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Cold Hardening of Spring and Winter Wheat and Rape Results in Differential Effects on Growth, Carbon Metabolism, and Carbohydrate Content[1]

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The effect of long-term (months) exposure to low temperature (5°C) on growth, photosynthesis, and carbon metabolism was studied in spring and winter cultivars of wheat (*Triticum aestivum*) and rape (*Brassica napus*). Cold-grown winter rape and winter wheat maintained higher net assimilation rates and higher in situ CO_2 exchange rates than the respective cold-grown spring cultivars. In particular, the relative growth rate of spring rape declined over time at low temperature, and this was associated with a 92% loss in in situ CO_2 exchange rates. Associated with the high photosynthetic rates of cold-grown winter cultivars was a 2-fold increase per unit of protein in both stromal and cytosolic fructose-1,6-bisphosphatase activity and a 1.5- to 2-fold increase in sucrose-phosphate synthase activity. Neither spring cultivar increased enzyme activity on a per unit of protein basis. We suggest that the recovery of photosynthetic capacity at low temperature and the regulation of enzymatic activity represent acclimation in winter cultivars. This allows these overwintering herbaceous annuals to maximize the production of sugars with possible cryoprotective function and to accumulate sufficient carbohydrate storage reserves to support basal metabolism and regrowth in the spring.

Rapid reduction of leaf temperature results in the accumulation of soluble carbohydrates (Azcón-Bieto, 1983; Brüggemann et al., 1992; Paul et al., 1992). In the short term, the accumulation of soluble carbohydrates suppresses photosynthesis by reducing Pi cycling and depleting ATP levels in the chloroplast (Mächler et al., 1984; Labate and Leegood, 1988; Labate et al., 1990). Inhibition of this sort is characterized by reduced sensitivity to O_2 at low temperature (Leegood and Furbank, 1986; Sage and Sharkey, 1987). In the longer term, Glc-feeding and cold-girdling experiments (Krapp et al., 1991, 1993; Krapp and Stitt, 1995) and data from transgenic tobacco (Stitt et al., 1990; von Schaewen et al., 1990) have shown that soluble carbohydrate accumulation can also suppress photosynthesis by down-regulating the levels of photosynthetic carbon reduction cycle enzymes and Chl *a*/*b*-binding proteins.

In contrast to these responses, long-term (months) acclimation of herbaceous plants to low growth temperatures results not only in the accumulation of large pools of soluble carbohydrates (Tognetti et al., 1990; Sagisaka et al., 1991; Koster and Lynch, 1992) but also in the enhancement of pathways involved in photosynthetic carbon metabolism (Guy et al., 1992; Holaday et al., 1992; Hurry et al., 1994, 1995a). The recovery of photosynthesis at low temperature is supported by increases in the activity of several enzymes of the photosynthetic carbon reduction cycle (Holaday et al., 1992; Hurry et al., 1995a) and by increases in the capacity for RuBP regeneration and in the pool sizes of phosphorylated intermediates (Hurry et al., 1994, 1995a). The increase in cellular Suc content during cold hardening also coincides with increases in the activity of cFru-1,6-BPase and SPS (Holaday et al., 1992; Hurry et al., 1995a) and in SPS protein content (Guy et al., 1992). Thus, during acclimation of cold-tolerant herbaceous plants to low temperature, the photosynthetic capacity that is normally lost during a shift to lower temperature is recovered, despite the accumulation of large, soluble carbohydrate pools (Hurry et al., 1994, 1995a).

Recent studies of photosynthetic acclimation of overwintering cereals have shown that freezing tolerance is strongly correlated with the capacity to increase photosynthesis and with the capacity to increase soluble carbohydrate pools during cold hardening (Tognetti et al., 1990; Öquist et al., 1993). Furthermore, field studies have shown that plants become predisposed to freezing injury when the fructan pool becomes depleted and simple sugars can no

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Abbreviations: CH, cold-hardened; cFru-1,6-BPase, cytosolic Fru-1,6-bisphosphatase; DHAP, dihydroxyacetone phosphate; F_m and F_m', fluorescence when all PSII reaction centers are closed in dark- and light-acclimated leaves, respectively; F_s, steady-state fluorescence in the light; F_v and F_v', variable fluorescence after dark acclimation and under light-acclimated conditions, respectively; K_T, thermal time calculated in °C days; NAR, net assimilation rate; NH, nonhardened; PGA, 3-phosphoglycerate; Q_{10}, ratio of rate at one temperature to the rate at a temperature 10°C lower; q_N, nonphotochemical quenching of fluorescence; Q_A, the primary, stable quinone acceptor of PSII; RGR, relative growth rate; RuBP, ribulose-1,5-bisP; sFru-1,6-BPase, stromal Fru-1,6-bisphosphatase; SPS, Suc-P synthase; $1\text{-}q_P$, proportion of reduced Q_A; Φ_{PSII}, quantum yield of PSII electron transport.

