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Stimulation of Phospholipid Biosynthesis during Frost Hardening of Winter Wheat[1]

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ABSTRACT

Lipids were labeled with ^{33}P during frost hardening of two varieties of winter wheat (*Triticum aestivum*), hardy Kharkov and much less hardy Champlein. The main labeled compounds were phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol. With time of incorporation the proportion of the radioactivity incorporated into the lipids increased in phosphatidylcholine, especially in Kharkov and at 1 C. During hardening, phospholipid synthesis was greatly stimulated in Kharkov, but much less in Champlein. The proportion of the phospholipids synthesized changed only little with hardening, with a trend towards an increase in phosphatidylcholine. Increased phospholipid synthesis does not seem to be a prerequisite to hardening in winter wheat. However, a high rate of phospholipid synthesis may be required to maintain frost resistance.

An increase in phospholipids has generally been observed during hardening of plants (4, 5, 7, 8, 11, 12, 14). It is not known whether this increase is a result of low temperature or whether it is part of the frost-hardening process. In an attempt to dissociate the two effects, phospholipid biosynthesis was measured at two temperatures during controlled hardening of a hardy and a less hardy variety of winter wheat.

MATERIALS AND METHODS

Growth and Hardening of Plants. Two varieties of winter wheat (*Triticum aestivum*), hardy Kharkov, and less hardy Champlein, were grown in 10-cm pots containing a 1:1 mixture of sand and vermiculite in a growth cabinet (Controlled Environment, Winnipeg) at a 20 C day and 15 C night temperature, a 16-hr photoperiod, a relative humidity of 60%, and a light intensity of 18,000 lux. The plants were watered with Hoagland No. 1 solution (9). After 12 days they were transferred to a hardening cabinet at a constant temperature of 1 C, an 8-hr photoperiod, and a light intensity of 8,000 lux. Before and during hardening the frost resistance of the plants was tested by controlled freezing (7, 13) in the pots at several temperatures. The temperature at which 50% of the plants were killed (killing temperature) was determined after 3 weeks of recovery.

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Labeling with ^{33}P. The roots of triplicate samples of two plants were covered with 2 ml of a radioactive solution containing 0.05 M citrate buffer pH 5 and 3 to 8 μCi of ^{33}P ($H_3{}^{33}PO_4$, 50 mCi/mmole, New England Nuclear) in 10-ml beakers for varying times at either 20 C or 1 C under continuous light at an intensity of 8,000 lux. During hardening experiments, feeding times were either 24 hr at 1 C or 12 hr at 20 C. At the end of the incorporation, the roots and crowns were rinsed, boiled for 3 min, covered with 6 ml of chloroform-methanol (1:2, v/v), and kept in a freezer under nitrogen. Uptake was determined by measuring radioactivity in medium combined with rinses. All data are means of measurements on triplicate samples. All experiments were repeated at least once.

Extraction, Separation, and Characterization of Lipids. Tissues were extracted within 3 days according to the method of Bligh and Dyer (1). After addition of 1.6 ml of water, tissue water included, the roots were homogenized (Polytron) for 30 sec. After centrifugation at 1500*g* for 5 min, the supernatant was decanted and the residue was re-extracted with 7.6 ml of the same mixture (chloroform-methanol-water, 2:4:1.6, v/v). The combined extracts were mixed first with 4 ml of chloroform, then with 4 ml of H_2O, and the phases were separated by centrifugation. The lower chloroform layer was evaporated under nitrogen and redissolved in a small volume of chloroform-methanol (2:1, v/v).

Lipids from all samples were separated by TLC on silica gel G in chloroform-methanol-acetic acid-water (85:12.5:12.5:2, v/v) (10). After measurement of the radioactivity, the lipids were detected by iodine vapors, Dragendorff's reagent (3), ninhydrin (0.25% in acetone-lutidine 9:1, v/v), and the Zinzadze reagent (6). Lipids from unhardened tissue, labeled at 20 C, were further characterized by cochromatography with reference compounds in the system described above and on Silica Gel H in three additional solvents: chloroform-methanol-butanol-acetic acid-water (40:15:40:7:7, v/v), chloroform-methanol-butanol-ammonium hydroxide (45:20:30:5, v/v) and butanol-acetic acid-water (80:25:15, v/v). The reference compounds used were, in order of increasing R_F levels in the first solvent: lysophosphatidylserine, lysophosphatidylcholine, phosphatidylinositol, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidyldimethylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidic acid, diphosphatidylglycerol (Applied Science, State College, Penna., and Analabs, North Haven, Conn.).

