Heat Tolerance of Cold Acclimated Puma Winter Rye Seedlings and the Effect of a Heat Shock on Freezing Tolerance

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An increase in tolerance to one form of abiotic stress often results in an increase in tolerance to another stress. The heat tolerance of Puma rye (Secale cereale) was determined for seedlings either not cold hardened or hardened under either controlled environmental or natural conditions. The heat tolerance was determined either as a function of time at 42°C or the ability to tolerate a maximum temperature. The seedlings were either not heat preconditioned or heat preconditioned before the heat stress. In all cases cold hardened seedlings were more heat tolerant than non or partially cold hardened seedlings. Heat preconditioning had no effect on the heat tolerance of naturally cold hardened seedlings. In contrast, seedlings cold hardened in a controlled environment chamber, then heat preconditioned, were more heat tolerant than non preconditioned seedlings. A heat shock of 36°C for 2 h increased the freezing tolerance of non hardened seedlings from −2.5°C to −4.5°C. Analysis of heat shock protein 70 (HSP70) gene expression indicated that the HSP70 gene was not induced by cold acclimation and therefore not directly involved in the increased thermo tolerance observed. A number of heat stable proteins, simple sugars and long chain carbohydrate polymers accumulated during the cold acclimation process and may have a role in increasing heat tolerance as well as freezing tolerance. These data suggest cold hardening increases heat tolerance, however, a heat shock to non acclimated seedlings only marginally increased the freezing tolerance of Puma rye seedlings.

Key words: Freezing — Heat — Rye — Secale cereale — Tolerance.

Cross-adaptation refers to the induction of enhanced stress tolerance to multiple stresses by exposure to a single stress. For example, enhanced heat tolerance can be induced by both low temperature (Palta et al. 1981) and saline treatments (Bonham-Smith et al. 1987, O’Connor et al. 1991). Moreover, freezing tolerance can be enhanced by subjecting plants to dehydration (Tyler et al. 1981, Siminovitch and Cloutier 1982), osmotic stress (Tyler 1979), heat shock (Guy et al. 1986, Lafuente et al. 1991), and salinization (Boussiba et al. 1975, Schmidt et al. 1986, Hincha 1994). Also, application of the phytohormone abscisic acid (ABA) to cell suspension cultures induces both heat and freezing tolerance (Robertson et al. 1994a).

Cross-adaptation indicates the existence of common systems for the development of resistance to unfavorable environmental factors (Boussiba et al. 1975, Levitt 1980, Hajela et al. 1990). Boussiba et al. (1975) subjected tobacco (Nicotiana tabacum) plants to salinization, dehydration and mineral deficiency and reported a two-fold increase in ABA levels and an increase in freezing tolerance. Accumulated evidence indicates common genes are expressed during various stresses and ABA treatment (Robertson et al. 1987, Mundy and Chua 1988, Guy et al. 1992, Neven et al. 1992, Arora and Wisniewski 1994). For instance, dehydrins, a group of proteins synthesized during dehydration of plants, accumulated during cold acclimation (Arora and Wisniewski 1994, Muthalif and Rowland 1994, Robertson et al. 1994b). Also, a spinach protein (CAP79), which increased in response to low temperatures, had a high degree of homology with maize HSP70 (Neven et al. 1992). Robertson et al. (1994a) reported that a set of ABA-responsive heat-stable proteins isolated from bromegrass cell suspension cultures protect heat sensitive proteins from heat-induced coagulation. This result suggests a molecular mechanism for cross-adaptation.

