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Factors contributing to enhanced freezing tolerance in wheat during frost hardening in the light

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Abstract

The interaction between light and temperature during the development of freezing tolerance was studied in winter wheat (*Triticum aestivum* L. var. Mv Emese). Ten-day-old plants were cold hardened at 5 °C for 12 days under normal (250 μmol m^{-2} s^{-1}) or low light (20 μmol m^{-2} s^{-1}) conditions. Some of the plants were kept at 20/18 °C for 12 days at high light intensity (500 μmol m^{-2} s^{-1}), which also increased the freezing tolerance of winter wheat. The freezing survival rate, the lipid composition, the antioxidant activity, and the salicylic acid content were investigated during frost hardening. The saturation level of hexadecanoic acid decreased not only in plants hardened at low temperature, but also, to a lesser extent, in plants kept under high light irradiation at normal growth temperature. The greatest induction of the enzymes glutathione reductase (EC 1.6.4.2.) and ascorbate peroxidase (EC 1.11.1.11.) occurred when the cold treatment was carried out in normal light, but high light intensity at normal, non-hardening temperature also increased the activity of these enzymes. The catalase (EC 1.11.1.6.) activity was also higher in plants grown at high light intensity than in the controls. The greatest level of induction in the activity of the guaiacol peroxidase (EC 1.11.1.7.) enzyme occurred under cold conditions with low light. The bound ortho-hydroxy-cinnamic acid increased by up to two orders of magnitude in plants that were cold hardened in normal light. Both high light intensity and low temperature hardening caused an increase in the free and bound salicylic acid content of the leaves. This increase was most pronounced in plants that were cold treated in normal light.

Keywords: *Triticum aestivum* L.; Antioxidant enzymes; Fatty acid composition; Frost tolerance; Salicylic acid

1. Introduction

Low temperature is one of the most important factors that limits the growth and distribution of plants. It is well known that even in frost-tolerant species, particularly in wheat plants, a certain period of growth at low, but non-freezing temperature, known as frost hardening, is required for the development of frost hardiness. In wheat plants this cold acclimation includes changes in a wide range of physical and biochemical processes that allow functioning at low temperatures (Shinozaki et al., 2003), such as the induction of antifreeze proteins (Yeh et al., 2000) and changes in the

Abbreviations: 16:0, hexadecanoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; APX, ascorbate peroxidase; CAT, catalase; DBI, double bond index; EDTA, ethylenediaminetetraacetic acid; FAME, fatty acid methyl esters; GR, glutathione reductase; GSH, reduced glutathione; HL, high light; LHC, light harvesting complex; LL, low light; NBT, nitro blue tetrazolium; NL, normal light; oHCA, *ortho*-hydroxy-cinnamic acid; PE, phosphatidylethanolamine; pHBA, *para*-hydroxy-benzoic acid; PG, phosphatidylglycerol; POD, guaiacol peroxidase; PS2, Photosystem 2; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; t16:1, *trans*-Δ3-hexadecenoic acid.

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membrane composition (Vigh et al., 1985; Huner et al., 1987; Wang et al., 2006). Growth at low temperature may also cause an excessive excitation of the electron transport systems, which may lead to an increase in the concentration of reactive oxygen species (ROS). If the plants are not able to control the intracellular ROS level, the membrane lipids, proteins and nucleic acids may suffer damage leading to the death of the cells (Suzuki and Mittler, 2006). Several studies have been conducted on changes in the antioxidant activity in plants under stress conditions. However, only a few data are available on changes in the antioxidant activity during cold hardening (Janda et al., 2003; Baek and Skinner, 2003), while very little is known about its role in the development of frost tolerance and the endogenous signals controlling these processes. Salicylic acid (SA) is an endogenous signal molecule, which may play a role in plant responses to abiotic stresses. Exogenous SA and certain related compounds were found to protect plants against heat stress (Dat et al., 1998) and chilling injury (Janda et al., 1999, 2000), to decrease the effect of paraquat on photosynthesis (Ananieva et al., 2002), and to be involved in the modulation of salt and osmotic stresses (Borsani et al., 2001; Al-Hakimi and Hamada, 2001; Hamada and Al-Hakimi, 2001). More recently it was also shown that SA treatment may enhance the freezing tolerance of wheat, since the ice nucleation activity of apoplastic proteins increased after SA treatment (Tasgín et al., 2003).

It was shown in winter rye and wheat plants that frost hardening under low light conditions was much less effective than under normal light conditions (Gray et al., 1997; Apostol et al., 2006). A certain level of freezing tolerance could also be induced in rye plants by high light treatment without low temperature. It was also shown that the induction of certain cold-stimulated genes in wheat is correlated with the relative reduction state of Photosystem 2 (PS 2) rather than with growth temperature or growth irradiance per se (Gray et al., 1997).

