Saline Growth Conditions Favour Supercooling and Increase the Freezing Tolerance of Leaves of Barley and Wheat

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Z. Naturforsch. **47 c,** 695–700 (1992); received May 14/July 9, 1992

Barley, Chlorophyll *a*, Fluorescence, Freezing Tolerance (Leaves), Salt Stress, Supercooling

When young plants of barley and wheat grown in hydroponic culture were subjected to salt stress, their freezing tolerance increased with increasing severity of salt stress. Detached leaves from salt-stressed plants also exhibited an increased ability to supercool. Avoidance of ice formation permitted leaf survival at subzero temperatures which were no longer tolerated when ice nucleation resulted in extracellular freezing. The increased freezing tolerance under salt stress is attributed to osmotic adjustment of the plants. Increased cellular solute concentrations decrease the extent of cellular dehydration at freezing temperatures, thereby decreasing mechanical and chemical stresses on biomembranes during freezing and thawing.

Introduction

More than 100 years ago, Müller-Thurgau observed that frost hardening of plants is usually accompanied by the accumulation of soluble sugars [1]. Later the significance of this early observation in regard to hardening was questioned on the basis of the simple fact that tissues of sugar cane or sugar beet accumulate sugars without ever becoming frost hardy [2]. A causal relationship between frost resistance and the accumulation of soluble sugars therefore appeared doubtful. Later, Ullrich and Heber [3] showed that soluble sugars prevent the formation of dark-green precipitates, when soluble green extracts from leaves are submitted to freeze-thaw cycles. An analysis of this observation revealed that the precipitated thylakoid membranes lost their capacity for photophosphorylation during freezing in the absence of sugars [4]. Inactivation is prevented by soluble sugars or other so-called membrane-compatible solutes such as glycine betaine or proline [5]. From this fact, the conclusion was drawn that the accumulation of sugars or other suitable solutes is indeed a relevant factor in the acquisition of frost hardiness, provided there is a close spatial relationship between these solutes and frost-sensitive biomembranes. In the case of sugar beet or sugar cane, sequestration of sugars, for instance in the vacuoles, might not permit the necessary interaction of membranes and sugars. On the other side, salts such as sodium chloride were found to interfere with membrane protection by sugars or other membrane-compatible solutes [6]. An excess of sugars in the membrane suspensions provided and an excess of salt prevented membrane protection during freezing.

An inhibitory effect of salt on the development of frost hardiness has repeatedly been reported [7–9]. It was therefore surprising that not only no decrease but actually an increase in frost hardiness was observed on raising the concentration of sodium chloride in the nutrient solution of hydroponically grown spinach [10]. This observation was confirmed by Larcher *et al.* [11]. Salinized cowpea revealed a lower susceptibility to severe temperature stress.

This acquisition of freezing tolerance no longer required lowering of the temperature which is normally needed to induce frost hardiness. Spinach tolerates NaCl concentrations as high as 300 mM in the growth medium. Osmotic water stress leads to transient wilting when salt is added at high concentrations to the nutrient solution. Salt is then taken up by the plant and contributes to the decrease in water potential which is necessary for the reestablishment of turgor. As high salt concentrations interfere with metabolism, salt is sequestered in the vacuoles. Osmotic balance between the vacuoles and the cytoplasm of the cells is assumed to be achieved by the synthesis and accumulation of membrane-compatible solutes in the cytoplasm. These mechanisms, *i.e.* sequestration of salt on one

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0939–5075/92/0900–0695 $ 01.30/0

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side and accumulation of protective solutes where membrane protection is needed when freezing results in cellular dehydration on the other side, are thought to account for the observed increase in frost hardiness.

We were interested to know whether uptake of salt generally increases the frost hardiness of plants. For this reason, we have extended the observations made by Schmidt *et al.* [10] with spinach. Barley is moderately halotolerant whereas wheat reacts much more sensitive to increased salt concentrations in the growth medium. During our work, different effects were found to contribute to the increased tolerance of subfreezing temperatures.