Heat tolerance is the ability of an organism to withstand an otherwise lethal heat treatment once it has been pre-treated with a nonlethal heat shock (Lindquist 1986, Nagao et al. 1990). The heat shock conditions that lead to the development of heat tolerance also induce the synthesis of heat shock proteins (HSP) (Gerner and Schneider 1975, Lindquist 1986, Nagao et al. 1990). HS proteins, especially HSP70, act as molecular chaperones (Ellis and van der Vies 1994, Muthalif and Rowland 1994, Robertson et al. 1994b) which facilitate protein folding and subunit assembly in unstressed cells (Pelham 1988, Ellis 1987, Hemmingsen et al. 1988) and stabilize cellular proteins during stress (Deshaies et al. 1988, Nagao et al. 1990). A heat shock also enhances chilling tolerance in cucumber (Lafuente et al. 1991) and salt resistance in tobacco cells (Harrington and Alm 1988), and cotton (Kuznetsov et al. 1990).
In the following studies, Puma winter rye seedlings were used to investigate: (i) The relationship between freezing tolerance and heat tolerance; and (ii) The effect of a sub-lethal heat treatment on seedling freezing tolerance.

Materials and Methods

Determination of heat tolerance—Heat tolerance was determined on Puma winter rye seedlings at varying levels of freezing tolerance. Puma winter rye plants were subjected to a decreasing temperature regime: 7 d at 7/5°C light/dark; 7 d 5/2°C; 14 d 2/0°C with a 14 h photoperiod. Seedlings were collected from the field in early November and stored at −4°C until used. Seedlings wrapped with moist paper towels in 25 × 140 mm glass test tubes were transferred to a shaking water bath. The test tubes were submerged in the water to 2/3 of their depth to ensure that the seedlings were exposed to the predetermined temperatures and then shaken at 30 rpm. Prior to the determination of heat tolerance, the plants were divided into two groups. Half of the seedlings were transferred directly from cold acclimating temperatures to high temperatures. This is defined as non-preconditioning. The remainder of the seedlings were subjected to nonlethal heat shock treatments using a procedure adapted from Moisyadi and Harrington (1989) with modifications. During heat preconditioning, the seedlings were exposed to gradually elevated temperatures as follows: 30°C for 1 h, 35°C for 1 h and then 40°C for 30 min. Heat tolerance of preconditioned and non-preconditioned Puma rye seedlings was then assessed by two assays, a temperature effect and a duration effect at 42°C. The temperature effect assay involved subjecting either non-preconditioned or preconditioned seedlings to the following temperatures for 2 h (38, 40, 42, 44, 46, 48, or 50°C). Heat tolerance (HT50) was defined as the temperature at which 50% of the seedlings survived. The duration effect assay involved treating the non-preconditioned and preconditioned seedlings at 42°C for 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, or 16 h. Heat duration tolerance (HDT50) was defined as the length of time at which 50% of the seedlings could survive at 42°C. After heat treatments the seedlings were transplanted to Redi-Earth™ to evaluate survival after three weeks of regrowth in a greenhouse at 20°C.

Deacclimation of Puma winter rye seedlings by a 42°C heat shock—Seedlings were cold acclimated using a decreasing temperature regime as described above. The cold-acclimated seedlings were either heat-preconditioned or non-preconditioned as described above and then incubated at 42°C for 1, 2 and 4 h. The heat-treated seedlings were kept at 23°C for 2 h (recovery period) and then subjected to a freeze test as described by Gusta et al. (1978). Regrowth conditions following the controlled freeze test were as described previously.

Induction of freezing tolerance by a heat shock at 42°C—Rye seedlings grown at 20°C with a 18 h photoperiod were subjected to a heat shock treatment and then evaluated for freezing tolerance. The seedlings were heat preconditioned at 30°C for 1 h and transferred to either 32, 34, 36, 38, 40, or 42°C for 2 h. The heat-treated seedlings were evaluated for freezing tolerance as described by Gusta et al. (1978).