The exact mechanisms of the contribution of light during the hardening period to the enhanced freezing tolerance of cereals are still poorly understood. Besides demonstrating the positive influence of high light intensity during growth on the freezing tolerance of wheat, the aim of the present work was to discover what biochemical changes were responsible for the enhanced freezing tolerance when hardening takes place in the light.

2. Results

2.1. Freezing tolerance after hardening under different conditions

To determine the effectiveness of the 12-day frost hardening under different light and temperature conditions, wheat plants were frozen at −10 and −12 °C at low light intensity (Table 1). All the unhardened plants died after 1 day of freezing even at −10 °C. Plants hardened under normal light conditions (250 μmol m^{-2} s^{-1}) had a survival rate of 100% after freezing either at −10 °C or at −12 °C. However, plants which were hardened either at high light intensity at normal growth temperature for 12 d or at low temperature with low light intensity (at 20 μmol m^{-2} s^{-1}) partially survived both freezing temperatures (Table 1).

Table 1
Freezing survival (%) of control plants of winter wheat (var. Mv Emese) grown at 20/18 °C under normal light conditions (250 μmol m^{-2} s^{-1}, NL; control), and of plants hardened for 12 days at 20/18 °C at high light intensity (500 μmol m^{-2} s^{-1}, HL) or at low temperature (5 °C) under normal or low (20 μmol m^{-2} s^{-1}, LL) light conditions

Treatment	Survival (%) after freezing	
	−10 °C	−12 °C
Control	0 ± 0	0 ± 0
20/18 °C HL	57 ± 33	1 ± 1
5 °C NL	100 ± 0	100 ± 0
5 °C LL	35 ± 8	1 ± 1

Plants were frozen for 1 day at −10 °C or at −12 °C. Means ± SD, $n = 4$.

2.2. Fatty acid composition

Table 2 shows the fatty acid composition of phosphatidylglycerol (PG) in total leaf extracts from control winter wheat plants and from plants hardened in the cold or at high light intensity. The most pronounced changes occurred in the saturation level of hexadecanoic acid: cold hardening, under both normal or low light conditions, caused a significant increase in the palmitic acid level (16:0), parallel with a decrease in the level of t16:1. Interestingly, these changes occurred not only in plants hardened at low temperature, but also, although in a lesser degree, in plants which were kept under high light irradiation. The saturation level of the C-18 lipid in PG from the leaves did not change substantially under these hardening conditions (Table 2).

Significant differences in the lipid composition of the PE fraction from the control plants occurred only when hardening was carried out at low temperature in the light. The 16:0 and 18:0 contents of PE decreased by about 30 mol% with a concomitant increase in the 18:3 content, leading to a significant increase in the DBI in plants which were hardened at 5 °C under normal light conditions (Table 3). The other treatments did not cause significant changes in this lipid class.

2.3. Antioxidant enzymes

To detect differences in the antioxidative capacity of wheat plants at the end of the hardening period the activities of five antioxidant enzymes were measured. Fig. 1a shows that the activity of the glutathione reductase (GR) enzyme was greatly affected by light: both low temperature hardening under normal light conditions and hardening at high light intensity at non-hardening temperature caused

Table 2
Fatty acid composition (mol%) of PG from total leaf extracts of winter wheat plants grown at 20/18 °C at normal light intensity (250 μmol m^{-2} s^{-1}, NL; control) and of plants hardened for 12 days at 20/18 °C at high light intensity (500 μmol m^{-2} s^{-1}, HL) or at low temperature (5 °C) under normal or low (20 μmol m^{-2} s^{-1}, LL) light conditions

Treatment	%16:0	%t16:1	%18:0	%18:1	%18:2	%18:3	DBI
Control	19.98 ± 2.24	19.47 ± 0.86	1.31 ± 0.61	1.38 ± 0.48	6.64 ± 0.23	51.21 ± 2.04	168.3 ± 6.1
20/18 °C HL	25.94 ± 3.33*	15.76 ± 3.22*	0.95 ± 0.09	1.34 ± 0.07	7.91 ± 0.81*	48.11 ± 1.24*	161.5 ± 3.5
5 °C NL	25.94 ± 1.36***	12.80 ± 0.96***	0.80 ± 0.25	1.07 ± 0.09	7.98 ± 0.50***	51.41 ± 0.65	171.3 ± 1.3
5 °C LL	28.67 ± 4.60**	13.81 ± 3.12**	1.17 ± 0.59	0.72 ± 0.31*	6.12 ± 0.59	49.52 ± 2.23	161.5 ± 6.1

Means ±SD, $n = 5$, *, **, *** significantly different from the control at the $p \leqslant 0.05$, 0.01 and 0.001 levels, respectively.

