PROTOPLAST PLASMALEMMA FLUIDITY OF HARDENED WHEATS CORRELATES WITH FROST RESISTANCE

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1. Introduction

The importance of the physico–chemical state of plant membranes in assuring survival at reduced temperatures has been emphasized [1]. The thermotropic phase transition temperature of the structural lipids in chilling-sensitive plants is at higher values than in chilling-resistant plants [2,3]. Chilling- and frost-resistant plants differ in several respects, one of these being their lower temperature limits. While the latter survive, after proper hardening, at rather low temperatures, the former suffer cellular damage at temperatures rather higher than the freezing point of the intracellular water. Extrapolation of the concept of homeoviscous adaptation of membrane fluidity, observed with both prokaryotic [4] and eukaryotic [5] cells, to frost-resistant plants, implies that membrane fluidity is increased in some way during the hardening process. However, senescence induces a decrease in the fluidity of membranes in almost every system studied [6–8]. Thus the questions arise:

(1) Whether the hardening plants can, in some way, compensate for the effects of senescence;
(2) To what extent these opposing trends in membrane fluidity affect the survival of such plants at reduced temperatures.

The key membrane involved in developing frost tolerance is the plasmalemma and to gain information about its physico–chemical state protoplasts were isolated from leaves of four cultivars of the wheat, *Triticum aestivum* L., of different hardiness, in order to follow the changes in microviscosity during hardening.

2. Materials and methods

2.1. *Chemicals*

Cellulase 'Onozuka' R-10 was obtained from Kinki mfg Co., Japan, while pectinase was from Sigma, and rhozyme from Rohm and Haas Ltd Philadelphia, PA. The spin-labeled fatty acid, 2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinoxyl (I[1,14]) was product of Syva. All the other reagents were of commercial laboratory use.

2.2. *Plants*

Four cultivars (Miranovskaja 808, Bezostaja 1, Short mexican and Penjamo 62) of wheat, *Triticum aestivum* L. are investigated here. The seeds were allowed to germinate at room temperature (25°C). The 6 days old seedlings were subjected to a hardening process, in which the illumination and environmental temperature were gradually decreased. Details of this program are described in [9] and also in fig.1.

2.3. *Preparation of protoplasts*

The leaves, collected at selected intervals during the hardening process, were cut in 0.5 mm pieces and incubated in a solution containing 0.5 M glucose and 1.4 mM $CaCl_2 \times H_2O$ at pH 6.0 for 3 × 20 min. The cell walls were digested by incubating the leaf pieces in the presence of 2% Onozuka R-10, 0.5% rhyzome, 1% pectinase, 3 mM MES, 0.6 mM NaH_2PO_4, 7 mM $CaCl_2 \times H_2O$, 0.5 M sorbitol in 100 ml at pH 6.4. The incubation proceeded at 25°C for 6 h. The sealed petri dishes were gently shaken during the incubation. The protoplasts formed were

then filtered into centrifuge tubes and spun at 1000 rev./min. The sedimented protoplasts were resuspended in the above solution, minus enzymes and spun again. The sediment was resuspended and the protoplasts counted. The average yield was 5×10^6 protoplast/g leaf.

2.4. *Incorporation of spin-labelled fatty acid into the plasmalemma*

The spin-labelled fatty acid I[1,14] was evaporated on the bottom of an Erlenmeyer flask. Sufficient incubation medium devoid of digesting enzymes, containing 5% bovine serum albumin, was then added to the flask and shaken for 16 h at room temperature. The concentration of the spin-labelled fatty acid was then set to 0.1%. Protoplasts (7.0×10^6) were incubated in 200 μl of this solution for 1 h. The unreacted spin-labelled fatty acid was removed by repeated mild centrifugation from 10.0 ml of the original buffer solution. ESR spectra were taken using a JEOL JES-PE-lx electron spin resonance spectrometer at 25°C. Rotation correlation times were calculated according to [10].

2.5. *Extraction and analysis of lipids*

Chloroform:methanol (2:1) 5 ml, was added to the protoplast suspension and lipids extracted and purified as in [11]. The extracted lipids were applied to silicagel G plates and developed using petrolether: ether:acetic acid (85:15:1) as solvent. The phospholipids, remaining at the origin, were removed into ampoulles and transesterified in the presence of 5% HCl in absolute methanol at 80°C under N_2. Gas chromatographic analyses of the fatty acid methyl esters were carried out using a JEOL 20 K instrument equipped with dual flame ionisation detector. The 2 m long stainless steel columns were filled with 10% Silar 10 C on Chromosorb W AW 100–120 mesh. Each probe was run in triplicate and quantitation was made by the triangulation technique.

3. Results and discussion

Evidence showing that the ESR signal originated from the plasmalemma and not from intracellular membranes of the cells have been presented [13]. The fluidity of the plasmalemma, as in the case of chloroplast lipids [12], was very similar at the beginning of the hardening regardless of the potential hardiness of the seedlings (fig.1). During the hardening there was an initial decrease in the microviscosity (i.e., increase in the rotational correlation time) up to a critical environmental condition. However, as the hardening continued the frost-resistant seedlings (Miranovskaja 808, Bezostaja 1) developed more fluid membranes while in the frost-sensitive seedlings (Penjamo 62, Short mexican) continuous rise of rigidity was observed. By the end of the hardening the plasmalemma fluidities of the 4 cultivars were in exactly the same order as their resistance to freezing temperatures. The initial decrease in microviscosity during the hardening evidently resulted from the ageing of the leaves. However, it is hard to believe that the seedlings underwent ageing at different rates. In fact, in control experiments with unhardened seedlings, the membranes lost fluidity at an identical rate [13]. Thus, if senescence alone was responsible for increasing microviscosities during hardening, all the seedlings should have had as rigid membranes as had Penjamo 62 by the end of the hardening. To resolve this contradiction we assume that a regulatory process was triggered, upon lowering the environmental temperature beyond a critical value, to re-establish at least the original fluidity of the plasmalemma. Apparently, the actual fluidity of the plasma membrane in hardening cells is the function of these two opposing processes. The differences in the courses of the fluidity curves can be interpreted as indicating that the ability to readjust the fluidity of the plasmalemma was the greatest in the most hardy cultivar (Miranovskaja 808) and smallest in the most sensitive one (Penjamo 62). Consequently, the seedlings faced subzero temperatures with survival chances depending on the acquired microviscosities of their plasma membranes.