RNA extraction and hybridization—RNA extraction and hybridization were performed as previously described (Robertson et al. 1994b) with the following modifications. Approximately 1 g FW of crown tissue was used per sample and the initial extraction was carried out in 3 ml 0.2 M sodium acetate (pH 5.2), 1 ml of 10% SDS and 3 ml of phenol equilibrated with 1 M Tris·Cl (pH 7.5). The RNA was separated by electrophoresis on 6% formaldehyde gel (1× MOPS, 1.2% agarose) and transferred to gene screen plus nylon membrane by capillary blotting using 20× SSC. Prehybridization was at 43°C for 16 h in 20 ml of prehybridization solution (50% deionized formamide, 5× SSPE, 1% SDS, 5× Denhardt’s solution) with 2 mg of yeast tRNA. Hybridization was performed overnight at 37°C in same solution following the addition of 50 ng of [32P]dCTP labelled soybean HSP70 cDNA (pSB70). The membrane was washed twice at room temperature for 15 min in 1× SSC, 1% SDS and once at 55°C in 1× SSC for 15 min before exposure to Kodak XAR 5 film.

Heat stable protein analysis—Heat stable proteins were isolated from 1 g of leaves of rye seedlings. The extraction, purification and sample preparation are as previously described (Robertson et al. 1994a). Purified protein samples (50 and 70 μg per lane) were separated by 10% SDS-PAGE and the proteins visualized by staining with Coomassie Blue.

Carbohydrate analysis—Rye crown tissue (1 g) was used for carbohydrate analysis. Extraction and analysis of carbohydrates in environmental growth cabinet raised material was done as previously described (Wilen et al. 1996).

Results

Heat tolerance of Puma winter rye seedlings—In general, seedlings of Puma winter rye acclimated for 4 weeks in a controlled environment or collected from the field in early November had more heat tolerance than non-acclimated seedlings. The heat tolerance of cold-acclimated Puma winter rye seedlings increased as cold acclimation progressed.
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(Fig. 1: mean of three experiments). Seedlings cold acclimated for 4 weeks in a controlled environment had a HT50 of 44°C versus 41.5°C for seedlings acclimated for 1 week. Seedlings collected from the field in early November were more heat tolerant (HT50 49.5°C) than the seedlings cold acclimated for 4 weeks in a controlled environment. Moreover, seedlings cold acclimated in a controlled environment tolerated higher temperatures after they received a heat-preconditioning treatment (Fig. 1). For example seedlings that were cold acclimated for 4 weeks, and then heat preconditioned had an HT50 of 48.5°C, 4.5°C more than the non preconditioned seedlings. However, field-acclimated seedlings did not show differences in heat tolerance whether the seedlings were heat-preconditioned or not.

Seedlings cold acclimated in a controlled environment tolerated a longer period at 42°C than non-acclimated seedlings (Fig. 2: mean of three experiments). Non-acclimated seedlings and seedlings acclimated for 1 week tolerated 42°C for 1.5 h, whereas seedlings cold acclimated for 4 weeks tolerated 42°C for 3.5 h (Fig. 2). The difference was especially pronounced if the plants were heat-preconditioned prior to the heat treatment (Fig. 2). Non-acclimated, heat-preconditioned seedlings had a HDT50 of 3.5 h at 42°C, whereas seedlings cold acclimated for 1 and 4 weeks had a HDT50 of 6 and 14 h at 42°C, respectively (Fig. 2).

The effect of a heat shock at 42°C on the freezing tolerance of Puma winter rye seedlings

The effect of a heat shock on freezing tolerance was determined by subjecting either cold-acclimated or non-acclimated Puma winter rye seedlings to 42°C. The seedlings received either a heat-preconditioning treatment or were transferred directly to 42°C. Seedlings cold acclimated for four weeks underwent an abrupt loss of freezing tolerance following exposure to 42°C for one hour for both non-preconditioned and heat-preconditioned seedlings (Fig. 3). However, seedlings that had been heat preconditioned before being placed at 42°C were more freezing tolerant than non conditioned seedling following the heat treatment. Even after 4 h at 42°C, the heat conditioned seedling exhibited up to 4°C more freezing tolerance than nonconditioned seedlings (Fig. 3).

