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Relative Insensitivity of Mitochondria in Hardened and Nonhardened Rye Coleoptile Cells to Freezing *in Situ*[1]

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ABSTRACT

Mitochondria were isolated from excised coleoptiles of hardened and nonhardened winter rye (*Secale cereale* L. cv. Puma) seedlings which had been frozen extracellularly to different temperatures. No significant differences in the respiratory functions (ADP/O and respiratory control) were observed between mitochondria isolated from nonlethally and lethally frozen cells of both the hardened and nonhardened rye. These results suggest that mitochondria *in situ* can retain their normal function even after the cell was killed by the dehydrative stresses of extracellular freezing. Presumably, a different level of sensitivity to freezing stresses exists between mitochondrial and other membranous elements in the cell.

Freezing studies of plant cells point to membranes at cell surfaces and in cell organelles as being the primary site of freezing injury (5, 7, 8, 11, 12). The correlation of increases in phospholipid content in tree cells with extreme hardening has also focused attention on the involvement of membranes in the adaptation of the plant cell to freezing (13, 14).

While evidence exists showing that injury to the plasma membrane occurs when the whole plant cell is damaged by the dehydrative stresses of extracellular freezing (12), evidence pointing to plant mitochondria as also the targets in such cell injury is based largely on observations made on the freezing of the isolated organelle (3, 4, 6, 7). Neither the important functional examination of the level of mitochondrial impairment sustained *in situ* during extracellular freezing of the plant cell nor whether the mitochondria, the cell, and other cellular membranes experience similar degrees of injury at freezing temperatures which are lethal to the cell has been reported.

In this communication, we present evidence for the relative insensitivity of plant mitochondria *in situ* to damage under extracellular freezing regimes which are clearly lethal to the cell.

MATERIALS AND METHODS

Plant Material. Coleoptiles (15-20 mm in length) were isolated from winter rye (*Secale cereale* L. cv. Puma) seedlings grown in the dark for 65 hr at 24 C (nonhardened) and from seedlings grown in the dark for 4 weeks at 2 C (hardened).

Freezing Procedure. Freezing experiments were performed on the excised coleoptiles as follows. One-g batches of coleoptiles were placed in polyethylene bags (10 × 15 cm) with moist cotton and sprayed with a fine mist of water before the bags were sealed with a 200 w bar sealer. The bags were first equilibrated at 0 C for 1 hr in a programmed freezer, the temperature was then allowed to drop at 1 C/hr to −20 C and thereafter at 3 C/hr until the desired temperature was attained. Cell survival after freezing was estimated by vital staining of the cells with neutral red or from observation of protoplasmic streaming. Survival of the isolated coleoptiles as a function of the freezing temperature was correlated with the survival of whole seedlings which had been subjected to identical freezing conditions and replanted on vermiculite flats as described by de la Roche *et al.* (2).

Fast freezing of the coleoptiles was performed by immersing the polyethylene bags containing the coleoptiles into a beaker containing ethylene glycol cooled to −20 C. A thermometer placed in the polyethylene bag indicated that approximately 30 sec were required to reach −20 C. Freezing of the coleoptile tissues at different freezing rates was also examined on a microscope freezing stage. At rates of freezing comparable to the fast freezing described above, complete intracellular freezing was observed whereas only extracellular freezing was noted when the slower freezing rate was employed. Cell survival after extracellular freezing was dependent on the degree of hardening and the temperature to which the coleoptiles were frozen. Intracellular freezing was always fatal to both hardened and nonhardened cells.

Isolation of Mitochondria. Coleoptiles, frozen to the desired temperature, were thawed immediately by immersion in 8 ml of 2 C mitochondrial isolation medium for 2 min. The mitochondria were then isolated immediately as described for wheat seedlings (10). O_2 uptake, using α-ketoglutarate as substrate, was measured polarographically with a conventional Clark-type O_2 electrode (10). The energy-coupling capacity (ADP/O), and RC[4] indices were calculated according to Chance and Williams (1). Mitochondrial protein and protein N were estimated by the methods of Lowry *et al.* (9) and Williams (16), respectively.

RESULTS

Table I compares the ADP/O, RC, and respiratory rates of mitochondria isolated from hardened and nonhardened rye coleoptiles of different lengths. The results indicate that equally functional mitochondria could be isolated from coleoptiles of different lengths and that only the yield of mitochondria on a fresh weight basis is different. These results are important in view of the fact that small variations in the length of the coleoptiles are unavoidable with the different germination and growth rates in the two temperature regimes.