Materials and Methods

Barley (*Hordeum vulgare cv.* Gerbel) and wheat (*Triticum sativum*) were grown in hydroponic culture in a growth chamber in 14 h light/10 h dark cycles (20 °C; 18 °C). The hydroponic medium contained salts as follows: 9 mM KNO_3, 6 mM $Ca(NO)_3)_2$, 3 mM $MgSO_4$, 1.5 mM KH_2PO_4, 126 μM NaFeEDTA, 69 μM H_3BO_3, 13.7 μM $MnCl_2$, 1.1 μM $ZnCl_2$, 0.5 μM $CuCl_2$, 0.2 μM Na_2MoO_4. The NaCl concentration was increased in steps of 50 mM per day when the plants were one week old. After two more days at the highest salt concentration, leaves of salt-treated or control (*i.e.* 15 days old) plants were used for the freezing experiments which were performed in electronically controlled freezers (lab-built control unit). Temperatures were decreased at a rate of 4 °C/h, kept constant at the lowest temperature as indicated for 2 h and then raised again at the same rate of 4 °C/h. The leaf temperature was recorded in some experiments with a thermocouple. Chlorophyll fluorescence was measured as described by Schreiber *et al.* [12] using the PAM 101 fluorometer (Walz, Effeltrich, Germany). A modulated (1.6 kHz) red measuring beam of extremely low intensity (<0.1 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) served to excite a basic level of fluorescence. At room temperature, this level, F_o, corresponds to the fluorescence which is emitted when the primary quinone acceptor Q_A in the reaction center of photosystem II of the chloroplast electron transport chain is fully oxidized. It should be noted that this may not longer be true at subzero temperatures, when oxidation of Q_A is slowed down. Q_A may become partially reduced in this situation by the low intensity measuring beam, and F_o increases to a steady state level F_s. Saturating light flashes of 1 s duration enable functional reaction centers to fully reduce Q_A and to increase fluorescence to its maximum level F_m. The ratio $(F_m - F_o)/F_m$ is a measure of the quantum yield of photosystem II [13, 14]. The closely related ratio F_m/F_o was used as an indicator of the functionality of photosystem II after freezing of the leaves when elevated temperatures had ascertained full oxidation of Q_A. The osmotic potential of the leaf sap was determined with an osmometer (Knaur, Oberursel, Germany). About 300 mg of leaf material were rapidly frozen in liquid nitrogen, thawed, homogenized and centrifuged at 12,000 × *g*. The clear supernatant was used for the determination of osmolarity [15].

Results

Barley and wheat plants were grown in hydroponic culture. Salt stress was applied by increasing the NaCl concentration in the growth media stepwise to 150 or 300 mM. Leaves were detached from the plants and cooled either with or without previous wetting at a rate of 4 °C/h to various subzero temperatures. After remaining for 2 h at the lowest temperature, they were slowly rewarmed to room temperature. Fig. 1 shows a typical experiment performed with two wheat leaves whose surface had not been wetted. In addition to recording leaf temperature, fluorescence excited with a low intensity modulated measuring beam was measured during the freeze-thaw cycle. Every 15 min a 1 s flash (about 4000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) was also applied to probe for the maximum fluorescence F_m. During lowering of the temperature to 0 °C, both F_m and F_o increased linearily with decreasing temperature. Below 0 °C, F_m remained constant while F_o increased further until flash freezing of the leaves occurred at about −12 °C. This is indicated by the two exotherms. Supercooling had prevented earlier freezing at higher temperatures. After freezing, the steady state fluorescence F_s increased rapidly. In contrast, the flash-induced fluorescence spikes decreased. Initially, the lowering of the spike levels was probably a simple consequence of damage of the frozen leaves. Later, spikes decreased as a consequence of damage to

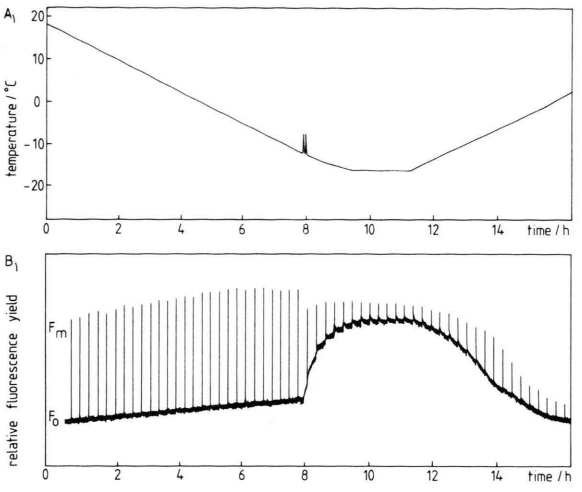

Fig. 1. Effect of a freeze-thaw cycle on chlorophyll *a* fluorescence of two detached wheat leaves. (A) Recording the temperature. Rates of cooling and warming were 4 °C/h. The lowest temperature was maintained for 2 h. Two exotherms at about −12 °C indicate flash freezing of the two leaves. (B) Recording chlorophyll fluorescence. Modulated fluorescence was excited by a modulated red measuring beam of extremely low intensity (<0.1 μmol quanta·m^{-2}·s^{-1}). F_o denotes the level of fluorescence emitted when the bound quinone Q_A in the reaction center of photosystem II was oxidized, and F_m when it was fully reduced by a 1 s saturating actinic flash (about 4000 μmol·m^{-2}·s^{-1}).