longer be released into the cytosol and intracellular liquid (Olien and Clark, 1993). This occurs earlier in the winter in nonhardy cultivars due both to the smaller soluble sugar reserves and to more rapid depletion of these reserves (Sagisaka et al., 1991). The accumulation of simple sugars in the cytosol (Koster and Lynch, 1992) suggests that they may be active cryoprotectants (Santarius, 1982; Anchordoguy et al., 1987). Fru is also present in the intracellular liquid, where it may decrease the adhesive energy that can develop from competition for interfacial liquid between hydrated plant surfaces and growing extracellular ice (Olien, 1974).

The aim of these experiments was to understand better the correlation observed between freezing tolerance, the accumulation of soluble carbohydrates, and the ability to recover photosynthetic capacity upon cold hardening. We have examined photosynthetic acclimation in leaves of spring and winter wheat (*Triticum aestivum*) and rape (*Brassica napus*) that developed during prolonged growth at low temperature (5°C). We show that winter cultivars of both wheat and rape increase the soluble/insoluble carbohydrate ratio and increase the level of extractable activity of photosynthetic and Suc biosynthetic enzymes. In contrast, spring cultivars show either a reduced soluble/insoluble carbohydrate ratio (spring wheat) or reduced carbohydrate accumulation (spring rape) and only small changes in enzymatic activity. The results are discussed with respect to regulation of enzymes involved in carbon metabolism and their possible role in the development of freezing tolerance.

MATERIALS AND METHODS

Plant Material

Seedlings of winter wheat (*Triticum aestivum* cv Portal), spring wheat (*T. aestivum* cv Dragon), spring rape (*Brassica napus* cv Paroll), and winter rape (*B. napus* cv Tor) (obtained from Svarlöf Norrlandsavdelningen, Umeå, Sweden) were grown five seedlings per 13-cm pot in coarse vermiculite. Water and nutrients were supplied as described previously (Hurry et al., 1993). Seeds were germinated under controlled-environment conditions: 300 μmol m^{-2} s^{-1} PPFD; day/night temperature regime 24/16°C; photoperiod 17 h. After 7 d, when the primary leaves had expanded, the wheat seedlings were cold hardened with a day/night temperature regime of 5/5°C and an 8-h photoperiod. The rape seedlings were cold hardened using identical conditions, but they were held at the warm temperature for 17 d, until leaf 3 had expanded and leaf 4 had started to appear. Except in studies of growth kinetics, all measurements were made on the fully expanded third leaves of 24- to 28-d-old NH and 65- to 70-d-old CH wheat plants and the fully expanded fifth leaves of 28- to 33-d-old NH and 56- to 63-d-old CH rape.

Growth Analysis

Both aerial and root portions of seedlings from one pot exposed to NH conditions were harvested for each cultivar every 4 d; those exposed to CH conditions were harvested every 10 d. The seedlings were dried to constant weight at 80°C. Growth coefficients were calculated from the slope of the natural log conversion of total plant dry weight versus time (Macdowall, 1974). Calculations of growth coefficients based on K_T were made assuming a threshold temperature for growth of 0°C according to Marsle et al. (1989), and Q_{10} values were calculated according to Sutcliffe (1977).

Measurement of CO_2 Exchange

In situ photosynthetic CO_2 exchange rates of NH and CH leaves were measured at 24 and 5°C, respectively, 3 to 4 h into the photoperiod using an open gas-exchange system (model LCA2; ADC Ltd., Hoddesdon, UK). Previous studies have shown that both NH and CH leaves sustain maximal photosynthetic rates 3 to 6 h into the photoperiod (Hurry et al., 1994). For the wheat cultivars, three to four leaves from different plants were combined to give a leaf area in the cuvette of 6 cm^2, and the measurements were repeated using four different pots to give four replicate measurements. Individual NH and CH rape leaves from four different pots were measured to give the four 6-cm^2 replicates.