Table 3
Fatty acid composition (mol%) of PE in total leaf extracts from winter wheat plants grown at 20/18 °C under normal light conditions (250 μmol m^{-2} s^{-1}, NL; control) and of plants hardened for 12 days at 20/18 °C at high light intensity (500 μmol m^{-2} s^{-1}, HL) or at low temperature (5 °C) under normal or low (20 μmol m^{-2} s^{-1}, LL) light conditions

Treatment	%16:0	%18:0	%18:1	%18:2	%18:3	DBI
Control	30.51 ± 2.07	1.87 ± 0.30	1.97 ± 0.15	31.29 ± 1.92	34.36 ± 4.28	167.6 ± 9.0
20/18 °C HL	28.20 ± 1.26	2.25 ± 0.97	2.19 ± 0.47	29.08 ± 2.09	38.28 ± 3.54	175.2 ± 7.1
5 °C NL	20.16 ± 1.80***	1.06 ± 0.46*	1.63 ± 0.67	28.83 ± 4.27	48.32 ± 5.74**	204.3 ± 9.2***
5 °C LL	27.77 ± 2.23	1.54 ± 0.66	1.64 ± 0.44	29.78 ± 2.22	39.27 ± 3.25	179.0 ± 8.2

Means ±SD, $n = 5$, *, **, *** significantly different from the control at the $p \leqslant 0.05$, 0.01 and 0.001 levels, respectively.

an increase in the activity. Low temperature at low light intensity only caused a slight, non-significant increase. Ascorbate peroxidase (APX), the other enzyme of the ascorbate glutathione cycle, showed a similar change, except that in this case low temperature hardening also caused a statistically significant increase at low light intensity (Fig. 1b). Comparing the data of catalase activity it can be seen that the highest activity was measured in plants treated with high light intensity for 12 days. Plants hardened at low temperature also exhibited slightly higher catalase activity than the control, unhardened plants (Fig. 1c). The most surprising changes occurred in the activity of the guaiacol peroxidase (POD) enzyme, as the most significant changes could be detected in plants hardened at low temperature under low light conditions (Fig. 1d). None of the treatments caused significant changes in the superoxide dismutase (SOD) activity (data not shown).

2.4. Effect of frost hardening on the ortho-hydroxy-cinnamic acid (ortho-coumaric acid) and salicylic acid contents in wheat leaves

For the determination of the *ortho*-hydroxy-cinnamic acid (oHCA) and salicylic acid (SA) levels, leaves of control and hardened plants were sampled after 12 days of treatment. While none of the hardening conditions used in the experiment caused any significant difference in the free oHCA content of wheat leaves after 12 days of hardening (Fig. 2a), the bound oHCA level increased by two orders of magnitude in plants cold-treated in normal light (Fig. 2b). The slight increase in the bound oHCA caused by high light intensity or cold hardening at low light intensity was not statistically significant. Both high light intensity and low temperature hardening caused an increase in the free SA content of the leaves (Fig. 2c). This increase was most pronounced in plants cold-treated in normal light. Bound SA also increased in a similar way under these conditions, although the rise was not significant in plants that were cold treated at low light intensity (Fig. 2d).

3. Discussion

In the present work, it was also shown that, as also experienced in rye (Gray et al., 1997), low temperature hardening in normal light is significantly more efficient in winter wheat plants than hardening under low light conditions; furthermore, growing at non-hardening temperature but under elevated light conditions may also increase the survival rate. In winter rye tolerance to low temperature-induced photoinhibition and the mRNA accumulation of certain cold-induced genes correlated with the excitation pressure of PS2 (Gray et al., 1997). However, the biochemical mechanisms by which light improves freezing tolerance are very poorly understood. The main aim of the present study was to provide further possible explanations for the advantage of hardening in the light.