Table II compares the survival of whole cells of hardened and nonhardened rye coleoptiles with the functional activity of

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[4] Abbreviation: RC: respiratory control.

mitochondria isolated from cells that have been subjected to slow (extracellular) freezing (1–3 C/hr) to various temperatures. Although the results indicate that there is a small impairment of respiratory control and phosphorylative capacities in mitochondria isolated from both hardened and nonhardened rye coleoptiles which have been subjected to freezing temperatures not lethal to the cell, subsequent freezing of the coleoptiles to lower lethal temperatures did not further change the functional respiratory capacities of the mitochondria isolated from these cells. Thus, mitochondria with high ADP/O and RC ratios can be isolated from hardened or nonhardened rye coleoptile cells which have been completely killed by freezing. Table II also shows that a greater degree of functional impairment of the mitochondria occurs *in situ* when the rye coleoptile cells are frozen at rates which are fast enough to cause complete intracellular freezing. Mitochondria in both hardened and nonhardened coleoptiles are damaged to the same degree after such intracellular freezing.

The functional activity of mitochondria isolated immediately from coleoptiles which have been frozen to lethal temperatures is compared with the activity of mitochondria from coleoptiles which have been frozen to the same temperature but which have been left at 2 C for 1.5 hr subsequent to thawing (Table III). These results indicate that inactivation of the functional respiratory capacities of the mitochondria can occur in the milieu of cells killed by freezing. Therefore, the isolation of fully functional mitochondria is contingent on their speedy removal from the thawed dead cells. However, once the mitochondria have been isolated from coleoptile cells that were

Table I. Comparison of respiratory and phosphorylative capacities between mitochondria isolated from rye coleoptiles of different lengths

Length of coleoptile, temperature and time of germination	RC	ADP/O	State 3 Respiration	mitochondrial protein
		Ratio	μ atom O/min·μg N x 10^{-3}	mg/g fresh wt.
15 mm 4 weeks at 2 C	4.2	2.5	10	0.62
15 mm 65 hr at 24 C	4.4	2.5	10	0.72
25 mm 80 hr at 24 C	4.2	2.4	12	0.36
50 mm 100 hr at 24 C	4.0	2.5	11	0.29

Table II. Comparison of mitochondrial function with cell and seedling survival of hardened (H) and non-hardened (NH) rye under different freezing conditions.

Values presented in this table represent the mean from 3 sets of experiments. The variation did not exceed ±15% of the mean value.

Treatment of Coleoptiles	RC		ADP/O		State 3 Respiration[b]		cell survival in excised coleoptiles		Regrowth of Whole seedlings on Replanting	
	H	NH	H	NH	N	NH	H	NH	H	NH
					μ atoms O/min·μg N x 10^{-3}		%		%	
No Freezing[a]	3.7	4.2	2.5	2.5	9	9	100	100	100	100
-4 C slow freezing	3.0	3.2	2.3	2.2	5	6	100	100	100	100
-10 C slow freezing	3.2	3.1	2.4	2.4	7	6	100	<10	100	<10
-14 C slow freezing	3.5	3.1	2.4	2.1	9	7	100	0	100	0
-22 C slow freezing	3.0	3.0	2.4	2.1	6	5	100	0	90	0
-34 C slow freezing	3.3	2.5	2.6	2.2	6	6	<10	0	0	0
-22 C fast freezing	1.8	2.1	1.9	1.9	3	3	0	0	0	0

[a] Excised coleoptiles were subjected to the same conditions as those prepared for freezing except that instead of being frozen they were kept at 2 C for the same duration of time.

[b] The yield of mitochondrial protein (as measured by protein of the final mitochondrial pellet) was 0.70 ± 0.11 mg/g fresh wt. of unhardened coleoptiles after they were killed by freezing to the indicated temperatures and 0.7 ± 0.13 mg/g fresh wt. of hardened coleoptiles which survived the same freezing.

Table III. Comparison of mitochondrial function with speed of isolation from nonhardened rye coleoptiles after slow freezing

Treatment of Coleoptiles	RC	ADP/O	State 3 Respiration	cell survival of coleoptiles
		Ratio	μ atoms O/min·μg N x 10^{-3}	%
No Freezing	3.6	2.5	9	100
Frozen to -16 C, thawed, isolated immediately	3.3	2.2	7	0
Frozen to -16 C, then allowed to stand at 2 C for 1½ h before isolation	1.6	1.6	2	0

killed by freezing, they do not deteriorate any faster than those isolated from either unfrozen or nonlethally frozen coleoptiles.