Measurement of Chl Fluorescence

Chl *a* fluorescence was measured using a modulated fluorometer (PAM Chl Fluorometer; Walz, Effeltrich, Germany) with the PAM 103 accessory and two Schott lamps (model KL 1500; Schott, Mainz, Germany) providing saturating flashes and actinic illumination. The experimental protocol is detailed elsewhere (Hurry et al., 1993). Briefly, fluorescence induction kinetics were monitored in humidified air containing 5% CO_2 at 24°C and at different PPFDs ranging from 10 to 2000 μmol m^{-2} s^{-1}. Fluorescence characteristics were evaluated when the steady-state F_s level was reached, which, depending on PPFD, occurred 20 to 40 min after the light was switched to the next higher level. Photochemical and nonphotochemical quenching coefficients, q_P and q_N, and the yield of electron transport were calculated according to Schreiber et al. (1994).

Enzyme Activities

At the time of the CO_2-exchange measurements, leaf material was frozen in the light using a freeze clamp chilled to the temperature of liquid N_2. The frozen material was ground to a fine powder at the temperature of liquid N_2 in a mortar and pestle. The powder was extracted in a glass-in-glass homogenizer containing 1 mL of ice-cold extraction medium that always contained 2% (w/v) PVP and 0.1% (v/v) Triton X-100. In addition, the medium for each enzyme extraction contained: for Rubisco, 100 mM Bicine (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, 15 mM 2-mercaptoethanol, 0.02% (w/v) BSA; for sFru-1,6-BPase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 15 mM 2-mercaptoethanol, 0.02% (w/v) BSA, 1 mM Fru-1,6-bisP; for cFru-1,6-BPase, 50 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA, 0.02% (w/v) BSA, 15 mM 2-mercaptoethanol; for SPS, 50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol.

An aliquot of the whole-leaf extract was taken to determine protein content (Bio-Rad DC), and the remainder, except for the SPS samples, was centrifuged for 10 s in a microfuge. Except for the SPS samples, enzyme activity was immediately assayed spectrophotometrically (model ZFP 22; Sigma Instruments, Berlin, Germany) at 25°C using whole-leaf extracts as described in detail previously (Hurry et al., 1995a). Care was taken to keep all solutions as close to 0°C as possible during the preparation of the samples for initial activity measurements. For the SPS samples, the remaining extract was split into three, 300-μL aliquots and immediately frozen in liquid N_2. These samples were subsequently thawed, and SPS activity was measured as described previously (Hurry et al., 1994).

Metabolites

At the time of the CO_2-exchange measurements, leaf material (6 cm^2) was frozen in the light using a freeze clamp chilled to the temperature of liquid N_2. Frozen leaf material was ground to a fine powder at the temperature of liquid N_2 with 1 mL of 10% (v/v) $HClO_4$, and metabolites were extracted and levels were determined spectrophotometrically (model ZFP 22; Sigma) as described previously (Stitt et al., 1989).

Starch and Soluble Sugars

Three hours into the photoperiod, leaf material (6 cm^2) was frozen in the light using a freeze clamp chilled to the temperature of liquid N_2. The frozen leaf material was ground to a fine powder in liquid N_2, and soluble sugars were extracted on ice for 15 min in 1 mL of a 0.2 M KOH solution containing 0.08% Triton X-100. The samples were then centrifuged in a bench-top microfuge at 17,000*g*. The supernatant was used directly to assay Fru and Glc content spectrophotometrically in a linked assay using the change in A_{340} that resulted from the reduction of $NADP^+$. The assay buffer contained 100 mM triethanolamine (pH 7.6), 10 mM ATP, and 3 mM $NADP^+$, to which was added: Glc, 16 μL of supernatant, 32 nkat of hexose phosphate kinase, and 16 nkat of Glc-6-P dehydrogenase. After this reaction reached completion, 233 nkat of phosphoglucose isomerase was added to the Glc reaction mix to measure Fru content. To assay for Suc, an aliquot of the supernatant was first incubated in a citrate buffer (200 mM, pH 4.6) containing 40 nkat of β-fructosidase at 37°C for 20 min to hydrolyze the Suc. Suc was then assayed in the same manner as Glc. For starch, the pellet was resuspended in 0.5 mL of citrate buffer (200 mM, pH 4.6) and boiled for 10 min, and the starch was digested by adding 100 nkat of amyloglucosidase and incubating at 55°C for 24 h. The samples were then boiled for 10 min and centrifuged for 3 min. The supernatant, containing the Glc released by starch digestion, was retained, and starch content was estimated as Glc equivalents. All enzymes were supplied by Boehringer Mannheim.