The membrane lipid composition in the leaves of the winter wheat variety Mv Emese was studied in control plants and in plants cold-hardened at low temperature at normal or low light intensity, or treated with high light intensity for 12 days. Monogalactosyldiacylglycerol and digalactosyldiacylglycerol usually show no significant change during hardening at low temperature (Huner et al., 1989). As previously observed in wheat crowns (Szalai et al., 2001), hardening caused a decrease in the 16:0 and 18:0 contents of PE in wheat leaves with a concomitant increase in the 18:3 content, leading to a significant increase

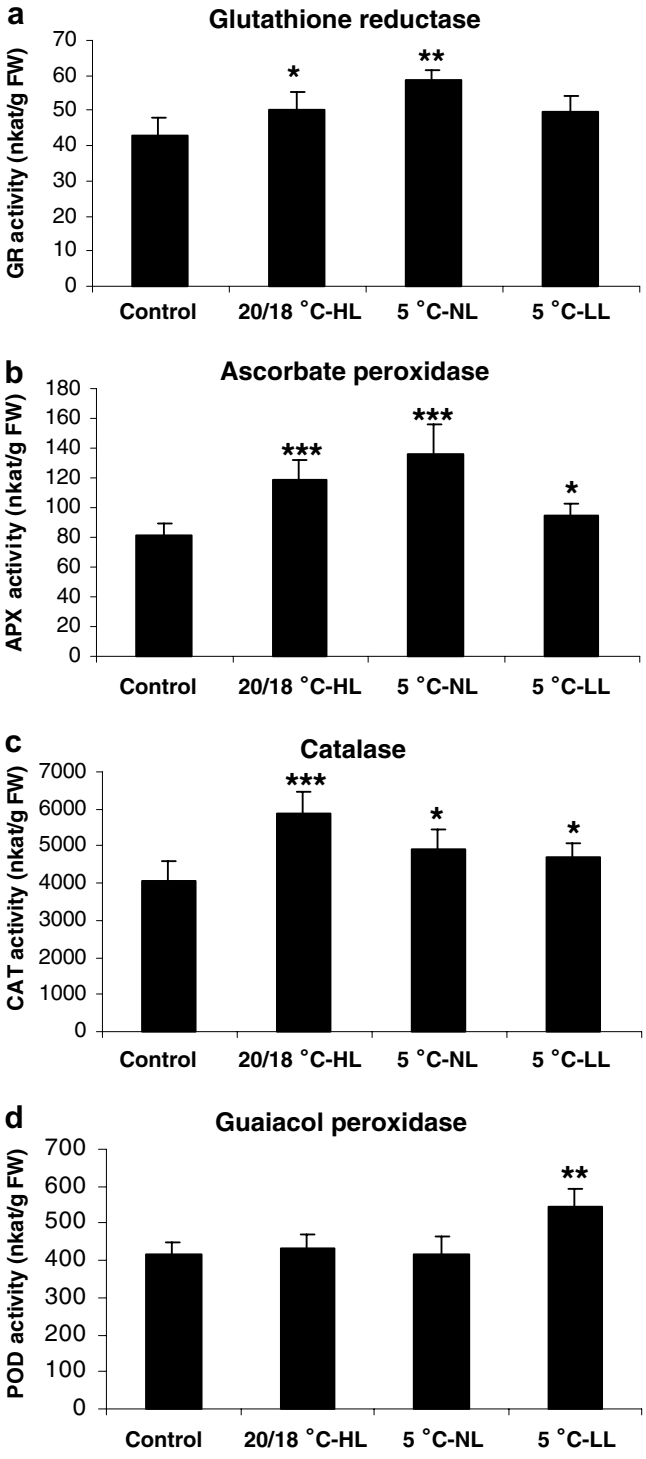

Fig. 1. Activities of antioxidant enzymes, namely glutathione reductase (a), ascorbate peroxidase (b), catalase (c) and guaiacol peroxidase (d), isolated from leaves of the winter wheat variety Mv Emese, which were either unhardened (control), or treated at high light intensity (HL; 500 μmol m^{-2} s^{-1}) or hardened at 5 °C for 12 days at normal (250 μmol m^{-2} s^{-1}, NL) or low (20 μmol m^{-2} s^{-1}, LL) light intensity. $n = 5$, *, **, *** significantly different from the control at the $p \leqslant 0.05$, 0.01 and 0.001 levels, respectively.

in the DBI. However, these changes were significant only when hardening was carried out at low temperature in the light.

