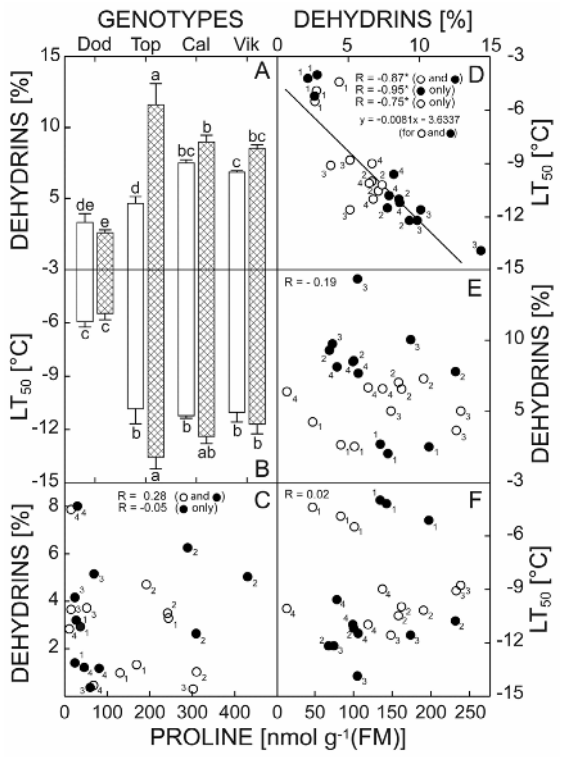

Fig. 2. Dehydrin content (*A*) and LT_{50} (*B*) in leaves of different cultivars, the relationship between callus dehydrin and proline contents (*C*), the relationship between LT_{50} and dehydrin content (*D*), between dehydrin and proline contents (*E*) and between LT_{50} and proline content (*F*) in leaves of plants exposed to cold for one month under two different irradiances. Calli were exposed to cold or cultivated under control conditions. In *A* and *B*, the columns denote means ± SE; means labelled with the same lower case letter do not differ significantly at $P = 0.05$ (Dod - cv. Dodolla; Top - cv. Topas; Cal - cv. Californium; Vik - cv. Viking). In *C*, *D*, *E* and *F*, *circles* denote values for each biological replicate (* - $P < 0.05$). In *C*, *empty circles* refer to the control treatment, *filled circles* to the cold treatment. In *A*, *B*, *D*, *E* or *F*, *empty columns* and *circles* refer to low irradiance, *hatched columns* and *filled circles* to high irradiance.

The correlation between the content of dehydrin in the leaves and the LT_{50} was highly significant ($P \leq 0.05$) and negative (-0.87, Fig. 2*D*). Within the HI treatment, the correlation was even higher (-0.95), while within the LI treatment, it was lower but still significant (-0.75, Fig. 2*D*). Because a higher FT is reflected by a more negative value of LT_{50}, this negative correlation implies a positive one between dehydrin content and FT. The experiments showed that the dehydrin content of the leaves, whether the plants were exposed to LI or HI, could be used to rank the entries with respect to FT.

Cold treated plants of three of the four entries (excluding cv. Topas) accumulated the similar or less amount of proline than the non-treated ones. Non-treated cv. Viking accumulated 142.3 nmol(proline) g^{-1}(f.m.), but only 88.7 and 93.3 nmol(proline) g^{-1}(f.m.) in the cold-treated plants exposed to LI and HI, respectively. Thus, there was no relationship between proline content and FT (Fig. 2*F*) or between the proline and dehydrin contents (Fig. 2*E*).

The effects of cold treatment, genotype and the cold treatment × genotype interaction on dehydrin content were all non-significant for the protoplast-derived calli. Dehydrin proteins were present in the SDS-PAGE profiles of some of the calli not exposed to cold treatment, but not in any of their cold-treated equivalents (Fig. 1). The highest dehydrin content was present in the cold treated calli of cv. Californium (4.7 %) and in the non-cold treated calli of cv. Viking (4.5 %). The lowest contents of dehydrin were present in cv. Dodolla calli (3.2 % in non-treated and 3.6 % in cold-treated). Similarly, for proline content, there was no significant cold treatment main effect, nor any significant cold treatment × genotype interaction. Although the genotype main effect was significant ($P < 0.05$), the content of proline in the calli of the non-cold treated plants was mostly higher than in the cold treated ones. Thus, the non-treated Dodolla calli accumulated proline up to 181.0 nmol g^{-1}(f.m.), but the cold treated ones accumulated only 29.3 nmol(proline) g^{-1}(f.m.). No correlation between proline and dehydrin content in the calli was evident (Fig. 2*C*), and the behaviour of the calli was overall too dissimilar to that of the leaves to contemplate an *in vitro* test of FT.