RESULTS

Growth Kinetics

Growth coefficients for total dry weight accumulation and RGRs were similar for winter and spring wheat, but the coefficients measured at 5°C were one-fourth to one-third those at 24°C (Table I; Fig. 1B). However, NARs of the winter wheat cultivar were higher under CH conditions than those of the spring cultivar, suggesting that the spring wheat plants required a greater leaf area to support dry matter accumulation (Fig. 1A). When the growth coefficients were calculated based on K_T rather than real time, the growth rates were equal for both winter and spring wheat grown at either 5 or 24°C. This suggests that the reduced growth rates at the lower temperature are a consequence of reduced thermal input. That the calculated Q_{10} values were close to 2 for both cultivars supports the conclusion that growth has been restricted by thermodynamic limitations (Table I). Similar trends for a range of growth parameters have been reported previously for winter rye (Griffith and McIntyre, 1993) and winter and spring wheat (Hurry and Huner, 1991) grown under similar conditions. Note that "growth rate" is used here to represent total biomass accumulation. As such, it incorporates not only resource allocation to growth but also to storage reserves. It also incorporates the accumulation of compounds that may occur simply because resource supply exceeds demand at this low temperature (Millard, 1988; Chapin et al., 1990). Therefore, although biomass accumulation is identical in winter and spring wheat cultivars at this low growth temperature, this may mask differences in relative allocation, as suggested by the differences in NAR (Fig. 1, A and C).

The growth coefficients and RGRs of both rape cultivars were lower than for either wheat cultivar under the CH regime (Table I; Fig. 1). Winter rape accumulated dry matter at only 15% of the rate at 5°C compared with 24°C, and consequently, dry matter accumulation for this cultivar had a Q_{10} of 2.7. Growth of the spring cultivar was even more severely restricted by the low temperature, resulting

Table I. *Estimated growth coefficients for total dry matter accumulation of winter and spring cultivars of wheat and rape*

Five seedlings from one pot for each cultivar grown under NH and CH conditions were harvested every 4 d and 10 d, respectively. Growth coefficients (k_1') were calculated from the plot of the natural log of total dry weight versus real time [k_1' (d^{-1})] and k_T [k_1' (°C d^{-1})].

Coefficient/Cultivar	NH	CH	Q_{10}
k_1' (d^{-1})			
Spring wheat	0.13	0.03	2.2
Winter wheat	0.12	0.03	2.1
Spring rape	0.20	0.01	4.8
Winter rape	0.20	0.03	2.7
k_1' (°C d^{-1})			
Spring wheat	0.006	0.006	
Winter wheat	0.006	0.006	
Spring rape	0.009	0.002	
Winter rape	0.009	0.006	

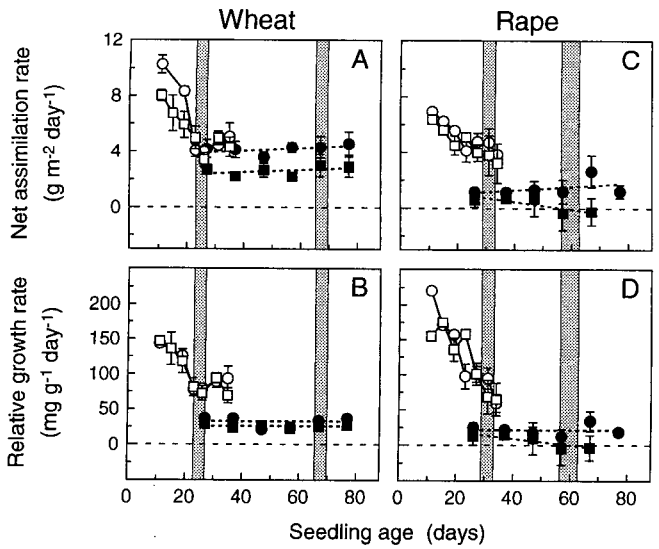

Figure 1. NAR (A, C) and RGR (B, D) for NH (○, □) and CH (●, ■) winter (○, ●) and spring (□, ■) wheat (A, B) and rape (C, D). Five seedlings from one pot for each cultivar grown under NH and CH conditions were harvested every 4 and 10 d, respectively. Growth rates were calculated from total dry matter accumulation over the preceding 4- or 10-d growth period. The vertical shaded bars represent the period for the subsequent measurements of photosynthetic characteristics; the left bars show the period for NH plants and the right bars show the period for CH plants. Each point (bar) represents the mean (±SD) of the five seedlings.

in a Q_{10} of 4.8 (Table I). Furthermore, because of overall tissue loss due to senescent leaves, the trend for both NAR and RGR was negative for the spring cultivar (Fig. 1C). Thus, the growth processes of spring rape are particularly sensitive to low temperature, and the resulting loss of vigor leads to occasional seedling death.