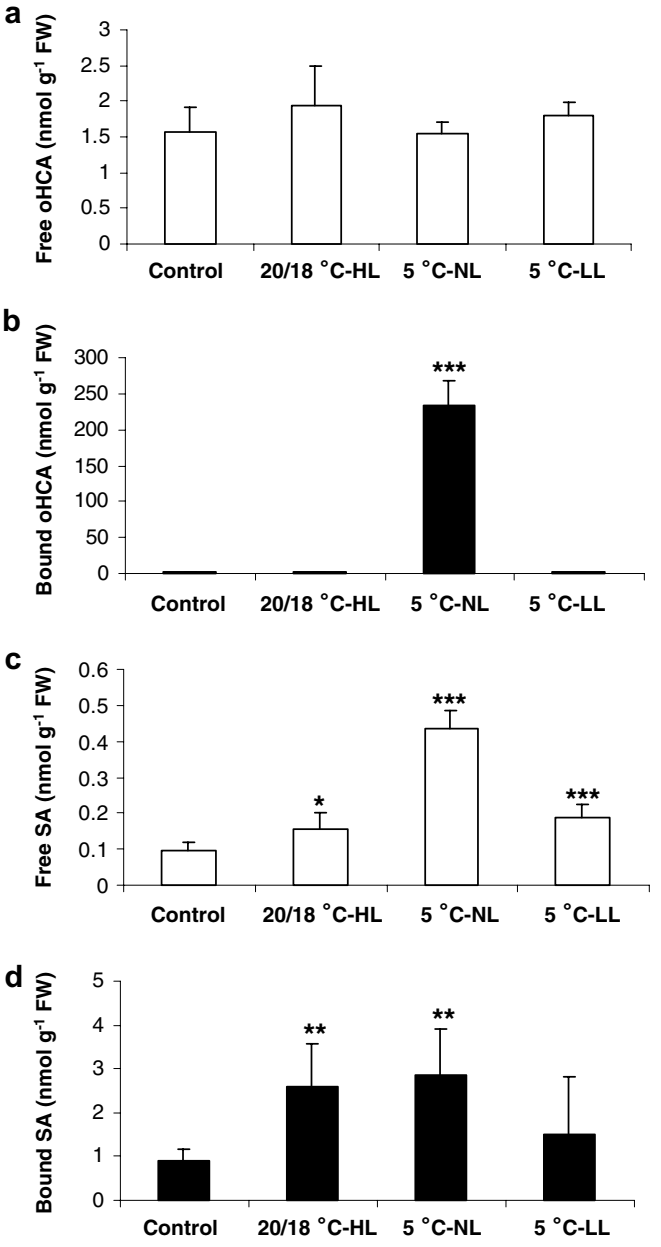

Fig. 2. Changes in free or bound oHCA (a, b) and free or bound SA (c, d) in the leaves of winter wheat variety Mv Emese, which were either unhardened (control), or treated at high light intensity (HL; 500 μmol m^{-2} s^{-1}) or hardened at 5 °C for 12 days at normal (250 μmol m^{-2} s^{-1}, NL) or low (20 μmol m^{-2} s^{-1}, LL) light intensity. $n = 5$, *, **, *** significantly different from the control at the $p \leqslant 0.05$, 0.01 and 0.001 levels, respectively.

Winter cereals must not only survive, but also need to grow and develop at low temperature to achieve maximum frost tolerance to survive the winter. Photosynthesis provides the energy necessary for the attainment of a cold-acclimated state and may contribute to achieving the highest potential freezing tolerance in winter cereals (Huner et al., 1998). Cyclic electron flow may have a role in regulating the quantum yield of PS 2, and may provide extra energy for CO_2 fixation (Cornic et al., 2000). Using

thermoluminescence and measurements of P700 relaxation kinetics it has recently been shown that growth at low, hardening temperatures in the light increased the rate of the cyclic photosynthetic electron transport, which may contribute to the higher frost tolerance observed after low temperature hardening in the light (Apostol et al., 2006). Cold hardening caused the most pronounced changes in the PG lipid fraction. PG, which includes t16:1, is involved in the oligomerization of the LHC (McCourt et al., 1985; Krupa et al., 1987) and the dimerization of PS 2 (Kruse et al., 2000). A correlation has also been demonstrated between the drop in the t16:1 content of thylakoid PG during hardening and the frost resistance of cereals (Huner et al., 1989; Szalai et al., 2001), so this drop in the t16:1 content can be used as a very sensitive indicator of the enhanced frost tolerance during the hardening period. In the present work it is shown that while the saturation level of the C-18 fatty acids in PGs extracted from the leaves did not change substantially under any of the hardening conditions used in these experiments, the saturation level of hexadecanoic acid decreased not only in plants hardened at low temperature, but also, to a lesser degree, in plants kept at high light intensity. This means that the regulation of LHC organization by changes in the t16:1 content of PG in cereals is caused not only by low temperature but also by excitation pressure, and may contribute to the enhanced freezing tolerance induced by high light at non-hardening temperatures.