Seedlings cold acclimated for one week deacclimated upon exposure to 42°C (Fig. 4: mean of three experiments). For example, after one hour at 42°C non-preconditioned seedlings had an LT50 of −5°C versus −11°C for the non-heated controls. The loss in freezing tolerance in seedlings acclimated for one week was less after heat-preconditioning than before heat preconditioning (Fig. 4). The loss in freezing tolerance of the heat-preconditioned seedlings occurred only after two hours at 42°C: LT50 of −8.5°C after heat preconditioning versus LT50 of −3°C before heat preconditioning and heating at 42°C for 2 h (Fig. 4).

The effect of a heat shock on freezing tolerance of non-acclimated Puma winter rye seedlings is shown in Figure 5 (mean of three experiments). Heat-preconditioned seedlings exposed to 36°C for 2 h were 1.5°C more freezing tolerant than the 20°C controls, while none of the other temperatures had any effect on freezing tolerance. 

Expression of HSP70 gene during cold acclimation

The expression of the 70 kDa heat shock protein (HSP70)
gene has been correlated with increased thermo tolerance (Nagao et al. 1990). From RNA blot analyses we determined that HSP70 transcript levels in rye did not change significantly during acclimation under natural conditions (Fig. 6), nor did subsequent dehardening of plants in the spring cause any change in HSP70 mRNA levels. Similar results were obtained in rye seedlings acclimated in controlled environments (data not shown). These results are similar to those reported for spinach plants grown in controlled environments (Neven et al. 1992).

Accumulation of heat-stable proteins during acclimation—Our previous work with a bromegrass cell culture has shown the importance of ABA-inducible heat stable proteins in conferring tolerance to freezing stress (Robertson et al. 1987, 1994a, b, Wilen et al. 1996), and that these proteins protect cellular components from heat denaturation (Robertson et al. 1994a). Soluble protein from non-acclimated and acclimated rye seedlings was heated to 90°C and analyzed by SDS-PAGE. A number of polypeptides (75-83 kDa) were found to accumulate in the seedlings that had been acclimated for 1 or 4 weeks in environmental growth cabinets (Fig. 7). A number of lower molecular mass proteins were also found to accumulate during the first week of acclimation, but disappeared by the end of the acclimation period. Heat stable proteins also accumulated in field acclimated rye seedlings. However, the molecular mass of the proteins which accumulated in the field acclimated material differed from that observed in the cabinet raised plants (Fig. 7). The field acclimated protein was enriched with a number of lower molecular mass polypeptides which were found to cross react with an antibody raised against a peptide based on the dehydrin carboxy terminal consensus sequence (data not shown; Robertson et al. 1994a).

Accumulation of carbohydrates during acclimation—We have previously reported the importance of sucrose and maltose in the acquisition of freezing tolerance (Wilen et al. 1996), and have also shown that sucrose can increase the effectiveness of the heat stable proteins in preventing pro-
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Fig. 7 The accumulation of heat-stable proteins in winter rye seedlings acclimated under controlled environment and natural conditions. Heat stable proteins from non acclimated seedlings, seedlings acclimated for 1 and 4 weeks in controlled environment cabinet and seedlings acclimated in the field were analyzed by SDS-PAGE on a 12% polyacrylamide gel. Twenty five 

protein denaturation (Robertson et al. 1994a). We therefore measured carbohydrate levels in acclimated and non acclimated rye seedlings. Consistent with the report of Koster and Lynch (1992), fructose and glucose increased during acclimation in both environmental growth cabinet (Fig. 8; peaks at RT 7.8, and 9.0 min) and field grown Puma rye (data not shown). Sucrose (RT 10.5 min) concentration increases were noted in field grown samples and in some, but not all of the environmental growth cabinet material. An increase was also noted in the accumulation of long chain polysaccharides (eluted at retention times between 33–44 min) during acclimation in growth cabinet raised plants.

Figure 8 is the HPLC profile of carbohydrates extracted from non acclimated rye seedlings (left), and from rye seedlings acclimated at 2°C for 7 d (right). The degree of polymerization of these unidentified polysaccharides was likely four and greater based on the retention time (RT) of the standards (maltotetraose RT=34 min; maltoheptaose RT = 42 min) and we are currently isolating these polymers for further analysis. We were unable to determine if a similar carbohydrates were present in field acclimated seedlings, likely due to the presence of interfering compounds.