CO_2 Exchange Rates

Following cold hardening, both winter cultivars increased their in situ net photosynthetic CO_2 exchange rates per unit area (Fig. 2). The photosynthetic rate of winter wheat increased 34% from 5.6 μmol CO_2 m^{-2} s^{-1} at 24°C to 7.5 μmol CO_2 m^{-2} s^{-1} at 5°C, and that of winter rape increased 21% from 7.5 μmol CO_2 m^{-2} s^{-1} at 24°C to 9.1 μmol CO_2 m^{-2} s^{-1} at 5°C. The increase displayed by the winter cultivars was similar to that reported previously for winter rye (Hurry et al., 1994). This recovery of photosynthetic capacity normally lost by a shift to lower growth temperatures has been associated with increased photosynthetic carbon reduction cycle capacity and increased pool sizes for phosphorylated intermediates (Hurry et al., 1994, 1995a). In contrast, the spring wheat photosynthetic rate remained constant following cold hardening, and the spring rape rate fell by 92% from 8.6 μmol CO_2 m^{-2} s^{-1} at 24°C to 0.7 μmol CO_2 m^{-2} s^{-1} at 5°C. The pronounced reduction in photosynthesis in the spring rape suggests a failure of this cultivar to cold harden, and this correlates with the restricted growth rates of these plants.

Chl Fluorescence

The light response of Chl *a* fluorescence parameters, measured at 24°C and in 5% CO_2, for NH and CH spring and winter wheats and rape is shown in Figure 3. Cold hardening of both spring and winter wheat increased the proportion of PSII reaction centers that remain open with increasing irradiance ($1-q_P$; Fig. 3A). Cold hardening also reduced the development of q_N (Fig. 3D) in both winter and spring wheat at low to intermediate irradiance. Associated with the lower level of PSII reaction center closure and lower q_N is an increase in antenna trapping efficiency (F_v'/F_m'; Fig. 3B) and Φ_{PSII} (Fig. 3C), which is indicative of the higher rates of photosynthesis in CH compared with NH leaves. Similar results have been reported for CH winter rye (Hurry et al., 1993). Cold hardening did not affect the dark-adapted F_v/F_m (0.80), but slightly increased the proportion of PSII reaction center closure (Fig. 3A) and lowered Φ_{PSII} (Fig. 3D) for the spring wheat cultivar compared with the winter cultivar, which is indicative of lower photosynthetic rates. This response agrees with the CO_2 exchange data presented in Figure 2 and it is similar to that reported previously for spring and winter wheat (Hurry et al., 1992).

Responses similar to those of winter and spring wheat can also be seen for CH winter rape (Fig. 3, E–H). However, in cold-treated spring rape leaves, approximately 75% of PSII reaction centers are closed at the growth irradiance of 300 μmol m^{-2} s^{-1} PPFD, even under the higher measurement temperature and in the presence of nonlimiting CO_2 concentrations (Fig. 3E). Trapping efficiency is also low in these leaves, even at low irradiance (Fig. 3F), and consequently, the yield of electron transport is initially low and declines rapidly to reach a minimum at around the growth irradiance (Fig. 3G). This is in accordance with their very low CO_2 exchange capacity (Fig. 2) and the reduction in the dark-adapted F_v/F_m from 0.82 to 0.62. Furthermore, q_N

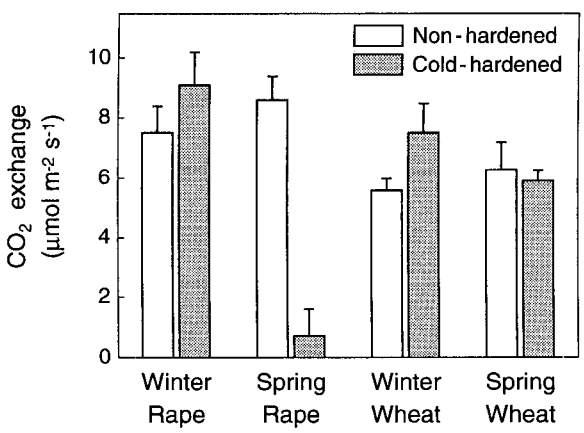

Figure 2. In situ net CO_2 exchange, measured 3 h into the photoperiod, and measured at 300 μmol m^{-2} s^{-1} PPFD and ambient temperature (surface temperatures were approximately 7 and 27°C for CH and NH leaves, respectively) and CO_2 NH (open bars) and CH (shaded bars) leaves of spring and winter wheat and rape. Each bar represents the mean (±SD) of four leaves.