Low temperature hardening in cereals may also act as a mild abiotic stress that induces generalized physiological processes including tolerance to freezing temperature, and resistance to photoinhibition or to various biotic stresses, such as snow moulds (Gaudet et al., 2003). It is a well-known fact that most stress factors, whether biotic or abiotic, are usually associated with oxidative stress and may activate the antioxidant systems. Antioxidant defence responses have long been associated mainly with enhanced antioxidant enzyme activity and increased levels of antioxidant metabolites (Hegedűs et al., 2001; Kocsy et al., 2001). In many cases the increased tolerance to certain stress factors can be attributed to the efficiency with which the plant is capable of neutralising ROS. In cereals a correlation was found between the development of tolerance to freezing and oxidative stress, which suggests that freezing tolerance at the subcellular level may be influenced by the ability to detoxify activated forms of oxygen (Bridger et al., 1994).

In the present work, it was shown that changes in the antioxidant enzyme activity during frost hardening depend not only on the temperature but also on the light intensity. The greatest induction of the GR and APX enzymes occurred when the cold treatment was carried out in normal light, but high light intensity at normal, non-hardening temperature also increased the activity of these enzymes. As members of the ascorbate-glutathione cycle, these enzymes play an important role in the detoxification of the ROS generated during photosynthetic electron transport in the chloroplasts. This is in agreement with previous results demonstrating a positive correlation between frost tolerance and the activity of APX in the leaves of cold-hardened plants of several cereal species, varieties and chromosome substitution lines (Janda et al., 2003). This supported the finding that APXs in the chloroplast play an important role in protecting the photosynthetic machinery against oxidative damage (Danna et al., 2003), thus keeping the photosynthetic processes efficient enough to provide energy for the development of frost tolerance. The catalase activity was also higher in plants grown at high light intensity than in the control. No difference in SOD activity was observed between plants treated under different temperature and light conditions. This is in agreement with results showing that SOD activity is not directly related to freezing tolerance (McKersie et al., 1999). In contrast to the enzymes GR, APX and catalase, the activity of the POD enzyme was induced to the greatest extent under cold conditions at low light intensity. This suggests that, although it may contribute to the detoxification of ROS, the activity of the POD enzyme is not a direct key factor in the freezing tolerance of wheat plants, as also found when investigating the chilling tolerance of maize plants (Janda et al., 2005). Since POD activity was also correlated with resistance to snow mould in wheat plants, it is possible that it has a major role in the development of resistance against pathogen attacks occurring during the winter season (Gaudet et al., 2003). It should be mentioned that other factors may also alter the activity of certain antioxidant enzymes. Preliminary experiments showed that aging under normal growth conditions might cause a decrease in the catalase activity and an increase in that of POD. This may explain the differences between the present results and earlier findings, which suggested that hardening caused a decrease in the activity of catalase (Janda et al., 2003). Furthermore, it must be emphasised that certain ROS, such as H_2O_2, act not only as harmful agents but also as signal molecules activating several specific and aspecific protective mechanisms, so the role of the components in the defence mechanism against oxidative damage is not only to remove ROS, but also to keep a balance between signalling and destructive effects (Suzuki and Mittler, 2006).

When applied at suitable concentrations, exogenous SA may cause transient oxidative stress in plants, which acts as a hardening process, increasing the antioxidative capacity of the plants (Dat et al., 1998; Janda et al., 1999) or inducing the synthesis of stabilising substances such as polyamines (Németh et al., 2002). It was also recently shown that SA spraying enhanced the freezing tolerance of wheat, parallel with increase in the ice nucleation activity of apoplastic proteins and with an increased antioxidant activity (Tasgín et al., 2003, 2006). There is evidence that not only does SA cause a rise in the quantity of ROS in the cell, but ROS may also lead to the accumulation of SA (León et al., 1995; Enyedi, 1999; Pál et al., 2005). Recent studies revealed that the SA-induced H_2O_2 accumulation in germinating wheat seedlings was not associated with the inhibition of catalase or APX, but the abiotic stress signal was

transduced via abscisic acid, Ca^{2+} and H_2O_2, which might be responsible for the activation of a common transcription factor associated with certain antioxidant enzymes (Agarwal et al., 2005). In the present experiment, changes in the endogenous SA content during the hardening period in the leaves of wheat plants were paralleled by the activity of APX and GR (Fig. 2c and d). Using *Arabidopsis* mutants it has recently been shown that an adequate level of SA is required to induce the required level of light acclimation processes (Mateo et al., 2006). It was shown that while *Arabidopsis* mutants with low foliar SA content were unable to increase photosynthetic electron transport efficiency or GSH levels to the same extent as wild type plants in response to transient exposure to high light intensity and were thus predisposed to oxidative stress, SA-accumulating mutants had lower PS2 efficiency than the wild type, but were able to increase their GSH contents. The role of the glutathione metabolism in the induction of cold tolerance has already been well described (Kocsy et al., 1997, 2000, 2004), and the glutathione level is closely correlated with the SA level in *Arabidopsis* (Mateo et al., 2006). The activity of GR may also be stimulated by SA, as shown in mustard and maize plants after exogenous SA treatments (Dat et al., 1998; Janda et al., 1999).

