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ANATOMICAL CHANGES IN LEAVES OF PUMA RYE IN RESPONSE TO GROWTH AT COLD-HARDENING TEMPERATURES[1]

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Rye plants were cold hardened by growth at 4/2 C day/night (D/N); unhardened control plants remained at 25/20 C D/N. Leaves from hardened plants contained 33% less water on a dry weight basis than those from unhardened plants. The osmolar concentration of expressed sap from hardened leaves was 1.5 times greater than that of unhardened leaves. The number of cell layers in fresh leaves remained constant, but leaves from hardened plants were about 1.5 times thicker than those from unhardened plants. Increased leaf thickness was caused by increased mesophyll cell size. The structure of the vascular bundle and both stomatal frequency and distribution patterns of the upper and lower epidermal surfaces were altered.

Introduction

BJORKMAN, BADGER, and ARMOND (1978) stated that there was "no evidence that growth temperature affects the kinetic properties of Ru-P_2 carboxylase." They also reported that growth of *Nerium oleander* at either 20/15 C or 45/32 C (day/night [D/N]) did not result in any changes in leaf thickness or gross anatomy. The effects on growth of *Secale cereale* 'Puma' at cold-hardening temperatures (4/2 C D/N) were extensively investigated at the molecular level by HUNER and MACDOWALL (1976*a*, 1976*b*, 1978, 1979*a*, 1979*b*), who showed that structural and functional changes in the CO_2-fixing enzyme, ribulose bisphosphate carboxylase-oxygenase, do indeed occur in response to growth temperature.

This report extends our observations on the effect of growth of Puma rye at cold-hardening temperatures to the anatomical and morphological levels. Unlike the results of BJORKMAN et al. (1978), our study clearly shows alterations in leaf thickness and gross anatomy upon adaptation to growth at low temperature and, thus, establishes that leaves of Puma rye not only undergo changes at the molecular level but also at the anatomical and morphological levels in response to changes in growth temperature.

Several investigators attempted to use anatomical and morphological characteristics as criteria for breeding in frost resistance. Small cell size (KOLKUNOV 1905, 1913; ROSA 1921; LEVITT and SCARTH 1936) and low stomatal frequency (HIRANO 1931) were reported to be associated with frost hardiness, but BARULINA (1923) and LEVITT (1956) concluded that there was no relationship between cell size and frost resistance. CHEN, LI, and CUNNINGHAM (1977) reported that an increase in the number of osmiophilic globuli and the disappearance of starch within the chloroplast were associated with the development of frost hardiness in leaf cells of cold-hardy *Solanum acaule*. PALTA and LI (1979) found a significant relationship between frost hardiness and the stomatal index of the upper leaf surface, the number of palisade parenchyma layers, and the thickness of the palisade layers. No relationship, however, was found between cell size and frost hardiness. Our evidence clearly supports the conclusion that small cell size is not a criterion for frost hardiness in cold-hardy Puma rye. A preliminary report of these results was presented by HUNER et al. (1979).

Material and methods

PLANT MATERIAL.—Rye (*Secale cereale* L. 'Puma') plants were germinated under the same conditions and then grown under either cold-hardening or non-cold-hardening conditions (HUNER and MACDOWALL 1976*a*). Germination and growth were performed in a growth chamber at 25/20 C D/N in pots containing vermiculite supplied with a modified Hoagland's solution (HUNER and MACDOWALL 1976*a*). Illumination was provided by daylight fluorescent light supplemented with incandescent light (a total radiation of 450 μE s^{-1} m^{-2} during a 16-h photoperiod). After 7 days, plants were transferred to 4/2 C D/N for 90 days under the same conditions. Control plants remained at 25/20 C D/N for an additional 3 wk. All plants were supplied with nutrient solution every other day. Plants grown under the low-temperature regime ultimately survived temperatures to −30 C; control plants tolerated temperatures of −4 C (HUNER and MACDOWALL 1976*a*). The plants grown at 4/2 C D/N are termed "hardened," and those at 25/20 C D/N, "unhardened."

