Polypeptide markers for low temperature stress during seed germination in sunflower

A. KUMAR and S.C. BHATLA[1]

Department of Botany, University of Delhi, Delhi-110007, India

Abstract

Sunflower seeds behaved as chilling and freezing sensitive and also exhibited acclimation under low seed moisture content (< 1 %). At high seed moisture content (approx. 22 %) they tolerated chilling stress but failed to acclimate under freezing temperatures. Pre-imbibitional chilling (5 °C) or freezing (-5 or -10 °C) stress significantly enhanced total soluble protein (TSP) content. Chilling treatment after imbibition (in contrast to pre-imbibition) enhanced germination and this was accompanied by increase in 30, 24 and 21.9 kDa TSPs content (3 d after germination). Freezing at -5 and -10 °C suppressed seed germination and increased content of 78 and 56.2 kDa wall bound proteins. Chilling acclimation decreased 35.4, 33.9, 29.5, 23.4 and 21.4 kDa TSPs.

Additional key words: chilling stress, freezing stress, *Helianthus annuus* L., protein.

Introduction

Sunflower grows well at temperatures of 20 - 30 °C with 27 - 28 °C being optimum. Sensitivity of sunflower seeds and seedlings to temperature variations and oxygen might be responsible for unsatisfactory field emergence under unfavourable sowing conditions such as low soil temperature or excess water in soil (Weiss 1999). At the time of harvest sunflower achenes are dormant and germinate poorly particularly at relatively low temperatures (Corbineau *et al*. 1990). They become non-dormant after long dry storage and germinate easily over a wide temperature range.

The ability to cold acclimate is a quantitative genetic trait in plants (Thomashow 1990). Well-known changes occurring during cold acclimation include changes in leaf ultrastructure (Ristic and Ashworth 1993), membrane composition (Lynch and Steponkus 1987, Miquel *et al.* 1993), protein composition (Raison 1973, Hughes and Dunn 1996, Karimzadeh *et al.* 2000), activities of enzymes, accumulation of sugars and polyamines (Levitt 1980, Strand *et al.* 1997) and activation of ion channels (Knight *et al.* 1996). Increase in soluble protein content and different protein patterns have been observed in cold acclimated winter wheat and cabbage species (Atici *et al.* 2003). Stress-induced variations in proteins have also been observed in *Parthenium argentatum* leaves (Sundar and Chaitanya 2003).

Present investigations were undertaken primarily to analyze sensitivity of sunflower seeds to low temperatures under low and high moisture contents. Differences in germination response due to low temperature stress have been analyzed in terms of polypeptides from cytosol and cell wall fractions which exhibit temperature-modulated variations in their expression.

Materials and methods

Temperature treatments and seed germination: Sunflower (*Helianthus annuus* L. cv. Morden) seeds were subjected to low temperature treatments in dry and imbibed condition. Dry seeds were exposed to different low temperature treatments viz. 5 °C, for 5 d, -10 °C for 2 d, and 5 °C for 5 d followed by -10 °C for 2 d . Control seeds were maintained at 25 ± 2 °C during this period. After temperature treatments seeds were imbibed for 4 h

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Abbreviations: SDS-PAGE - sodiumdodecyl sulphate - polyacrylamide gel electrophoresis; TSP - total soluble protein; WBP - wall bound protein.

[1] Author for correspondence; Fax: +91 11 27666744; e-mail: scbhatla@hotmail.com

in water and germination was achieved on moist germination paper in three sets of Petri dishes (25 seeds in each Petri dish) in dark at 25 ± 2 °C. In the second condition, seeds were first imbibed for 4 h in water and then exposed to above said low temperature treatments. Seeds were regarded as germinated with the rupture of seed coat and emergence of radical. Cotyledons were harvested from fresh seeds (ungerminated; 0-d stage) and 3-d-old seedlings for various analyses. Percentage germination response was calculated from three sets of 25 seeds each for every treatment. Fresh mass data was determined from 15 pairs of cotyledons. Dry mass and moisture content of cotyledons were calculated after drying in an oven at 80 °C.