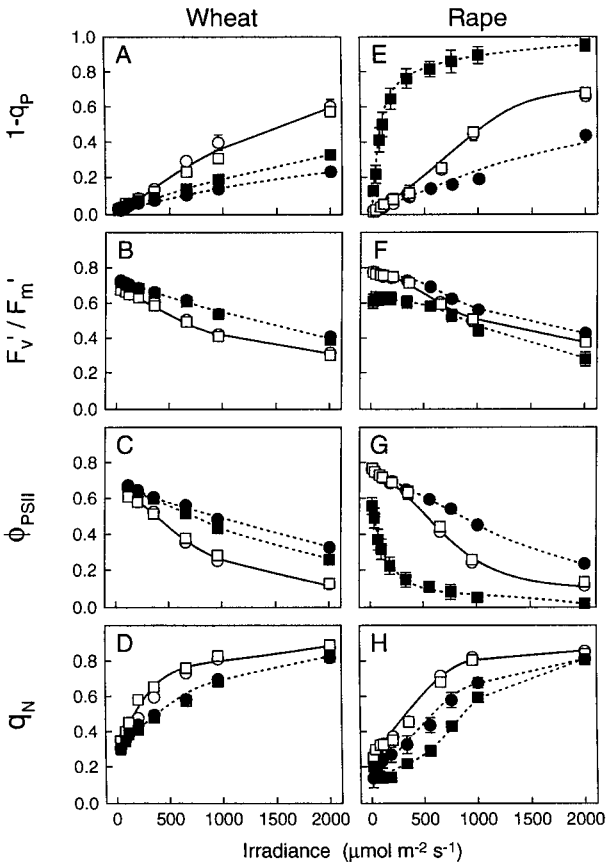

Figure 3. Chl *a* fluorescence quenching coefficient light-response curves for NH (○, □) and CH (●, ■) winter (○, ●) and spring (□, ■) wheat (A–D) and rape (E–H), measured at 24°C in 5% CO_2. The initial F_v/F_m of the measured leaves was 0.80 ± 0.01 for both NH and CH leaves of both spring and winter wheat cultivars and 0.82 ± 0.02 and 0.62 ± 0.06 for NH and CH leaves, respectively, of the spring rape cultivar and 0.83 ± 0.01 and 0.81 ± 0.01 for NH and CH leaves, respectively, of the winter rape cultivar. Each point (bar) represents the mean (±SD) of four replicate samples.

develops very slowly in these leaves, not reaching appreciable levels until irradiance is above 500 μmol m^{-2} s^{-1} PPFD (Fig. 3H). The high proportion of closed PSII centers and low q_N at low irradiance suggest that these PSII reaction centers are unable to transfer electrons efficiently even at low photon fluence. One consequence of this inability to dissipate electrons is that the transthylakoid pH gradient, and therefore high-energy-state quenching, develops slowly in these leaves. This is distinct from the response of CH winter rape leaves, in which the lower q_N is a consequence of increased dissipation of the proton gradient because of faster ATP consumption by the photosynthetic carbon reduction cycle. These data show that the lesion in CH spring rape photosynthesis may be associated with the consumption of reducing equivalents, and therefore with carbon metabolism. A similar conclusion was drawn from earlier studies of a range of spring wheats grown at low temperature (Hurry and Huner, 1991).

Enzyme Activity

Activity per unit area and the activation state of several enzymes of the photosynthetic carbon reduction cycle and of the cytosolic pathway for Suc synthesis are shown in Table II. Spring rape did not modulate the total extractable activity per unit area of any of the enzymes measured. In contrast, winter rape increased both the activity and activation state of Rubisco and sFru-1,6-BPase such that the in vivo activity of Rubisco increased 2-fold and the activity of sFru-1,6-BPase increased 6-fold (Table II). Similarly, CH winter rape showed a 3-fold increase in cFru-1,6-BPase activity and showed increases in both activity and activation state of SPS such that the limiting, or in vivo, activity increased 5-fold.

Both wheat cultivars increased the activity and maintained high activation states of Rubisco and sFru-1,6-BPase to a similar degree, but to a lesser extent than was seen in winter rape. Total extractable Rubisco activity increased by approximately 30% on an area basis, and total sFru-1,6-BPase activity increased by 140%. However, the activation state of SPS for the spring wheat fell from 52 to 23%, and the in vivo activity remained unchanged. In contrast, CH winter wheat increased total activity and maintained the high activation state so that the in vivo activity of SPS increased 2-fold (Table II).