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LEAF CROSS SECTIONS.—Samples for the anatomical study were prepared by infiltrating excised leaf blades with tap water by using a faucet aspirator. For uniform physiological condition, only the uppermost, fully expanded leaf blades were used. Leaf cross sections, 75 μm thick, of fresh material were made perpendicular to the midrib from the central portion of the leaf with an Oxford Vibratome (Model G, Oxford Co., San Mateo, California). The average length and width of the mesophyll cells were measured by direct microscopic observations (Reichert, ×40 objective and ×10 eyepiece) with an eyepiece micrometer. Measurements were made at 10 different locations in each cross section. Photographs were taken with a Robot (Recorder 24 ME) camera mounted on the microscope.

LEAF SURFACE IMPRINTS.—Plastic imprints were made of the adaxial and abaxial leaf surfaces (SINCLAIR and DUNN 1961) and were observed under the microscope for stomatal counting and stomatal distribution patterns at 20 different locations on each leaf surface.

LEAF DRY WEIGHT DETERMINATION.—Leaf samples were dried in an oven at 82 C for 72 h and at 101 C for an additional 24 h. All samples were brought to room temperature in a desiccator for final weighing.

DETERMINATION OF LEAF CELL SAP CONCENTRATION.—The osmolar concentration of the expressed sap of the leaves of hardened and unhardened Puma rye was determined with a Hewlett-Packard vapor pressure osmometer (Model 302B). The sap was extracted by quickly freezing the leaf material in liquid nitrogen immediately upon excision, and then grinding it in a mortar and pestle. The homogenate was centrifuged at 30,000 *g* for 15 min at 4 C to remove particulate matter, and the osmolality of the supernatant was determined by comparison with standard solutions of known osmolality.

CHLOROPHYLL DETERMINATION.—Chlorophyll content of leaf extracts prepared by boiling samples in methanol was determined by the method of ARNON (1949).

ABSORPTION SPECTRA.—Pigments from leaves of cold-hardened and unhardened plants were scanned at 25 C in 80% acetone using a Beckman Acta III recording spectrophotometer at a rate of 0.5 nm s^{-1}.

Results

When Puma rye plants were grown under the cold-hardening conditions, a consistent increase in leaf thickness was observed (fig. 1*A*,*B*). Leaves grown at the low temperature were about 1.5 times thicker than those grown under the control conditions. The number of cell layers in these leaf cross sections was the same in both hardened and unhardened plants. To account for the increase in leaf thickness upon low-temperature acclimation, mesophyll cell dimensions (length × width) were measured at 10 different locations and were: for hardened leaves, 36.0 ± 2.8 × 23.6 ± 3.0 μm; for unhardened leaves, 23.6 ± 4.8 × 19.6 ± 1.0 μm. Therefore, the length of the mesophyll cells of leaves from cold-hardened plants increased by a factor of 1.5. The width of the cells, however, was not significantly different, which was consistent with our visual observation that leaf width was not significantly affected by growth at cold-hardening temperatures. In addition, the epidermal cell walls of leaves of hardened plants were thicker than those of unhardened plants.

Closer examination of leaf cross sections revealed alterations in the structure of the vascular bundles: a differential proportion of phloem and xylem in leaves of hardened and unhardened rye (fig. 1*C*,*D*). The hardened leaves had equal proportions of phloem and xylem; the unhardened, relatively more phloem than xylem. Also, the cell walls of the mestome sheath (O'BRIEN and CARR 1970) nearest the conducting elements and leaves of hardened rye (fig. 1*C*) were much thicker than those in leaves of unhardened plants (fig. 1*D*). These anatomical differences were confirmed in three separate experiments.

The adaxial and abaxial epidermal surfaces of leaves from hardened and unhardened Puma rye had marked changes in stomatal frequency (table 1). Regardless of surface, the stomatal frequency of leaves from unhardened plants was about twice that of hardened plants. These results are consistent with those of HIRANO (1931). The stomatal frequency of the abaxial surface of leaves from unhardened plants was significantly higher than that of the adaxial surface. In contrast, there was no significant difference between the stomatal frequency of the abaxial and adaxial surfaces of leaves from cold-hardened plants.