Measurement of Radioactivity. Radioactivity of solutions was assayed by scintillation counting (Unilux II, Nuclear Chicago) using Bray's solution (2). Efficiency was 80% for unquenched samples. Counts were corrected for quenching by external standard channels ratio. Radioactivity was mea-

sured directly on thin layer plates by means of a scanner (Actigraph II, Nuclear-Chicago) and the results were expressed as percentage of lipid radioactivity. The efficiency of the scanner was approximately 20% and its reproducibility ±5%.

RESULTS

Hardening of Winter Wheat. Before hardening, the variety Kharkov was slightly more frost-resistant than Champlein (Fig. 1). Killing temperature for Kharkov decreased rapidly from −8.1 C to −18.7 C in 4 days of hardening, and reached −22.1 C after 21 days. The killing temperature for Champlein decreased slowly throughout the experiment to reach −13.2 C after 21 days.

Incorporation of ^{33}P into Lipids at 20 C and 1 C. Figure 2 shows the incorporation of ^{33}P into the lipids of both varieties at 20 C and 1 C. After chromatography in chloroform-methanol-acetic acid-water (85:12.5:12.5:2, v/v), four well separated main peaks of radioactivity were detected. They were characterized as phosphatidylinositol, phosphatidylcholine, phosphatidylglycerol, and phosphatidylethanolamine. Of the other reference compounds tested, only two cochromatographed with more than traces of radioactivity in this solvent: phosphatidylserine cochromatographed with phosphatidylcholine, and phosphatidic acid with phosphatidylethanolamine. Chromatography in the other solvents showed that they were not significantly labeled. Traces of radioactivity were also detected at the origin, ahead of phosphatidylethanolamine and at the solvent front.

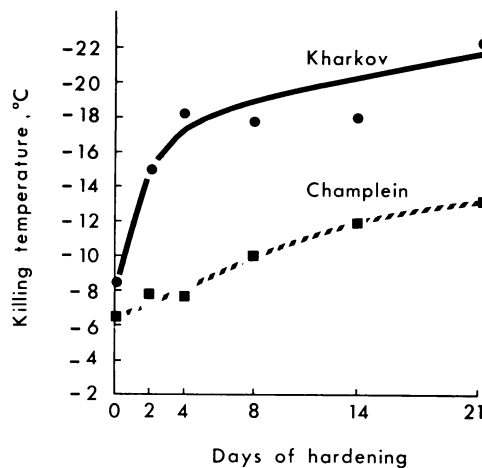

FIG. 1. Killing temperatures for two varieties of winter wheat during hardening.

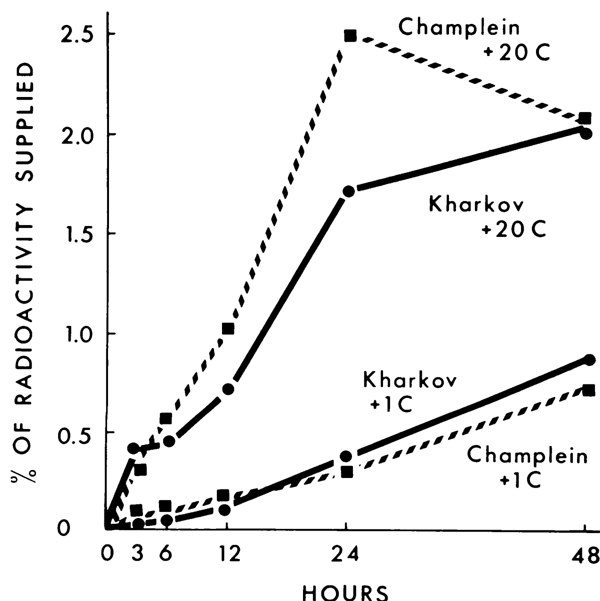

FIG. 2. Incorporation of ^{33}P into lipids of winter wheat at 20 C and 1 C (1% represents 180,000 dpm).