SDS-PAGE analysis of polypeptides: Cotyledons (1 g fresh mass) were harvested from seeds at different stages of germination after being subjected to low temperature stress prior to and after imbibition. Tissue was ground to fine powder in liquid nitrogen and homogenized with cold Tris-sucrose homogenization buffer [0.1 M Tris pH 7.5; 0.4 M sucrose, 10 mM KCl, 1 mM $MgSO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 % v/v 2-mercaptoethanol] in a proportion of 2 $cm^3 g^{-1}$(f.m.) using pre-chilled pestle and mortar. The homogenate was filtered through five layers of muslin cloth and centrifuged at 10 000 *g* for 30 min at 4 °C. Total soluble protein (TSP) in the supernatant was used for subsequent analysis. For wall bound protein fraction, the pellet was incubated overnight in 2 M NaCl. NaCl - extracted proteins were dialysed against Tris-EDTA (pH 7.5) for 8 h followed by acetone precipitation. Protein pellet was re-dissolved in grinding buffer. SDS-PAGE was carried out according to Laemmli (1970) using a 5 % stacking gel and 10 to 20 % linear gradient resolving gel (Sadeghipour and Bhatla 2002). Total protein content was determined according to Bradford (1976).

Results

Effect of low temperature stress on seed germination: Seeds exposed to varying temperatures prior to imbibition exhibited remarkable variations in germination response with reference to low temperature stress. Seeds exposed to low temperature in dry condition (prior to imbibition) behaved as chilling sensitive since seed germination percentage was reduced in seeds pre-exposed to 5 °C. Thus, the response was 20 % less in these seeds compared to control seeds (25 °C) 3 d after treatment (Table 1). Freezing temperature of -5 or -10 °C evoked similar reduction in germination response. Thus sunflower seeds were also freezing sensitive under low seed moisture content, however, the germination response normalized and became similar to that in control sets if the seeds were pre-exposed to non-freezing temperature of 5 °C followed by an exposure to freezing temperature of -5 or -10 °C. The observed differences in seed germination response due to chilling or freezing stress (as compared to control) were statistically significant (CD at 5 % level = 13.976; Table 1).

Table 1. Effect of low temperature stress on germination percentage of sunflower seeds determined 3 d following temperature treatments. Seeds were exposed to temperature treatments before or after imbibition. Each treatment consisted of 25 seeds at a time. Data represent mean from three sets of independent experiments along with standard deviations (SD). CD at 5 % level = 13.976.

Treatments	Prior to imbibition	After imbibition
25 °C (control)	68.0 ± 3	68.0 ± 3
5 °C	48.0 ± 10	70.6 ± 10
-5 °C	46.6 ± 8	0
-10 °C	52.0 ± 7	0
5 °C followed by -5 °C	64.6 ± 5	0
5 °C followed by -10 °C	69.3 ± 9	0

Alterations in total protein content: Pre-imbibitional low temperature stress (5 °C, -5 °C and -10 °C) significantly enhanced total soluble protein (TSP) content in the cotyledons of seeds 3 d after treatment (CD at 5 % level = 3.09; Fig. 1). No such increase in TSP content was detected in seeds subjected to low temperature stress after imbibition and the protein contents remained unaltered during 3 d of treatments. Cell wall bound protein (WBP) fraction largely remained unaffected by low temperature stress both prior to and after imbibition (CD at 5 % level = 1.93).

Expression of polypeptides (wall bound and cytosolic) in response to low temperature stress: Chilling sensitivity of sunflower seeds was profusely affected by seed moisture content. It was reflected in altered germination response and accompanying changes in TSPs and WBPs. Greater germination response at 5 °C in post-imbibed seeds was accompanied with enhanced expression of TSPs, particularly 30, 24, and 21.9 kDa, and their down regulation in other treatments (Fig. 2; *lane* 2). In contrast to changes in TSP, several WBPs revealed down regulation in seeds at 5 °C following imbibition, in contrast to other treatments. These particularly include 38, 32.8, 17.2, and 12.3 kDa polypeptides (Fig. 3; *lane* 2). Up regulation of these polypeptides was commensurated with no seed germination response at other temperatures.