photosystem II. As long as ice was present in the leaves, *i.e.* as long the temperature did not rise above about −1 °C, the spikes exhibited two kinetic phases, a fast and a slow one. Apparently, after a fast phase of oxidation, Q_A^- was slowly oxidized after the flashes. It remained partially reduced in the presence of the measuring beam. During warming, F_s decreased to the low level F_o originally observed at the beginning of the experiment. The fluorescence peaks F_m induced by the flashes first increased in relation to F_s, but decreased at temperatures higher than −6 °C. When the room temperature was finally reached, $(F_m - F_o)/F_m$ and F_m/F_o were much lower than they had been at the beginning of the experiment.

In unstressed leaves which had been predarkened for a sufficient long time, $(F_m - F_o)/F_m$ values are usually close to 0.8 [16]. Lower levels indicate a decreased quantum efficiency of energy conversion in photosystem II. This may be either a consequence of regulation or of the damage caused by a stress treatment. The fluorescence kinetics of Fig. 1 indicate that photosystem II became progressively inhibited during warming. Inhibition is attributed to biomembrane rupture and the loss of cellular compartmentation. The leaves did not survive the freezing experiment of Fig. 1. Turgor was completely lost.

In other experiments, observed fluorescence phenomena were similar in principle to those shown in Fig. 1 when freezing of the leaves occurred except that initial $(F_m - F_o)/F_m$ ratios, or F_m/F_o ratios, were more or less recovered depending on the extent of damage cells suffered during freezing and thawing. When F_m/F_o ratios recovered after freezing, the leaves regained full turgor. Because cellular disruption is much more difficult to quantitate than damage to the reaction centers of photosystem II in the thylakoid membranes which results in fluorescence changes, the fluorescence ratio F_m/F_o was taken to indicate membrane damage as a result of freezing. Before freezing, ratios were usually between 4 and 5. When after freezing, ratios had dropped below 1.66, it was arbitrarily, but in agreement with the appearance of the leaves after thawing, assumed that the leaves had suffered lethal damage (see also [10]).

Based on these criteria, detached leaves from barley plants grown in the absence of NaCl tolerated subzero temperatures down to −5 °C without changes in F_m/F_o (Fig. 2). These leaves did not appear to have been frozen. However, they were completely damaged at −10 °C after freezing had occurred. A decrease of F_m/F_o to about 3 which defines 50% damage was observed between −6 and −7 °C. Large error bars in this temperature range show that leaves had either survived (had not been frozen) or were killed by freezing. Treatment with 150 mM NaCl shifted the lethal temperature from −10 to −12.5 °C and the 50% damage level from −6.5 to −10 °C. Once again, surviving leaves which had retained pre-freezing F_m/F_o ratios appeared to be unfrozen throughout the experiment. When plants were stressed with 300 mM NaCl, the lethal temperature was not further decreased, but the 50% level was below −11 °C. Clearly, salting the growth medium increased leaf survival during exposure to subzero temperatures far beyond effects expected from decreased osmotic potentials and the accompanying freezing point depressions. Osmotic potentials increased from