Differences in plasmalemma microviscosities of the hardened seedlings are unlikely to be attributed to phospholipid fatty acid compositions. Table 1 indicates a non-specific increase in the level of linolenic acid during the hardening process, which is in agreement with observations [12,14]. Whether the hardiness-dependent augmentation of membranes [9,15,16] and accumulation of phosphatidyl choline [9,15] alone, or favourable changes in the ratio of phospholipids to steroids together with some unknown factors, is involved in adapting membrane fluidity to

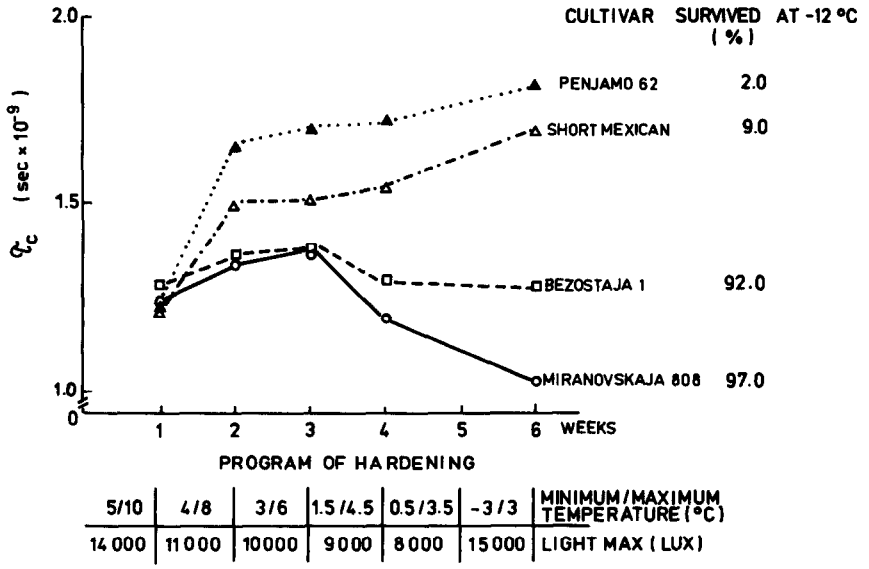

Fig.1. Changes in plasmalemma fluidity during hardening. Seeds of 4 cultivars of the wheat, *Triticum aestivum L.* were sown in soil and germinated in a phytotron at 20°C day (16 h) and 12°C night (8 h) temperature. The 6 days old seedlings were subjected to a hardening process; the phytotron programme is given below the abscissa. At the end of the hardening, the temperature was lowered to −12°C with a cooling rate of 2°C/h and the seedlings were exposed to this temperature for 6 h. After this time it was raised to −2°C, the leaves were cut and the regeneration of the seedlings was observed. Survival values displayed alongside their respective curves, are expressed as the percentage of the seedlings regenerated. The leaf protoplasts were isolated as in section 2. (7.0×10^6) protoplasts were used for spin label studies.

Table 1
Phospholipid fatty acid composition of protoplasts of leaves obtained from unhardened and hardened wheat seedlings

Cultivar	treatment	Fatty acids (wt %)					
		16:0	16:1	18:0	18:1	18:2	18:3
Miranovskaja	UH	23.05	7.07	0.73	4.27	19.02	45.85
	H	18.71	5.95	1.13	2.83	19.28	52.09
Bezosztaja	UH	21.11	9.38	0.86	4.20	20.00	44.44
	H	24.30	2.41	0.63	2.01	21.83	48.82
Short Mexican	UH	20.09	9.09	0.74	3.95	18.18	47.76
	H	22.99	5.41	0.36	1.35	18.03	51.85
Penjamo	UH	22.85	6.65	0.54	3.08	17.17	49.70
	H	25.25	2.82	1.25	2.72	22.79	59.59

Hardening took place as described in the footnote of fig.1: UH, unhardened (25°C grown); H, hardened

low temperature remains the task of further investigations. Efforts are also being made to artificially modify the plasmalemma of these cultivars to see whether frost resistance is affected.

The data [12,17–19] on transitional states of plant membranes provide indirect information about the situation prevailing in the plasma membrane itself. The recently described technique of spin-labelling of protoplast plasma membrane is suitable to furnish direct insight into actual physico–chemical state of these structures. The present observation, along with those made on chloroplast [12] and artichoke-tuber lipids [19], strongly suggests that plants are able to control the fluidity of their membranes while exposed to decreasing temperatures, and raises the possibility that their actual survival at low temperatures will be determined also by their success to adjust this fluidity to the new temperatures. The ability to adapt membrane fluidity to temperature might be one of the major factors distinguishing frost-resistant from chilling-sensitive plants.

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