Differences in stomatal distribution patterns were also observed. All stomata were in single rows parallel to the midrib of the leaf. The most drastic difference between leaves of hardened (fig. 2*A*) and unhardened rye plants (fig. 2*B*) was observed on the abaxial surface. Leaves from hardened and unhardened plants differed in the number of rows of epidermal cells between each successive row of stomata (table 2). For the abaxial surface of leaves of hardened plants, *a* was about seven and *b* was about 11 epidermal rows, whereas in leaves of unhardened

TABLE 1

ESTIMATION OF STOMATAL FREQUENCY OF LEAVES FROM HARDENED AND UNHARDENED PUMA RYE

	No. of stomates/unit area	
	Abaxial surface	Adaxial surface
Hardened rye.....	5.4±1.1	3.9±1.0
Unhardened rye.....	12.8±1.5	7.9± .9

NOTE.—All counts were performed at a magnification of × 160 such that a maximum number of stomatal rows was contained within a square field of view (.55 × .55 mm). Stomatal frequencies are the averages of 20 measurements made at different locations on the leaf surfaces. All values are the averages of four leaves from two separate experiments and are ± SD. The unit area was .303 mm^2.

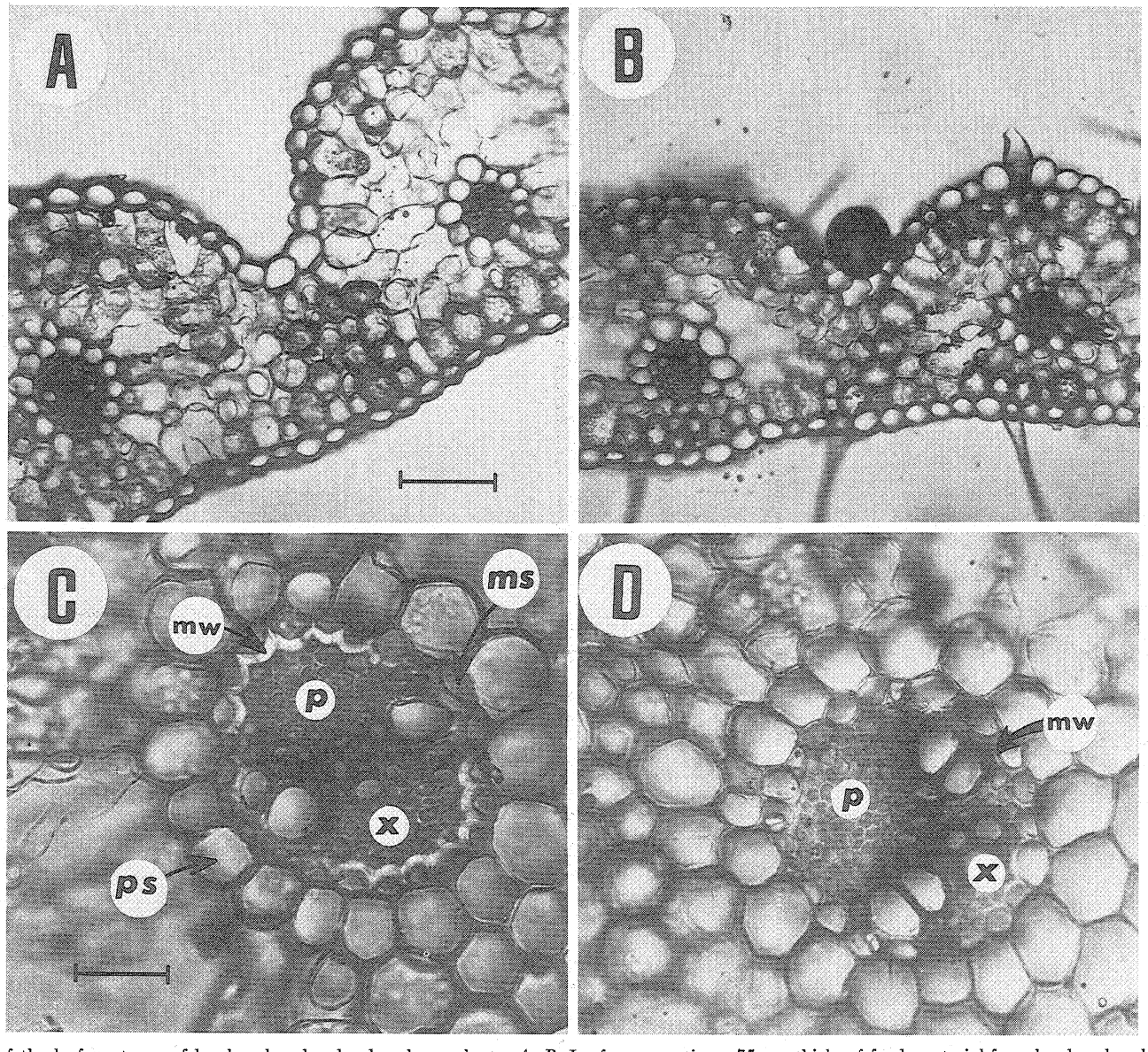

FIG. 1.—Comparison of the leaf anatomy of hardened and unhardened rye plants. *A*, *B*, Leaf cross sections, 75 μm thick, of fresh material from hardened and unhardened rye, respectively; both × 136. Bar in *A* = 50 μm. *C*, *D*, Cross sections of the vascular bundles of leaves from hardened and unhardened rye, respectively; both × 400. Bar in *C* = 12.5 μm. *p* = phloem; *x* = xylem; *ps* = parenchymal sheath; *ms* = mestome sheath; *mw* = mestome cell wall.