Effect of freezing stress (-5 and -10 °C) on altering polypeptide pattern was particularly evident soon after treatment of imbibed seeds (0-d stage) and it scaled down at 3-d stage. This included up regulation of 78 and

56.2 kDa WBPs in -5 and -10 °C and 12.3 and 11.5 kDa WBPs in -5 °C only. These up regulations can be regarded as freezing-specific changes. While these freezing-induced up regulations of polypeptides were maintained at 3-d stage in -5 °C, they reverted back at -10 °C to WBP pattern evident at 25 °C. Dry seeds subjected to these freezing temperatures also revealed similar changes in WBPs particularly at 3-d stage. Thus, an up regulation of 56.2, 50.1 and 38 kDa WBPs were clearly evident. No changes in TSPs were found in response to freezing stress (Fig. 3).

With regard to chilling acclimation, a down regulation of 35.4, 33.9, 29.5, 23.4 and 21.4 kDa TSPs was evident in imbibed seeds at 0-d (Fig. 2; *lanes* 5,6). No such changes were detected in dry seeds at 0-d and in dry or imbibed seeds at 3-d stage in response to chilling followed by freezing. Up regulation of WBPs was observed at 56.2 kDa in dry seeds at 0-d in seeds subjected to 5 °C followed by -5 °C (Fig. 3; *lane* 5). Similar up regulation was also evident at freezing temperature of -5 and -10 °C in imbibed seeds, particularly at 0-d stage (Fig 3; *lanes* 3,4).

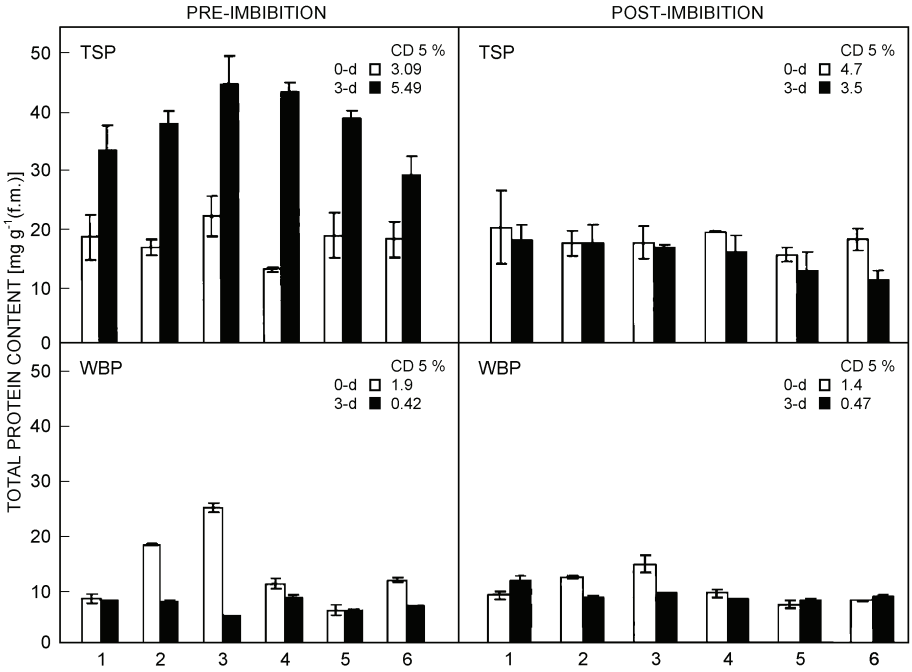

Fig. 1. Changes in total protein content in the cytosol (total soluble proteins; TSP) and cell wall fraction (wall bound proteins; WBP) of cotyledons from sunflower seeds subjected to low temperatures. Treatments: 25 °C (1, control), 5 °C (2), -5 °C (3), -10 °C (4), 5 °C followed by -5 °C (5) , 5 °C followed by -10 °C (6).