Other studies of dehydrin induction in cold treated oilseed rape have identified a 31 kDa ERD10 dehydrin (Deng *et al*. 2005) and a 19.2 kDa BnDHN1 (Yao *et al*. 2005), but the ~47 kDa products detected in the present experiments have not been previously observed.

Irradiance during frost hardening was thought to be an important factor in the development of FT in winter rye and wheat, since hardening under low irradiance was less effective than under natural irradiance (Gray *et al.* 1997, Janda *et al*. 2007). A similar results were obtained here with oilseed rape, while the somewhat reduced FT (and leaf dehydrin content) of the Ethiopian mustard cultivar in response to HI was likely an effect of photoinhibition. HI of 400 μmol m^{-2} s^{-1} combined with a low temperature may inhibited the activity of the photosynthetic centres, leading to some inhibition of overall metabolism, and hence of FT. On the other hand, the greater FT and higher leaf dehydrin content of the spring oilseed rape cultivar Topas could be explained by the fact that this cultivar appeared to be more capable than were the winter ones of exploiting higher irradiance. The lesser FT of the latter

may also reflect the longer period needed by winter oilseed rape to acquire FT, a difference noted in the cereals by Fowler (2008). Different rates of FT development, as well as differences in the levels of dehydrin expression and accumulation, may be a pleiotropic effect of vernalization genes, as suggested by Kosová *et al*. (2007). Although Rapacz (1999) also showed that the FT of spring oilseed rape cultivars was comparable to that of winter ones, this does not necessarily imply that spring types become as (or even more) winter hardened than winter ones. When a plant reaches its reproductive stage, its ability to achieve a high level of FT becomes substantially reduced. In vernalization requiring plants, this stage is reached only once the vernalization (as well as any photoperiod) requirement has been fulfilled, whereas in spring types, the timing of the switch depends on the rate of the plant development. Thus, unlike spring types, winter ones are able to re-acclimate during the winter season before they have reached a relatively advanced stage of development (Prášil *et al.* 2005). The ability to re-acclimate is usually an important factor for successful over-wintering.

The relationship between leaf dehydrin content and FT we have reported agrees well with what has been observed for barley (Kosová *et al.* 2008, 2010), wheat (Vítámvás *et al.* 2007, 2010) and olive (Cansev *et al.* 2009). Although proline was not significantly accumulated as a result of cold treatment in our experiments, the literature contains a number of reports that cold treatment does induce the accumulation of proline (Atici *et al*. 2003) and that there is a positive correlation between the level of proline in the leaf and FT (McClinchey and Kott 2008, Dörffling *et al*. 2009, Walker *et al*. 2010). However, in cauliflower, Fuller *et al.* (2006) suggested the opposite trend, specifically that FT was associated with lower proline contents. Janská *et al.* (2010) have described an ambiguous relationship between the proline content of *in vitro* selected hydroxyproline resistant winter oilseed rape and FT, with some lines accumulating less proline after cold treatment than when not exposed to any cold treatment. Bhattarai and Fettig (2005) have suggested that dehydrin accumulation forms part of the response of many plant species to various stresses. Thus the presence of dehydrins (and the elevated content of proline) in the non-cold treated calli is indicative that other stress factors were probably operating in these cell lines. In blueberry cell cultures, Parmentier-Line *et al.* (2002) have shown that a number of dehydrins are produced under supposedly non-stressful conditions. Our findings agree with those of Parmentier-Line *et al*. (2002), who showed that the responses of *in vitro* grown cells was very different from that of whole plants, thus making cell culture unsuitable for the study of dehydrin expression in whole plants.

We conclude that FT in oilseed rape and Ethiopian mustard can be predicted on the basis of the dehydrin content of the leaves of cold treated plants, and that there is potential to develop cold-induced dehydrins as protein markers for the improvement of FT in *Brassica* spp.

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