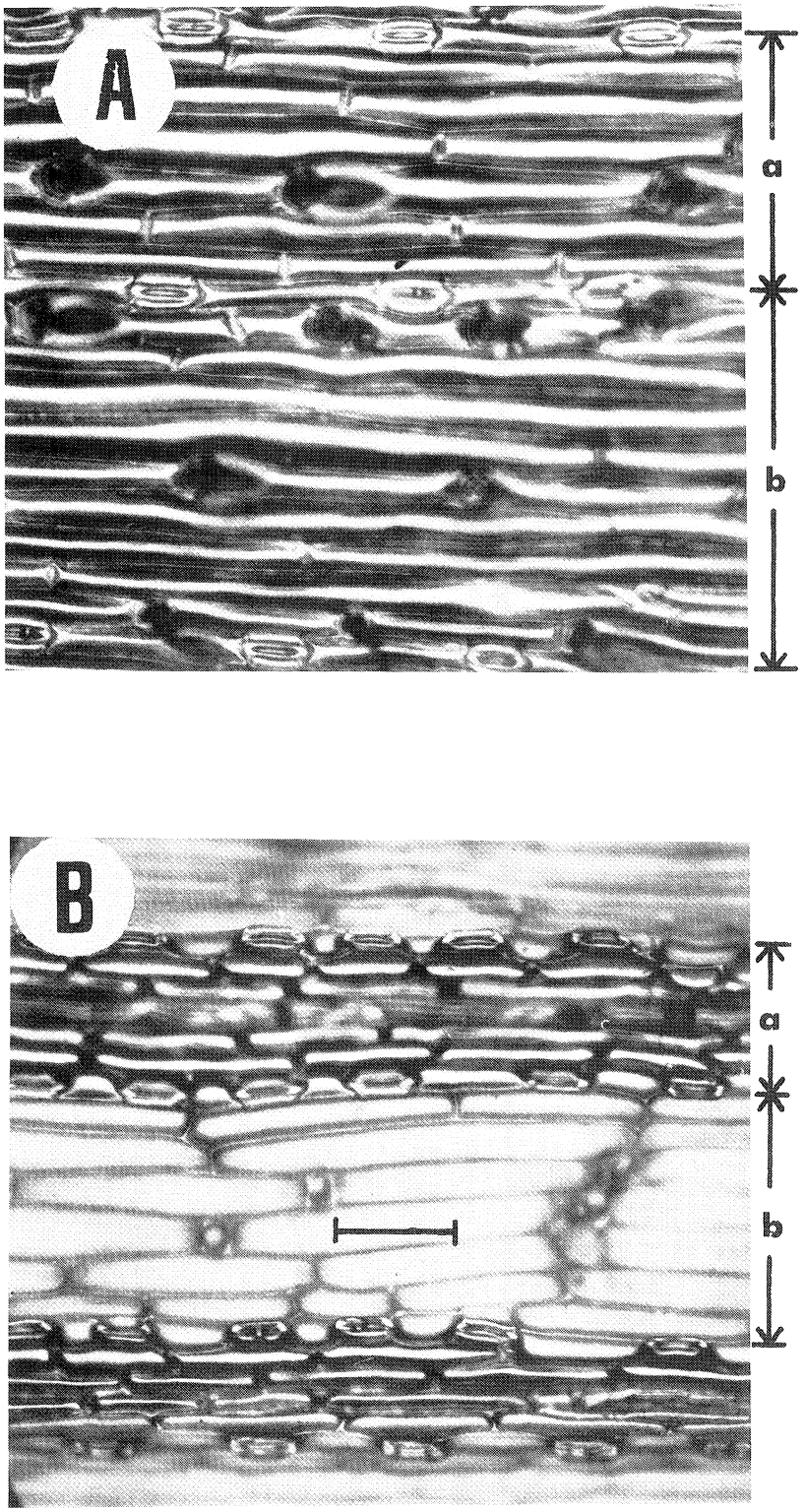

FIG. 2.—Comparison of the stomatal distribution patterns on the abaxial surface of leaves from hardened (*A*) and unhardened (*B*) rye plants. *A*, *B*, Photographs of plastic imprints of the abaxial surfaces; both × 160. Bar in *B* = 50 μm. *a*, *b*, = no. of rows of epidermal cells between successive rows of stomata.

TABLE 2

COMPARISON OF THE STOMATAL DISTRIBUTION PATTERNS OF LEAVES FROM HARDENED AND UNHARDENED RYE PLANTS

	Rows of epidermal cells between stomatal rows (no.)			
	Abaxial		Adaxial	
	a	*b*	*a*	*b*
Hardened rye.....	6.6±1.1	10.6±1.5	6.0±1.0	10.8±1.6
Unhardened rye.....	3.5± .5	6.6± .5	6.9± .8	6.9± .8

NOTE.—Plastic imprints of the abaxial and adaxial leaf surfaces were observed and the number of rows of epidermal cells between stomatal rows counted at 15 different locations on each leaf surface. Magnification was × 160. All values are the average of four leaves from different plants in two separate experiments and are presented as ±SD. Distances *a* and *b* are as shown in fig. 2.

TABLE 3

COMPARISON OF WATER CONTENT OF LEAVES OF HARDENED AND UNHARDENED PUMA RYE

	Water content (%)	g dry wt / g fresh wt	g water / g dry wt	Expressed sap concentration (osmolar)
Hardened rye.....	80.6±1.7	.194± .020	4.19± .42	.74
Unhardened rye.....	87.1± .9	.129± .011	6.77± .28	.45

NOTE.—Percentage of water content was calculated as g fresh wt − g dry wt/g fresh wt × 100%. Values for the expressed sap concentration are the results of single experiments; all other values are the averages of four experiments ± SD.

plants, *a* was about four and *b* about seven epidermal rows. These patterns were repeated (*ababab*) across the surface of each leaf.

On the adaxial surface, the stomatal distribution pattern ($a = 7$, $b = 11$) of leaves of hardened plants was the same as that on the abaxial surface (table 2). However, the distribution pattern on the adaxial surface of leaves of unhardened plants ($a = b = 7$) differed from that on the abaxial surface ($a = 4$, $b = 7$).