It was also suggested that not only SA but also other related compounds, such as oHCA, could provide protection against low temperature in young maize plants (Janda et al., 2000; Horváth et al., 2002). Since the antioxidant ability of the hydroxycinnamic acids, including oHCA, has been demonstrated with respect to their ability to quench singlet molecular oxygen, it is suggested that an increase in the oHCA content was induced independently of the SA biosynthesis, and may play a role in the antioxidative response (Foley et al., 1999).

It can be concluded from these results that the light intensity during the frost hardening of wheat plants is a key factor in the development of frost tolerance, and several processes, including lipid metabolism and antioxidant activity, may contribute to the enhanced freezing tolerance. It was also shown that the salicylic acid metabolism is involved in the light mediated development of freezing tolerance in wheat.

4. Experimental

4.1. Plant material

Seeds of winter wheat (*Triticum aestivum* L. variety Mv Emese, Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary) were sown in plastic pots containing 3:1 (v:v) loamy soil and sand. The plants were grown for 10 d (3-leaf stage) in a Conviron PGR 15 growth chamber with a 16 h/8 h light/dark period, at 20/18 °C (day/night) with 75% relative humidity and 250 μmol m^{-2} s^{-1} (control, normal light; NL) photosynthetic photon flux density (PPFD) at the leaf level provided by metal halide lamps. Low temperature hardening was carried out in a chamber of the same type at a constant 5 °C either under the light conditions of normal growth (light-hardened plants) or at 20 μmol m^{-2} s^{-1} PPFD (low light-hardened plants; LL). For the high light intensity treatment (HL) the plants were lifted closer to the light source of the growth chamber, where the PPFD was approx. 500 μmol m^{-2} s^{-1}. The measurements were carried out after the 12th day of hardening. Samples were taken from the middle part of the youngest fully developed leaves in the middle of the light period.

4.2. Determination of freezing tolerance

To determine the ability of plants to tolerate freezing, pots containing approx. 40 plants were put in a frost chamber for 1 day at −10 or −12 °C in the dark. Then the frozen seedlings were cut off at ground level and the regrowth of the surviving plants was evaluated after 2 weeks under normal growth conditions (20/18 °C, NL). At the end of the 2nd week, plants, which had survived freezing and had begun to develop were clearly distinguishable from those which had died (Veisz et al., 1997). The percentage of plants able to grow after freezing is given as the survival rate.

4.3. Lipid analysis

The lipids were extracted according to the method of Bligh and Dyer (1959) using 1 g plant tissue. Leaves were ground in liquid nitrogen and the frozen powder was extracted with 5 ml chloroform:methanol mixture (1:2 v/v), then 5 ml distilled water, 5 ml chloroform and 5 ml 2% (m/v %) NaCl were added. After centrifugation (5000*g*, 10 min) the chloroformic phase was transferred to a vial. The pellet was resuspended in 5 ml chloroform, vortexed and centrifuged as above. The chloroformic phases were combined, evaporated to dryness under a vacuum and resuspended in toluol:ethanol (4:1 v/v). The various lipid classes were separated by TLC on silica gel plates, after which the fatty acids were transmethylated (Pham-Quoc et al., 1994). The GC–SIM–MS analysis of fatty acid methyl esters (FAME) was carried out with a Shimadzu Model GCMS-QP2010 system (Shimadzu Co., Kyoto, Japan) fitted with an SP-2380 capillary column (30 m × 0.25 mm I.D., $d_f = 0.20$ μm) supplied by Supelco/Sigma–Aldrich Co. (St. Louis, MO, USA). For the GC–MS analysis of FAME, the injector and initial oven temperatures were 200 and 175 °C, respectively. After 8 min, the oven temperature was increased to 240 °C at a rate of 50 °C/min and kept at this level for 5 min. Samples were injected in split mode with a 1/5 split ratio. The He carrier flow-rate was 37.8 cm/s. For the MS quantification heptadecanoic acid was used as internal standard. Ions with *m*/*z* 74, 55, 67, and 79 were monitored for saturated, monounsaturated, diunsaturated, and triunsaturated FAME, respectively. The MS ion source and interface temperature

were 200 °C, and the solvent cut time was 2 min. Integration was performed using GCMS solution software 2.10 (Shimadzu Co., Kyoto, Japan). The double bond index (DBI) was calculated using the following formula: DBI = 1*(%18:1)+2*(%18:2)+3*(%18:3).