Discussion

The induction of heat tolerance by conditions that increase cold acclimation has been reported in leaf tissue of Solanum (Palta et al. 1981). In contrast, Chen et al. (1982) and Guy and Haskell (1987) reported that cold acclimation had no effect on heat tolerance. In this study, seedlings either at different stages of cold acclimation or acclimated under different environmental conditions, were heated for 2 h at various temperatures or held at 42°C for various lengths of time. In general, the heat tolerance of Puma winter rye seedlings was positively associated with the freezing tolerance of the seedlings. Puma winter rye seedlings cold acclimated for 4 weeks in a controlled environment were

Fig. 8 The accumulation of polysaccharides during acclimation of Puma rye seedlings. HPLC profile of the methanol extract of nonacclimated Puma rye seedlings (left profile) and Puma rye seedlings acclimated for 7 d at 2°C in an environmental growth cabinet (right profile).
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more heat tolerant than seedlings acclimated for 1 week (Fig. 1, 2). Moreover, fully cold hardened seedlings collected in early November from the field exhibited higher levels of freezing tolerance and heat tolerance than seedlings acclimated in a controlled environment (Fig. 1). The increased hardness of the field material may reflect the imposition of additional stresses besides low temperature (e.g., drought, wind, freeze-thaw).

Heat-stable proteins accumulate in response to cold (Lin et al. 1990, Houde et al. 1992), dehydration (Close et al. 1989) and exogenous ABA (Robertson et al. 1994a). Recently, it was shown that a set of ABA-responsive heat-stable proteins isolated from bromegrass cell suspension cultures increased the heat stability of heat-sensitive proteins isolated from control cells (Robertson et al. 1994a). As ABA-treated bromegrass cells were more heat tolerant than non-treated cells, it was postulated that heat stable ABA-responsive proteins were involved in conferring heat tolerance. In the present study, cold acclimation of Puma winter rye seedlings resulted in increased accumulation of heat-stable proteins (Fig. 7). These heat-stable proteins may help confer the observed heat tolerance to cold-acclimated Puma winter rye seedlings.

The difference noted between the heat stable polypeptides that accumulated in field-raised rye versus controlled environment-acclimated seedlings could be due to the presence of additional stresses during the acclimation period in the field-grown plants. Plants grown in the field are subject to temperature fluctuation, freeze-thaw, drought, and wind stress. Robertson et al. (1994b) noted a difference in the accumulation of dehydrin mRNA between rye plants acclimated in the field and in growth cabinets, with a greater accumulation of low molecular mass dehydrin transcripts detected in the field-raised plants. It was also noted that similar, but not identical, heat-stable proteins could also be induced by drought, cold acclimation or exogenous ABA in rye seedlings grown in environmental growth cabinets (Fu 1995).

HSP70, a family of 70 kDa heat shock proteins, may assist protein folding and assembly under unfavorable environmental conditions (Deshaiies et al. 1988, Nagao et al. 1990). Recently, a spinach cDNA encoding a cold acclimation-induced protein (CAP79) displayed a high degree of sequence homology with HSP70 (Neven et al. 1992). A 70 kDa heat shock cognate (HSC70) isolated from cold-acclimated spinach seedlings, exhibited both ATP and protein binding, indicating that it may be involved in conformational changes of other proteins (Anderson et al. 1994). However, the authors were not able to demonstrate a corresponding increase in HSP70 during the cold acclimation treatment. Therefore, whether HSP70 was involved in general stress tolerance is in question. Our results also indicate that HSP70 is not up regulated during acclimation under natural cold acclimating conditions (Fig. 6).