The water content of Puma rye leaves was affected by growth at cold-hardening temperatures (table 3). The water content was lower in leaves of hardened plants than in those of unhardened plants. Expressed on a dry weight basis, leaves of hardened plants had 1.5 times less water than those of unhardened plants. The dry weight and the sap concentration were about 1.5 times higher in leaves of cold-hardened plants.

The absorption spectra (fig. 3) indicate that the proportions and the types of chlorophyll in leaf extracts of cold-hardened and unhardened plants were virtually identical. This is confirmed by the results of chlorophyll analyses (table 4), which indicate a chlorophyll *a*/chlorophyll *b* ratio of 3 for leaves from both hardened and unhardened plants. This value is consistent with that for other species (KOK 1976). The leaves of hardened and unhardened plants differed in chlorophyll content by a factor of 1.4 on a fresh weight basis, which was consistent with our visual observations. On a dry weight basis, however, no significant differences were observed.

TABLE 4

CHLOROPHYLL DETERMINATIONS OF LEAVES FROM HARDENED AND UNHARDENED PUMA RYE

	Chl *a* / Chl *b*	mg chl / g fresh wt	mg chl / g dry wt
Hardened rye.....	3.17± .31	3.35± .14	17.9±1.1
Unhardened rye.....	3.07± .30	2.46± .17	19.2±1.3

NOTE.—Mg chlorophyll/g dry wt was calculated on the basis of g dry wt/g fresh wt (data in table 3). All values are the averages of four experiments ± SD; chl = chlorophyll.

Discussion

Changes in leaf anatomy and morphology in response to environmental stress occur in a variety of plants. ZALENSKI (1904) noted that, in general, increasing water deficit resulted in a decrease in the size of the epidermal, stomatal, and mesophyll cells and an increase in the number of stomata, the thickness of the outer epidermal cell walls, and the number of palisade parenchyma layers. VASIL'YEV (1956) noted that leaves of all winter cereals "were more nearly horizontal in the fall than spring grains sown at the same time." PALTA and LI (1979) reported that frost-hardy species of potato had thicker leaves than nonhardy species. This was a consequence of a

double palisade layer in frost-hardy species contrasted with a single layer in nonhardy species. LONGSTRETH and NOBEL (1979) showed that salt stress increased leaf thickness, a result of increased mesophyll cell size in *Atriplex patula*, a salt-tolerant species. We have shown that leaves of Puma rye, a frost-hardy species, increased in thickness in response to growth at low temperature and that this increase reflected an increase in mesophyll cell length by a factor of 1.5 rather than an increase in the number of cells. This is similar to the increase found by LONGSTRETH and NOBEL (1979) for *A. patula* in response to salt adaptation.

It is generally agreed that the smaller the cell size, the hardier the plant is to frost and drought stress (ZALENSKI 1904; KOLKUNOV 1905, 1913; LEVITT 1956). However, it has not been regarded as a factor to account for the increase in frost hardiness during cold adaptation of plants (LEVITT 1956). Our results clearly establish that an increase in frost hardiness of Puma rye upon cold adaptation is not correlated with a smaller cell size but rather with an increase in cellular dimensions. Thus, we conclude that there is no mandatory relationship between plant cell size and the degree of frost hardiness.