Discussion

Temperature and degree of imbibition are two important conditions which influence seed germination. At harvest time sunflower seeds are dormant and germinate poorly. This is the result of embryo dormancy and the inhibitory action of seed coat. Embryo dormancy is mainly responsible for seed sensitivity to low temperature (Corbineau *et al*. 1990). Following initial water uptake, germinating seeds are characterized by relatively little change in seed water content until it is terminated by the initial embryo growth (Bradford *et al*. 2000). Different processes have been suggested to be involved in the loss of viability of seeds in different water content ranges. At low hydration level, water molecules associated with macromolecular structures are non-freezable and their removal can lead to conformational changes that may be irreversible and damaging. At intermediate water contents, uncontrolled oxidative reactions can lead to damage. It is possible to identify a specific water content (or water potential) corresponding to desiccation damage (Boyer 2001). Sunflower seeds (present work) behave as chilling and freezing sensitive and also exhibit acclimation under low moisture content. At high moisture content, they tolerate chilling conditions but fail to acclimate under freezing temperatures.

There exists little information about the genes responsible for the initiation of embryo growth. Pre-sowing hydration of seeds is reported to be associated with an increase in protein synthesis (Job *et al*. 2000). Onset of germination is accompanied with a change in the pattern of gene expression which quickly switches from a developmental mode to a germination mode. Proteins/polypeptides associated with cell enlargement

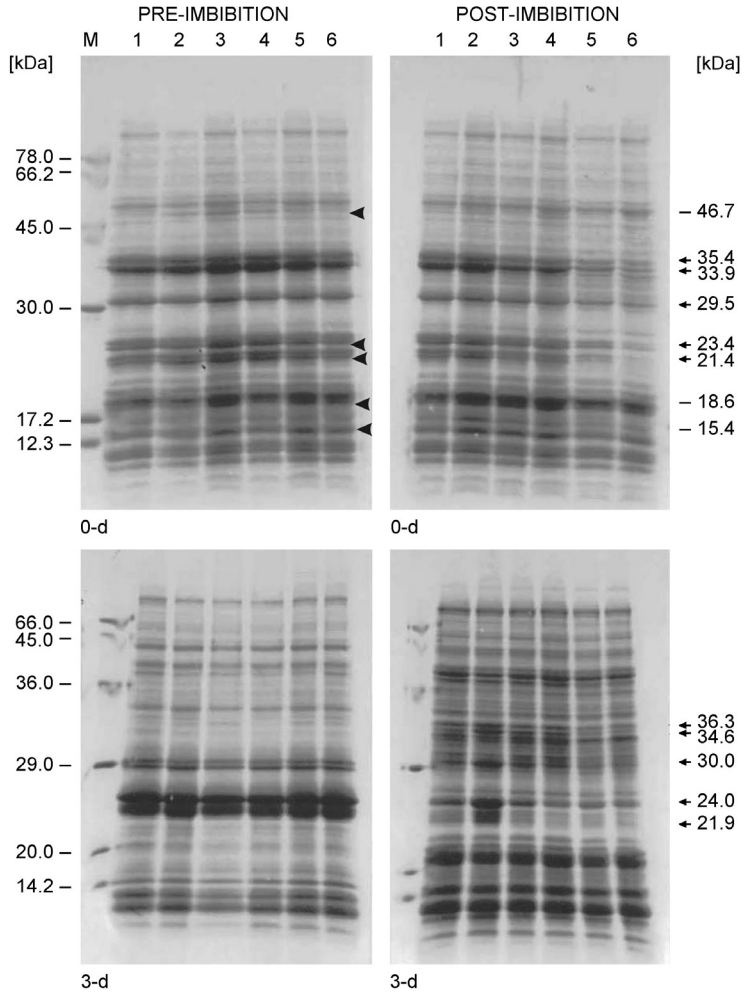

Fig. 2. Changes in the polypeptide pattern of total soluble proteins (TSP) from the cotyledons of sunflower seeds subjected to low temperatures. Treatments: 25 °C, (1, control). 5 °C (2), -5 °C (3), -10 °C (4), 5 °C followed by -5 °C (5), 5 °C followed by -10 °C (6).