DISCUSSION

Our results show that differences in the tolerances to the stresses of extracellular freezing between hardened and nonhardened rye cells cannot be accounted for by differences in the responses of their respective respiratory apparatus to freezing stress. The mitochondria *in situ* retained most of their normal functional capacity even after the cell was subjected to lethal extracellular freezing conditions. However, extended retention of the mitochondria in the milieu of the lethally damaged cell subsequent to thawing resulted in increased injury (Table III). This suggested that any impairment to mitochondrial function must take into account secondary effects resulting from retention of the organelle in the milieu of the dead cells prior to isolation.

In contrast to the relative insensitivity of mitochondria *in situ* to the dehydrative effects of slow or extracellular freezing, mitochondrial function was severely impaired in hardened and nonhardened rye cells which were killed by intracellular ice formed during fast freezing (Table II). Sensitivity of mitochondria to intracellular freezing may be attributed to direct mechanical disruption of the organelle by intracellular ice crystals. It is noteworthy that isolated rye mitochondria, in pellet form, were very sensitive to even slow freezing conditions (unpublished). Damage of isolated mitochondria to freezing in the absence of cryoprotectants had also been reported with other plant and animal tissues (3, 4, 6, 15).

The results in this paper did not imply that mitochondria *in situ* were insensitive to extracellular freezing damage (*i.e.* at temperatures below 34 C) or that different susceptibilities to this damage did not exist between mitochondria of hardened and nonhardened rye cells. They indicated, however, that extracellular freezing damage to rye mitochondria *in situ* was not apparent even at lethal freezing temperatures. In this respect, rye mitochondria *in situ* did not share the same sensitivity as the rye cell to extracellular freezing stress.

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LITERATURE CITED

1. CHANCE B, GR WILLIAMS 1965 The respiratory chain and oxidative phosphorylation. Adv Enzymol 17: 65-134
2. DE LA ROCHE IA, CJ ANDREWS, MK POMEROY, P WEINBERGER, M KATES 1972 Lipid changes in winter wheat seedlings (*Triticum aestivum* L.) at temperatures inducing cold hardiness. Can J Bot 50: 2401-2409
3. DICKINSON DB, MJ MISCH, RE DRURY 1967 Dimethyl sulfoxide protects tightly coupled mitochondria from freezing damage. Science 156: 1738-1739
4. FISHBEIN WH, JL GRIFFEN 1975 External-internal ice and functional-structural damage in mouse liver mitochondria as a function of cooling rate. Cryobiology 12: 569-570
5. GARBER MP, PL STEPONKUS 1976 Alterations in chloroplast thylakoids during cold acclimation. Plant Physiol 57: 681-686
6. GREIFF D, M MYERS 1961 Effect of dimethyl sulphoxide on the cryotolerance of mitochondria. Nature 190: 1202-1204
7. HEBER U, KA SANTARIUS 1964 Loss of adenosine triphosphate synthesis caused by freezing and its relationship to frost hardiness problems. Plant Physiol 39: 712-719
8. LEVITT J 1972 Responses of Plants to Environmental Stress. Academic Press, New York, pp 208-228
9. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275
10. POMEROY MK 1974 Studies on the respiratory properties of mitochondria isolated from developing winter wheat seedlings. Plant Physiol 53: 653-657
11. SHERMAN JK 1972 Comparison of *in vitro* and *in situ* ultrastructural cryoinjury and cryoprotection of mitochondria. Cryobiology 9: 112-122
12. SIMINOVITCH D, J LEVITT 1941 The relation between frost resistance and the physical state of protoplasm. II. The protoplasmic surface. Can J Res C 19: 9-20
13. SIMINOVITCH D, J SINGH, IA DE LA ROCHE 1975 Studies on membranes in plant cells resistant to extreme freezing. I. Augmentation of phospholipids and membrane substance without changes in unsaturation of fatty acids during hardening of black locust bark. Cryobiology 12: 144-153
14. SINGH J, IA DE LA ROCHE, D SIMINOVITCH 1975 Membrane augmentation in freezing tolerance of plant cells. Nature 257: 669-670
15. WALTON KG, M KERVINA, S FLEISCHER, DS DOW 1970 Prolonged storage of mitochondria by freezing: retention of respiratory control and energized swelling. Bioenergetics 1: 3-8
16. WILLIAMS RJ, HT MERYMAN 1970 Freezing injury and resistance in spinach chloroplast grana. Plant Physiol 45: 752-755