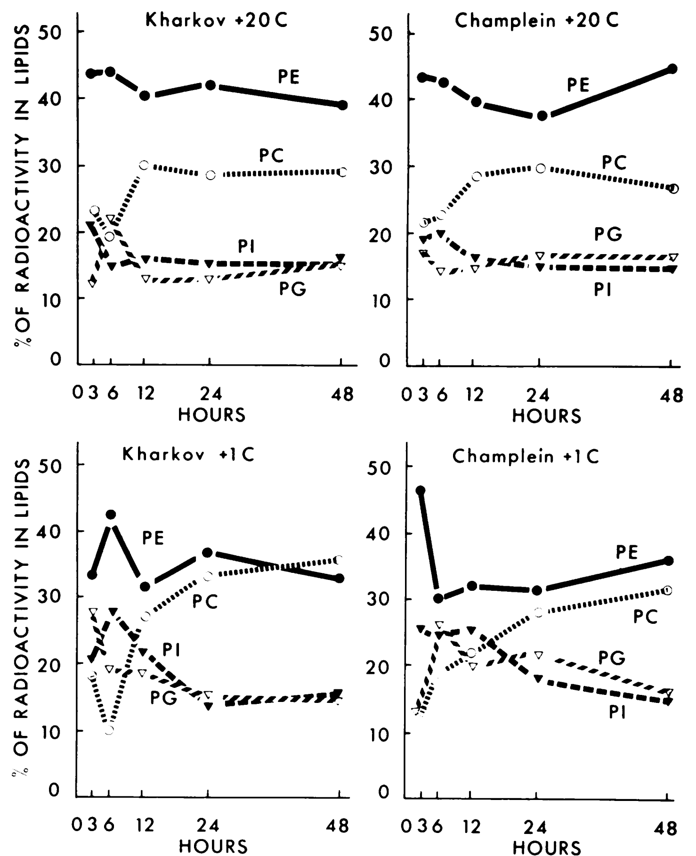

FIG. 3. Incorporation of ^{33}P into individual phospholipids of winter wheat at 20 C and 1 C. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PG: phosphatidylglycerol.

With time of incorporation the proportion of the radioactivity incorporated into the lipids increased in phosphatidylcholine while it decreased in phosphatidylinositol and phosphatidylglycerol at both temperatures (Fig. 3). The proportion of label in phosphatidylethanolamine was lower at 1 C than at 20 C. There was little difference between the relative labeling in the various phospholipids in the two varieties. However, at 1 C the increase in phosphatidylcholine occurred sooner and was greater in Kharkov than in Champlein.

Uptake of ^{33}P during Hardening. At both temperatures and in both varieties there was a large temporary increase in rate of uptake of ^{33}P (*i.e.*, radioactivity absorbed during the incorporation, 12 hr at 20 C and 24 hr at 1 C, at different times of hardening) after 2 days of hardening (Fig. 4). The rate of uptake which was initially observed in unhardened tissue was almost completely restored after approximately 4 days of hardening. After this recovery, the rate of uptake increased again upon further hardening.

Synthesis of Phospholipids during Hardening. Incorporation of ^{33}P into lipids at 20 C was markedly stimulated in both varieties early in the hardening period (Fig. 5). This increase

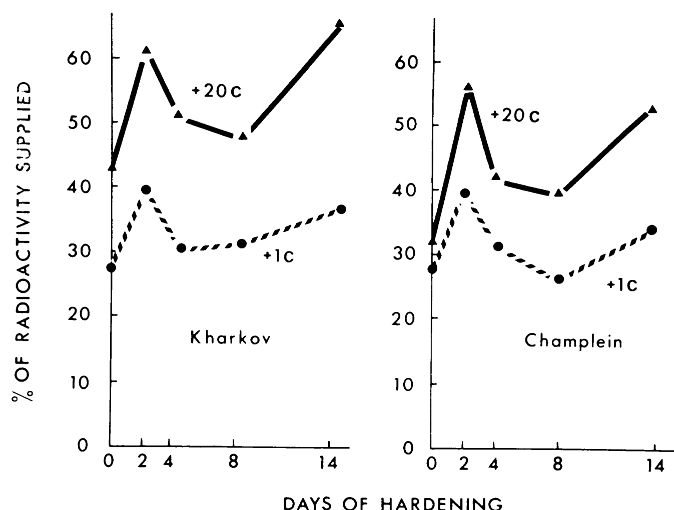

FIG. 4. Uptake of ^{33}P during 12 hr at 20 C and during 24 hr at 1 C at different times of hardening of winter wheat.

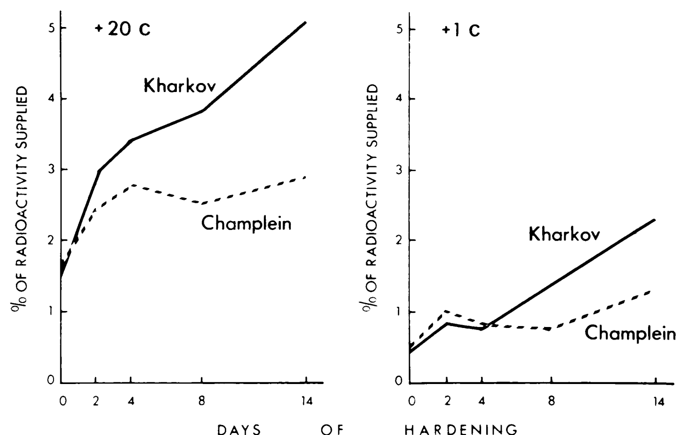

FIG. 5. Incorporation of ^{33}P into lipids during 12 hr at 20 C and during 24 hr at 1 C at different times of hardening of winter wheat, expressed as percent of radioactivity supplied (1% represents 70,000 dpm).

was greater in Kharkov than in Champlein. After 4 days of hardening, incorporation was not further enhanced in Champlein while in Kharkov it increased almost linearly throughout the 14 days of the experiment. When ^{33}P was incorporated at 1 C, stimulation was much less. It was again greater in Kharkov but occurred only after a delay of several days.