are good candidates as markers since the initial protrusion of embryo from the seed is due to water uptake and cell expansion. Messenger RNAs for a number of cell wall hydrolases (polygalactouranase, arabinosidase, β-1,3-glucanase and chitinase) are up regulated following imbibition (Bradford *et al.* 2000). During periods of cold acclimation, some plants accumulate apoplastic antifreeze proteins, such as endochitinases, β-endoglucanases and osmotin, leading to structural adaptations for protecting cells from freezing stress (Bray *et al.* 2000). Expression of specific forms of aquaporins could be another important protein modulated by low temperature stress. It is evident from the present observations that polypeptides with molecular mass between 25 to 30 kDa (equivalent to molecular mass of aquaporin proteins) do exhibit differential expression in relation to chilling or freezing stress at 0-d and 3-d stages. It may be mentioned at this point that genes for the expression of aquaporins are known to be up regulated by GA and ABA and also differentially affected by drought. (Sanders and Bethke 2000). Several other genes induced by low temperature are also induced by water deficit or ABA. Thus, a category of late embryogenesis abundant (LEA) proteins, specifically LE 25 reported from tomato seeds, are known to confer freezing tolerance (Bray *et al. 2000*).

To sum up, distinct changes in polypeptides of wall fraction and buffer soluble tissue homogenates were evident in response to chilling stress, freezing stress and during chilling acclimation. Expression patterns of these changes were also modulated differently in dry and imbibed seeds. Lastly, these modulations of polypeptides were expressed as early (0-d stage) or late stage (3-d) responses to varying temperature stresses. Present work has highlighted the significant polypeptide markers of low temperature stress expressed in cytosol and cell wall fractions of cotyledons. Their molecular mass are close to some of the polypeptides expected to be regulated during low temperature stress. Further work is likely to confirm whether different isoforms of hydrolases and aquaporins are expressed at different stages of germination.

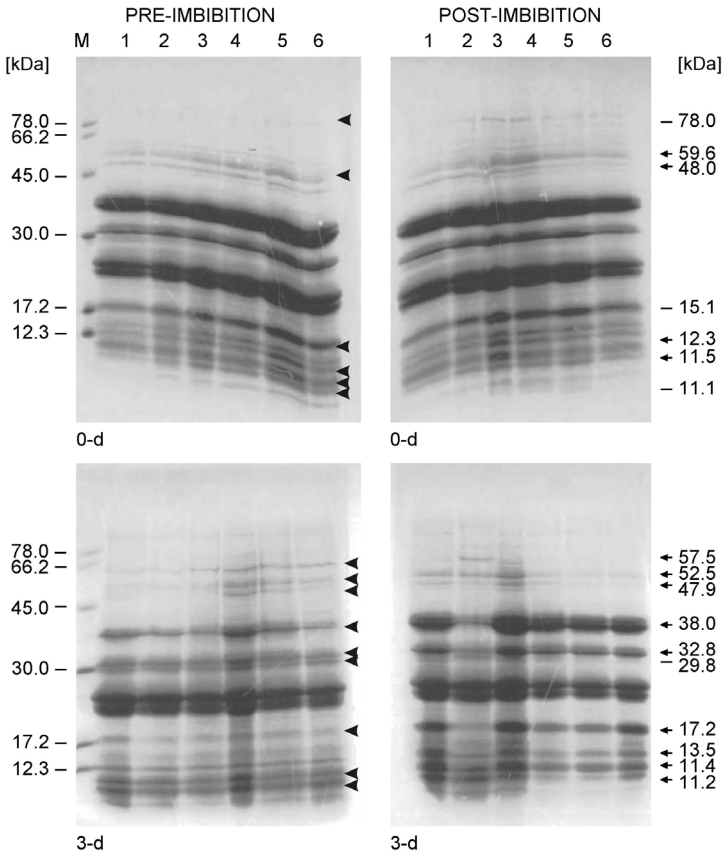

Fig. 3. Changes in the polypeptide pattern of salt soluble, wall bound proteins from the cotyledon homogenates of sunflower seeds subjected to low temperatures. Treatments: 25 °C (1, control), 5 °C (2), -5 °C (3), -10 °C (4), 5 °C followed by -5 °C (5), 5 °C followed by -10 °C (6).

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