Although there was no significant increase in leaf

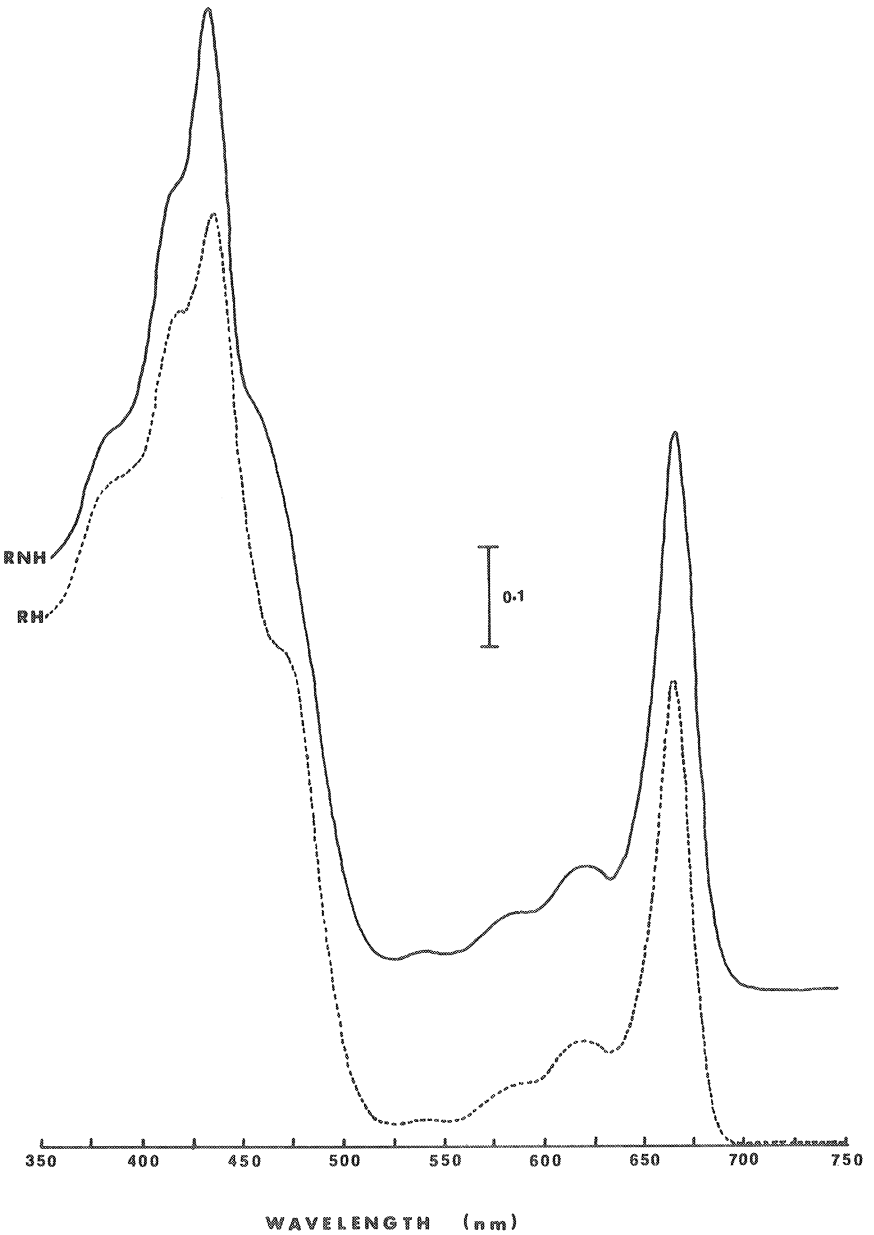

FIG. 3.—Comparison of the absorption spectra of pigments extracted from leaves of hardened (*RH*) and unhardened (*RNH*) leaves of Puma rye. The spectra were obtained with the pigments dissolved in 80% acetone. Bar = .1 absorbance unit.

and mesophyll cell width, the stomatal frequencies in the abaxial and adaxial surfaces of leaves from hardened and unhardened plants were different. The stomatal frequency was lower in leaves of hardened plants than in those of unhardened plants, regardless of the epidermal surface investigated. This difference in stomatal frequency can be explained, in part, by the difference in the stomatal distribution patterns. In general, the stomata within any single row, as well as the individual rows of stomata, are closer together in leaves of unhardened plants than in those of hardened plants. Since there was no significant difference in leaf width, this would necessitate a greater stomatal frequency in leaves of unhardened than in those of hardened plants. The change in stomatal frequency is thus most likely the result of temperature effects on the differentiation of epidermal cells.