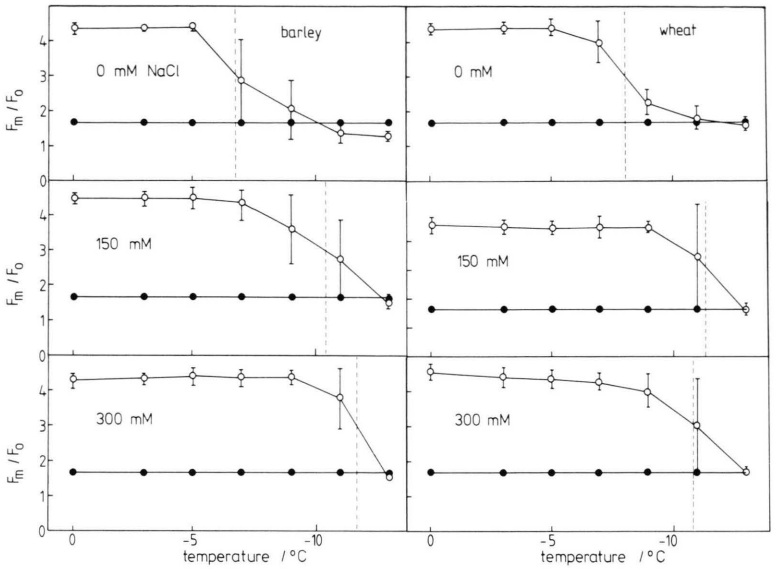

Fig. 2. F_m/F_o ratios of barley and wheat leaves after exposure to subzero temperatures. Barley leaves (left figures) and wheat (right figures) were detached from control plants (no NaCl added to the growth medium, upper figures), from plants growing in 150 mM NaCl (figures in the middle) and from plants grown in 300 mM NaCl (lowest figures). They were subjected to temperature cycles similar to the one shown in Fig. 1. The lowest temperatures of exposure were as indicated on the abscissa. After the experiments, F_o and F_m were measured. The vertical line gives the value for 50% damage. The closed symbols indicate a F_m/F_o ratio of 1.66 which corresponds to lethal damage.

380 ± 30 mosmol·l^{-1} in control leaves grown without NaCl to 910 ± 90 mosmol·l^{-1} in leaves of barley exposed to 300 mM NaCl. The osmolar freezing point depression is −1.86 °C.

Similar results were obtained with wheat (Fig. 2). Control leaves were frozen and killed at −12 °C (Fig. 1). The 50% damage level was observed at −8 °C. Once again, effects of salting were similar for 150 and 300 mM NaCl in the culture medium but the 50% damage level was shifted to −11 °C and the killing temperature to −13 °C.

In the following experiments, leaves maintained contact with water during the freeze/thaw cycle. This ensured early freezing. At −2 °C, leaves were frozen stiff. Fig. 3 shows the results of freezing leaves of barley and wheat, respectively. Damage to the leaves occurred at higher subzero temperatures compared to the supercooling experiments of

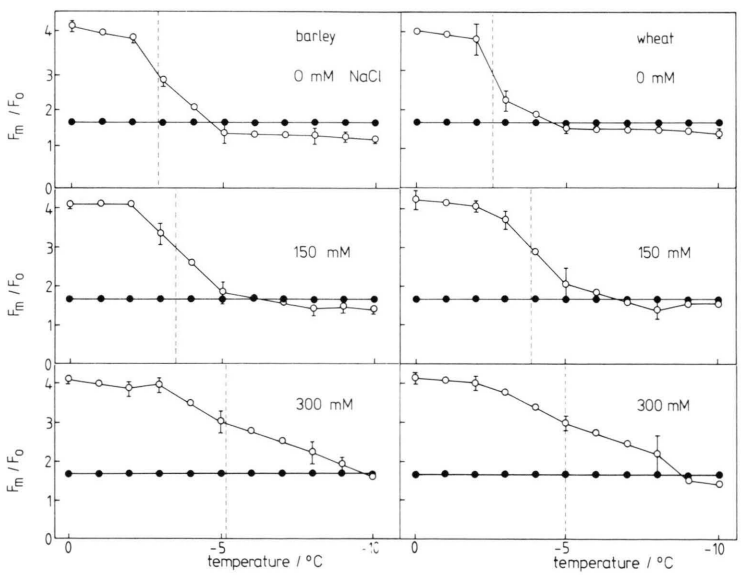

Fig. 3. F_m/F_o ratios of barley and wheat leaves after freezing to different temperatures. The barley leaves (left figures) and wheat leaves (right figures) were in contact with water to facilitate ice nucleation. They were either taken from control plants (no NaCl in the growth medium), from plants grown in the presence of 150 mM NaCl or from plants with 300 mM in the growth medium. For further information, see legend to Fig. 2.

Fig. 2. In barley, the lethal temperature of leaves as defined by $F_m/F_o = 1.66$ was shifted from −4.5 °C in the controls to −6 °C in leaves from plants salted with 150 mM NaCl and further to −9.5 °C, when 300 mM NaCl had been used for salting. 50% was at −2.5 °C (controls), −3.5 °C (150 mM NaCl) and −5 °C (300 mM NaCl). It should be noted that error bars are small. Reproducibility of the observations was good.

In wheat, the corresponding values for lethal damage were −4.5 °C (controls), −6.5 °C (150 mM NaCl) and −9 °C (300 mM NaCl). Values for 50% damage were similar to those observed with barley. In contrast to barley, whose growth was only slowed down in 300 mM NaCl, wheat creased growing in 300 mM NaCl.