When total enzyme activity was measured per unit of protein, no change in activity was found in spring rape. Spring wheat showed no change in either of the Fru-1,6-BPases or SPS; however, there was a 35% reduction in total Rubisco activity. In contrast, in the winter cultivars the activity of both of the Fru-1,6-BPases and SPS increased (Table II). It is interesting that all three actively growing cultivars (winter and spring wheat and winter rape) showed a decrease in Rubisco activity per unit of protein. Similar decreases in Rubisco activity and increases in Rubisco activation were found in transgenic tobacco and spinach leaves that were either fed Glc or cold girdled (Stitt et al., 1990; Krapp et al., 1991, 1993; Krapp and Stitt, 1995). This response was associated with increased accumulation of soluble carbohydrates. Soluble carbohydrates also accumulate in cereals (Tognetti et al., 1990; Koster and Lynch, 1992) and rape (Paul et al., 1992) at low growth temperatures. However, in the experiments with spinach and tobacco, the loss of Rubisco activity was also associated with the loss of other photosynthetic carbon reduction cycle enzymes and with a suppression of photosynthesis. In contrast, the decrease in relative Rubisco activity in the two winter cultivars was associated with increases in the activity of both Fru-1,6-BPases and, to a lesser extent, SPS and in the recovery of photosynthesis. Thus, these data show that in CH leaves, the accumulation of soluble carbohydrates in winter cultivars (see below) does not lead to the general suppression of photosynthetic enzymes. Furthermore, the relative loss of Rubisco activity may be a consequence of the accumulation of a large pool of inactive Rubisco as a nitrogen store under this growth-limiting condition (Millard, 1988). However, this remains to be determined.

Table II. *Activity and activation state of photosynthetic carbon reduction cycle and Suc synthesis enzymes in NH and CH leaves of winter and spring cultivars of wheat and rape*

Three hours into the light period, leaves (6 cm^2) were frozen in the light at the temperature of liquid N_2 using a freeze clamp. Each value represents the mean ± SD of three leaves.

Enzyme	Activity	Rape				Wheat			
		Winter		Spring		Winter		Spring	
		NH	CH	NH	CH	NH	CH	NH	CH
Rubisco	Total[a]	39 ± 4	63 ± 3	43 ± 3	48 ± 6	36 ± 3	47 ± 1	35 ± 2	45 ± 1
	Initial[a]	29 ± 4	59 ± 2	34 ± 2	44 ± 6	24 ± 1	44 ± 1	21 ± 1	37 ± 3
	Percent	73 ± 8	92 ± 2	79 ± 2	90 ± 3	69 ± 5	94 ± 2	60 ± 3	84 ± 6
sFru-1,6-BPase	Total[a]	10 ± 3	40 ± 2	11 ± 1	14 ± 1	11 ± 2	27 ± 3	11 ± 2	27 ± 2
	Initial[a]	4 ± 1	25 ± 5	5 ± 1	5 ± 1	8 ± 1	20 ± 1	9 ± 1	22 ± 2
	Percent	39 ± 12	66 ± 11	47 ± 10	35 ± 8	72 ± 4	73 ± 5	83 ± 14	82 ± 1
cFru-1,6-BPase	Total[a]	2.1 ± 0.2	7.8 ± 2.0	2.3 ± 0.1	3.4 ± 0.2	2.1 ± 0.4	7.1 ± 0.6	2.3 ± 0.4	6.0 ± 1.0
SPS	V_{max}[a]	1.9 ± 0.1	5.9 ± 0.8	2.7 ± 0.1	3.4 ± 1.0	2.3 ± 0.2	4.8 ± 0.2	2.5 ± 0.4	4.7 ± 0.1
	Limit[a]	0.5 ± 0.1	2.3 ± 0.6	0.5 ± 0.2	0.8 ± 0.1	1.3 ± 0.1	2.8 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
	Percent	23 ± 5	38 ± 7	19 ± 8	16 ± 1	57 ± 4	58 ± 1	52 ± 3	23 ± 3
Rubisco	Total[b]	28.1 ± 3.0	21.6 ± 0.6	24.5 ± 1.7	24.5 ± 4.5	35.6 ± 3.0	19.5 ± 1.2	32.3 ± 4.0	18.1 ± 0.6
sFru-1,6-BPase	Total[b]	3.7 ± 1.0	8.5 ± 0.4	3.9 ± 0.2	4.0 ± 0.2	7.5 ± 1.5	13.0 ± 1.3	9.0 ± 1.8	10.5 ± 1.0
cFru-1,6-BPase	Total[b]	0.8 ± 0.1	1.8 ± 0.4	0.8 ± 0.1	1.0 ± 0.1	1.5 ± 0.3	3.4 ± 0.3	1.9 ± 0.3	2.3 ± 0.4
SPS	V_{max}[b]	0.7 ± 0.1	1.5 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.6 ± 0.2	2.3 ± 0.1	1.9 ± 0.3	2.0 ± 0.1
Chl	–[c]	451 ± 6	683 ± 49	464 ± 21	302 ± 63	347 ± 9	394 ± 24	304 ± 9	397 ± 16