4.4. Enzyme assays

For the analysis of antioxidant enzyme activity 0.5 g of the leaves was homogenized in 2.5 ml ice-cold Tris buffer (0.5 M, pH 7.5) containing 3 mM $MgCl_2$ and 1 mM EDTA. The catalase (EC 1.11.1.6.) activity of the extract was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm according to Janda et al. (1999). The reaction mixture contained 0.44 M Tris buffer (pH 7.4), 0.0375% H_2O_2 and enzyme extract ($\varepsilon = 0.0436\ mM^{-1}\ cm^{-1}$). The ascorbate peroxidase (APX) (EC 1.11.1.11.) activity was determined in the presence of 0.2 M Tris buffer (pH 7.8) and 5.625 mM ascorbic acid according to Janda et al. (1999). The reaction was started with 0.042% H_2O_2. The decrease in absorbance at 290 nm was monitored ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). The guaiacol peroxidase (POD) (EC 1.11.1.7.) activity was measured at 470 nm as described by Ádám et al. (1995). The reaction mixture consisted of 88 mM Na–acetate buffer (pH 5.5), 0.88 mM guaiacol, 0.0375% H_2O_2 and enzyme extract ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). The glutathione reductase (GR) (EC 1.6.4.2.) activity was determined at 412 nm according to Smith et al. (1988). The reaction mixture contained 75 mM Na–phosphate buffer (pH 7.5), 0.15 mM diethylenetriamine–pentaacetic acid, 0.75 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM NADPH, 0.5 mM oxidized glutathione and 50 μl plant extract in a total volume of 1 ml ($\varepsilon = 14.15\ mM^{-1}\ cm^{-1}$). The activities were expressed in nkat/g FW. The superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to Yordanova et al. (2004). Briefly, 0.5 g leaf samples were homogenized in four volumes (w/v) of an ice-cold buffer containing 0.1 M Tris–HCl (pH 7.8), 0.1 mM EDTA and 0.05% Triton X-100 and centrifuged at 4 °C for 30 min at 15 000*g*. The crude extracts were dialyzed for 24 h against half-strength extraction buffer without Triton X-100. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.053 mM NBT, 10 mM methionine, 0.0053 mM riboflavin and an appropriate aliquot of enzyme extract. The reaction was started by switching on the light and was allowed to run for 7 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT as monitored at 560 nm.

4.5. Salicylic acid extraction and analytical procedure

SA and oHCA were measured according to Meuwly and Métraux (1993) and Pál et al. (2005). 1.5 g of the leaves were ground in liquid nitrogen in a mortal and pestle, in the presence of 0.75 g quartz sand. The tissue powder was transferred to a centrifugation tube and mixed with 2 ml of 70% methanol containing 250 ng *ortho*-anisic acid (oANI) (used as internal standard) and 25 μg *para*-hydroxy-benzoic acid (pHBA) (used as extraction carrier). The extract was centrifuged at 10 000*g* for 20 min. The pellet was resuspended in 2 ml 90% methanol, vortexed and centrifuged as above. The methanol content was evaporated from 2 ml of the mixed supernatants at room temperature under a vacuum. 1 ml of 5% (w/v) TCA was added to the residual aqueous phase and the mixture was centrifuged in an Eppendorf centrifuge at 15 000*g* for 10 min. The supernatant was gently partitioned twice against 3 ml of a 1:1 (v/v) mixture of ethyl acetate/cyclohexane. The upper organic layers contained the free phenolic portion. The aqueous phases containing the methanol-soluble bound phenolics were acid hydrolysed. 250 ng oANI, 25 μg pHBA and 1.3 ml 8 N HCl were added to the aqueous phase and incubated for 60 min at 80 °C before partitioning twice as above. Just prior to the HPLC analysis, the organic phases were evaporated to dryness under a vacuum and resuspended in 1000 μl of the HPLC starting mobile phase. SA and oHCA were quantified fluorimetrically (W474 scanning fluorescence detector, Waters, USA), with excitation at 317 nm and emission at 436 nm for oHCA, followed by excitation at 305 nm and emission at 407 nm for SA. The detection limits for oHCA and SA were 5 and 0.25 ng, respectively with a 5/1 signal/noise ratio.

4.6. Statistical analysis

The experiments were repeated several times and representative data are shown. The results were the means of five measurements for lipids, SA metabolites and antioxidant enzymes and four for freezing survival rate analysis. The data were statistically evaluated using the standard deviation and *T*-test methods.

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