Experiments similar to those described for detached leaves were also perfomed with intact plants growing in soil. As in the case of detached leaves, salting increased the tolerance of freezing. However, no supercooling comparable to that seen with detached leaves could be observed. Watering the soil with salt solutions had no measurable effect on the temperatures of first ice formation inside the leaves. It appears that ice nucleation starting from the soil/root system interfered with the ability of attached leaves to supercool.

Discussion

Intracellular ice formation as it may occur when extensive supercooling finally leads to flash freezing, invariably kills fully hydrated leaf cells. Extracellular freezing, on the other hand, may or may not be lethal depending on the extent of cellular dehydration and frost hardiness. The plants used in the present work are in principle capable of frost-hardening. In our experiments, however, they had not undergone any conventional hardening treatment which usually requires prolonged exposure to temperatures below 10 °C under short-day conditions [15].

Extensive dehydration by freezing of non-hardy cells is lethal for several reasons. Cellular compartments shrink and solute concentrations increase. Simultaneously, cellular activities which maintain gradients (such as the pH gradient across the tonoplast, *cf.* [18]) are slowed down or cease to function under freezing conditions. Compartmentation may be disturbed even if changes in membrane permeability do not occur and membrane rupture can be avoided. However, shrinkage of osmotically active compartments during freezing and their re-expansion during thawing also exert stresses on biomembranes which have to adjust their areas to altered volumes. Membrane rupture is often likely to be responsible for cell death during thawing [19]. Solute accumulation in the cells during dehydration may exert chemical stress on biomembranes. Non-compatible solutes such as NaCl cause, if sufficiently concentrated, dissociation of proteins from biomembranes causing the loss of membrane functions [20].

In the experiment reported above, dehydration during freezing was extensive. When, before salting, the osmolarity of the cell sap was below 0.4 osmol, more than 50% of available water had left the cells to form ice in the intercellular space at a freezing temperature of −1.86 °C (the molal freezing point depression) and more than 75% at a temperature of −3.72 °C. After salting, when the osmolarity had increased to almost 1 osmol·l^{-1}, only about 50%, of the available water was frozen out at −3.72 °C. The increase in cellular solute concentration caused by salting had, at comparable freezing temperatures, reduced cellular dehydration and the accompanying stresses.

Most of the increase in intracellular solute concentration caused in leaves by adding 300 mM NaCl to the nutrient solution of barley plants (more than 500 mosmol·l^{-1}) is brought about by the uptake of NaCl. Freezing of thylakoids in the presence of similarly high NaCl concentrations causes the rapid loss of photophosphorylation which is due to the dissociation of polypeptides of the ATP synthetase from the membrane and to transient membrane rupture which results in the loss of plastocyanin from the thylakoid interior [21]. However, in the experiments reported here, as in the work on spinach reported by Schmidt *et al.* [10], the accumulation of NaCl was not only not damaging but actually increased freezing tolerance. It is known that mechanisms responsible for cytoplasmic ion homeostasis prevent the accumulation of chloride in the cytoplasm of leaf cells [22]. Excess NaCl is actively pumped into the vacuoles [23, 24]. Osmotic homeostasis requires the synthesis of isoosmolar concentrations of presumably membrane-compatible solutes in the cytoplasm. Thus decreased dehydration of leaf cells during

freezing which is caused by solute synthesis and salt uptake is thought to account for most, if not all, of the observed increase in freezing tolerance of barley and wheat plants subjected to salt stress. In the experiments of Schmidt *et al.* [10] with salt-stressed spinach, cells were killed when extracellular ice formation reduced their unfrozen volume to 15% of the initial volume. Similar values are reported by Beck *et al.* [25] for frost stress in general. They correspond to the value of 20% reported to be necessary to maintain cellular viability under drought conditions [15, 26]. In the experiments reported here, unfrozen cellular volumes were between 15 and 18% of initial values at the freezing temperatures in Fig. 3 where $F_m/F_o = 1.66$. All these observations stress the importance of solute accumulation as one of several mechanisms leading to increased dehydration and freezing tolerance.

Unexpectedly, uptake of salt under salt stress also increased the ability of the leaves to supercool. In the absence of artificial ice nucleation, lethal flash freezing occurred in detached leaves from salt-stressed plants at much lower temperature than in leaves from control plants (Fig. 2). It is known that supercooling permits various plants to endure subzero temperatures [17]. However, in our experiments increased supercooling was a property of salt-stressed detached leaves. We failed to observe increased supercooling, when potted plants of barley and wheat were subjected to freezing conditions. Therefore, the importance of supercooling for survival of barley and wheat plants in the natural environment at freezing temperatures is not clear.

Acknowledgements

This work was performed within the Sonderforschungsbereich 176.

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