When the data were expressed as percentages of the radioactivity absorbed by the plants, to minimize the effect of changes in rate of uptake, phospholipid synthesis was shown to be greatly stimulated in Kharkov at both temperatures of incorporation, while stimulation was much less in Champlein (Fig. 6).

Little change was observed in the pattern of phospholipids synthesized during hardening. The proportion of ^{33}P incorporated into phosphatidylcholine tended to increase with hardening in both varieties and at both temperatures.

DISCUSSION

Hardening causes a great stimulation of phospholipid synthesis in the hardy variety Kharkov. Stimulation occurs at both temperatures of incorporation and is much less pronounced in Champlein (Figs. 5 and 6). It is therefore not a temperature effect but an actual part of the hardening process.

Figure 6 shows that although uptake is affected by the hardening process (Fig. 4) and although the changes in rate of uptake influence lipid synthesis, they do not account for more than a small part of the increase in phospholipid synthesis observed in Figure 5. Particularly, the large increase in phospholipid synthesis at 1 C in Kharkov after 4 days of hardening is not matched by a large increase in uptake of ^{33}P.

The apparent decrease in rate of phospholipid synthesis in Champlein at the beginning of the hardening period when ^{33}P is incorporated at 20 C (Fig. 6) reflects the rapid increase in uptake and the less rapid increase in phospholipid synthesis at that time. The slowing down of phospholipid synthesis at 20 C in Kharkov after 14 days of hardening is also only apparent. There is, however, actual decrease in phospholipid synthesis at 20 C in Champlein after 14 days of hardening.

From Figures 1 and 4 it can be concluded that rate of uptake of ^{33}P does not correlate with frost resistance. Although the two varieties differ greatly with respect to frost hardening, they behave almost identically with respect to uptake of ^{33}P during hardening. The sudden and temporary increase in rate of uptake at the beginning of the hardening period may be interpreted as the result of an initial cold shock which increased cell permeability. This cold shock may have influenced the hardening response.

The increase in the rate of phospholipid synthesis does not correlate with the increase in frost resistance during hardening (Figs. 5 and 6). When incorporations were made at 1 C, which is the hardening temperature, the most rapid increase in the rate of phospholipid synthesis came after Kharkov had acquired most of its frost resistance. When incorporations were made at 20 C, the rate of phospholipid synthesis went on increasing for a long time after almost maximal frost resistance had been reached in Kharkov. Increased phospholipid synthesis is, therefore, not a prerequisite to hardening in winter wheat. However, a high rate of phospholipid synthesis may be required to maintain the high level of frost resistance attained in Kharkov. It may become the limiting factor in Champlein after a few days of hardening.

This increased rate of phospholipid synthesis with little qualitative change may reflect repair from damages incurred

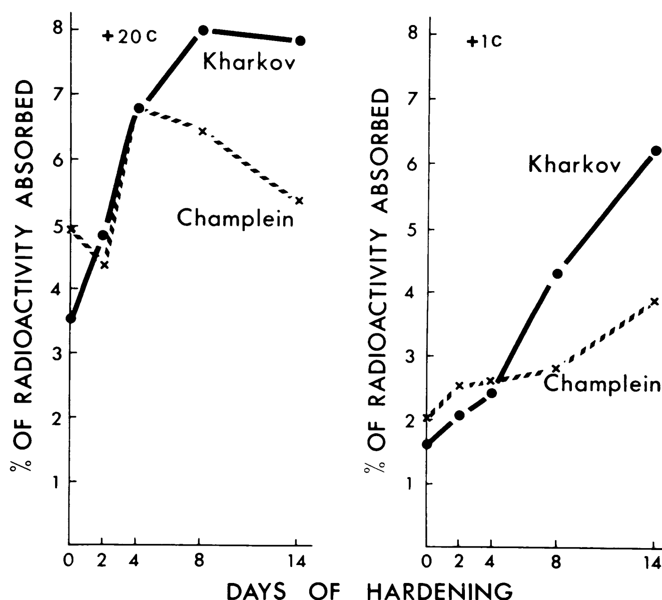

FIG. 6. Incorporation of ^{33}P into lipids during 12 hr at 20 C and during 24 hr at 1 C at different times of hardening of winter wheat, expressed as percent of radioactivity absorbed by the plants.

by the cell membranes at low temperature during hardening. It may also be interpreted as either the incorporation into the membranes of phospholipids which will help the membranes to function at 1 C (*e.g.*, phospholipids with less saturated fatty acids), or the multiplication of existing membranes without qualitative changes, or an increase in the proportion of phospholipids to other components in the membranes.

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