During cold acclimation other changes may also occur within the cells, e.g., carbohydrate accumulation, cell wall thickening, etc., which render the cells more tolerant to a heat stress. Both heat stable proteins and carbohydrates accumulated during cold acclimation of rye seedlings (Fig. 7, 8). We have shown previously that both heat stable proteins and carbohydrates are necessary for full expression of freezing tolerance (Wilen et al. 1996). It was also shown that the addition of heat stable proteins or sucrose prevented the denaturation of cellular proteins by heat (Robertson et al., 1994a). The most effective protection occurred when both heat stable proteins and sucrose were used in combination.

Heat-preconditioning (heat shock) induces increased heat tolerance (Lindquist 1986) and synthesis of HSP (Li and Hahn 1980, Lindquist 1986, Nagao et al. 1990). In this study, higher heat tolerance was evident in seedlings subjected to heat preconditioning, especially in seedlings that were cold acclimated in growth cabinets. Thus, the heat preconditioning used in this study likely induced synthesis of heat protectants (possibly heat shock proteins) that are involved in the development of heat tolerance.

Dehardening of woody perennials has been extensively studied (Howell and Weiser 1970, Gusta and Weiser 1972, Coleman 1985). However, very little is known about the dehardening of winter cereals. Gusta and Fowler (1976a, b) demonstrated a decrease in the hardiness of winter wheat and winter rye exposed to warm temperatures in both controlled environments and during spring growth in the field. In these studies, the rate of dehardening was strongly influenced by temperature. Crowns of winter wheat and winter rye dehardened at a faster rate at 20°C compared to 10 or 15°C. The same authors reported that increasing spring soil temperatures resulted in a reduction in LT50, an increase in crown water content and a decrease in dry weight; conditions which are associated with the resumption of active growth (Fowler and Gusta 1977). To the authors' knowledge, no studies on the effect of high temperatures on the dehardening of winter rye have been reported previously. In the studies by Gusta and Fowler (1976a, b), deacclimation of winter wheat plants at 20°C occurred over several days. In the present study, winter rye plants lost up to 12°C of freezing tolerance after one hour at 42°C. In seedlings acclimated for 4 weeks, the rapid loss in freezing tolerance occurred irrespective of whether the tissue was heat preconditioned or not. This suggests that heat shock factors or HSP are not capable of repairing freeze-induced protein damage despite their ability to protect against heat-induced protein damage (Nagao 1990, Ellis and van der Vies 1991). Robertson (unpublished results), studying bromegrass suspension culture cells, also observed that a heat shock rapidly dehardened freezing-tolerant bromegrass cells. Further analyses of protein fractions isolated from freezing-tolerant cells revealed that specific proteins...
coagulated at heat shock temperatures (42°C). This finding may suggest that these heat sensitive proteins play a role in freezing tolerance.

It is well established that the fluidity of the membrane is temperature dependent. However, it is not known if membrane composition can change within one hour at 42°C. Previous research has established that protein synthesis decreased during the heat shock response (Nagao et al. 1990). If protein synthesis is inhibited at 42°C, then the enzymes involved in changing the fluidity of the membrane may not be synthesized. Further work is required to establish this. In addition, elevated temperatures increase respiration (Leenders et al. 1974, Nickell and Browder 1988) which could result in a rapid loss of cryoprotective carbohydrates and freezing tolerance of the seedlings.

Freezing tolerance was only marginally increased by a heat shock at 36°C. Heat-shock-induced tolerance to low temperatures has been reported in chilling sensitive species, such as cucumbers (Lafuente et al. 1991). Exposure of cucumber cotyledons to 37°C before chilling reduced the rate of ion leakage by 60% compared to non-heat-shocked controls. A 4- to 5-fold increase in freezing survival was also reported for heat shocked conidia spores of Neurospora crassa (Guy et al. 1986). Heat shock-induced freezing tolerance has not been observed in freezing tolerant species, such as spinach seedlings (Guy and Haskell 1987) and Brassica suspension culture cells (Johnson-Flanagan and Singh 1987). Therefore, if a heat shock response leads to the development of freezing tolerance, the inductive conditions could be a critical factor determining the level of tolerance induced.

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References


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