The mestome and parenchymatous sheath surrounding the vascular bundles of leaves was first noted by DUVAL-JOUVE (1875) and subsequently thoroughly characterized by the pioneering work of SCHWENDENER (1890), who studied more than 100 species of grasses, including *Secale cereale*. In grasses with both sheaths, which includes *S. cereale* (VON WETTSTEIN 1962), SCHWENDENER noted that the cell walls of the mestome sheath were similar to the endodermal cell walls of roots. This observation was confirmed by VAN FLEET (1950) and O'BRIEN and CARR (1970), who showed that the mestome cell walls of *Triticum aestivum* and *Avena byzantina* were thicker on the side adjacent to the conducting elements. More importantly, SCHWENDENER noted that drought stress resulted in the development of asymmetrically thickened mestome sheath cell walls. The mestome sheath in *S. cereale* and the environmental influence on the development of asymmetric thickening of the mestome cell wall (fig. 1*C*,*D*) agree with the work of SCHWENDENER. However, our results cannot be due to drought stress since both hardened and unhardened plants were watered every other day with nutrient solution. We are unaware of any previous report indicating that plant growth at low temperature results in an increased asymmetric thickening of the mestome sheath cell wall of grasses (fig. 1*C*).

Changes in dry matter and water content occur after cold-hardening of various cultivars of wheat (LEVITT 1966; GUSTA, BURKE, and KAPOOR 1975) and potato (PALTA and LI, unpublished results). Our results for Puma rye (table 2) agree with their observations. Water content decreased on a dry weight basis by a factor of 1.5 with a concomitant increase in dry matter on a fresh weight basis upon growth at cold-hardening temperatures. The osmolar concentration of the expressed sap of rye leaves also increased from 0.45 to 0.74 in response to low growth temperature. However, the increase in the sap concentration by a factor of 1.5 does not account for the increase in frost hardiness of Puma rye from −3 to −30 C (LEVITT 1956).

A morphological change consistently observed in Puma rye was the darker green leaf color after cold acclimation, in agreement with observations reported by SINZ (1914) for wheat. The chlorophyll analyses (table 4) corroborate this visual observation since, on a fresh weight basis, leaves of hardened plants had 35% more chlorophyll than those of unhardened plants. However, this increase in chlorophyll content could be accounted for by the increase in dry weight upon low-temperature acclimation.

Chlorophyll was much more easily extracted from leaves of unhardened plants than from those of hardened plants. Leaf segments of hardened plants had to be ground in a mortar and pestle before all of the chlorophyll was removed. This difference in extractability may be due to the observed thickening in the epidermal cell walls as a result of growth at low temperature (fig. 1*A*,*B*).

It is difficult to ensure that leaves of plants of exactly the same physiological age are compared. To minimize this discrepancy, we carefully selected only mature, fully expanded leaves for study. Furthermore, when hardened and unhardened rye plants of identical chronological age were examined, the same changes in leaf anatomy and morphology were observed.

BJORKMAN et al. (1978) reported no apparent differences in leaf thickness or anatomy in *Nerium oleander* grown at either 20 or 45 C. The characteristic changes in leaf morphology and gross anatomy of Puma rye in response to growth at cold-hardening temperatures may be a property of the specific plant and/or the low-temperature regime at which the plants were grown.

In conclusion, our results establish that growth of Puma rye at low temperature involves changes at the cellular level and, thus, they supplement published results at the molecular level (HUNER and MACDOWALL 1976*a*, 1976*b*, 1978, 1979*a*, 1979*b*). More importantly, we can now unequivocally state that growth temperature does indeed affect both the "leaf thickness and gross anatomy" as well as the kinetic properties of ribulose bisphosphate carboxylase-oxygenase of Puma rye.

Although certain anatomical changes, such as mesophyll cell size, stomatal distribution patterns, and proportion of phloem of xylem, are significant, their relevance to the ability of Puma rye to acclimate to low temperature is not obvious. As STOCKER (1960) emphasized, certain stress effects may be "purely causal processes and thus cannot be considered teleologically." However, further investigations concerning the mestome sheath support the thesis that this structure may represent a barrier to ice

propagation during freezing and thus may play an important role in adaptation to cold-hardening temperatures (LINDSTROM, HUNER, and CARTER 1980).

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