[a] $\mu mol\ m^{-2}\ s^{-1}$. [b] $\mu mol\ mg^{-1}$ protein h^{-1}. [c] $mg\ m^{-2}$.

Metabolite Levels

Associated with these changes in enzymatic activity, the pool of photosynthetic carbon reduction cycle intermediates also increased variably depending on whether the plants were winter or spring cultivars (Fig. 4). Cold hardening increased RuBP content in leaves of both winter cultivars but not spring wheat. In contrast, RuBP content decreased 4-fold in spring rape leaves following long-term growth at low temperature (Fig. 4A). Similarly, PGA content increased with cold hardening in leaves of both winter cultivars and spring wheat but did not increase in spring rape (Fig. 4B). Consequently, the RuBP/PGA ratio of winter wheat remained unchanged with cold hardening. However, the RuBP/PGA ratio fell by 30% in winter rape leaves, 50% in spring wheat, and 70% in spring rape (Fig. 4E). The response of the two spring cultivars to long-term growth at low temperature suggested a reduction in the capacity to regenerate RuBP in the CH leaves of these cultivars but did not suggest any problem with RuBP carboxylation.

Similarly, CH leaves of winter and spring wheat and winter rape showed approximately 3-fold increases in DHAP, both wheat cultivars showed 3-fold increases in Fru-1,6-bisP, and winter rape showed 7-fold increases in Fru-1,6-bisP (Fig. 4, C and D). As with PGA and RuBP, the spring rape cultivar did not respond to growth at the lower temperature by accumulating larger pools of either DHAP or Fru-1,6-bisP. However, there was a pronounced accumulation of DHAP compared with RuBP in spring rape and in spring wheat (Fig. 4F). This increase in the DHAP/RuBP ratio correlated with the lower RuBP/PGA ratios in these cultivars (Fig. 4E) and was also indicative of a lower capacity for RuBP regeneration. The apparent reduction in the capacity for RuBP regeneration in spring rape and spring wheat after long-term acclimation does not appear to be due to a Pi limitation similar to that experienced by leaves exposed to elevated CO_2 (Stitt, 1986) or low temperatures over the short term (Labate and Leegood, 1988; Labate et al., 1990). Under these long-term cold-hardening conditions, the PGA/DHAP ratio either dropped or remained constant (Fig. 4G), showing no limitation in chloroplastic ATP. Furthermore, it should be noted that CO_2 exchange is limited only at 5°C in CH spring rape (Fig. 2). Thus, these indications of decreased RuBP regeneration may be limiting to CO_2 exchange only in the extreme case of spring rape. In the other cultivars, the general increases in metabolite pool sizes (Fig. 4) may be an acclimation response to support the high rates of photosynthesis maintained at the low temperature.

Cold hardening also resulted in 2- to 3-fold increases in hexose phosphates in both wheat cultivars and in winter rape but resulted in only minor changes in spring rape (Fig. 5). Of these metabolites, only UDP-Glc is exclusively cytosolic, whereas the others are present in both the cytosol and the stroma to varying degrees (Gerhardt et al., 1987). The accumulation of phosphorylated intermediates in leaves exposed to low temperatures for short periods is associated with inhibition of photosynthesis because of suboptimal stromal Pi status (Mächler et al., 1984; Labate et al., 1990). In contrast to these earlier findings, those cultivars that sustained high rates of photosynthesis following cold acclimation were those that accumulated large hexose phosphate pools. Furthermore, the Glc-6-P/Fru-6-P ratio, which suggests the partitioning of these hexose phosphates between the cytosol and the stroma, was not greatly altered by cold hardening in any of the cultivars (Fig. 5E). Similar increases in hexose phosphates (Hurry et al., 1994) and adenylate pools (Hurry et al., 1995b) have been reported

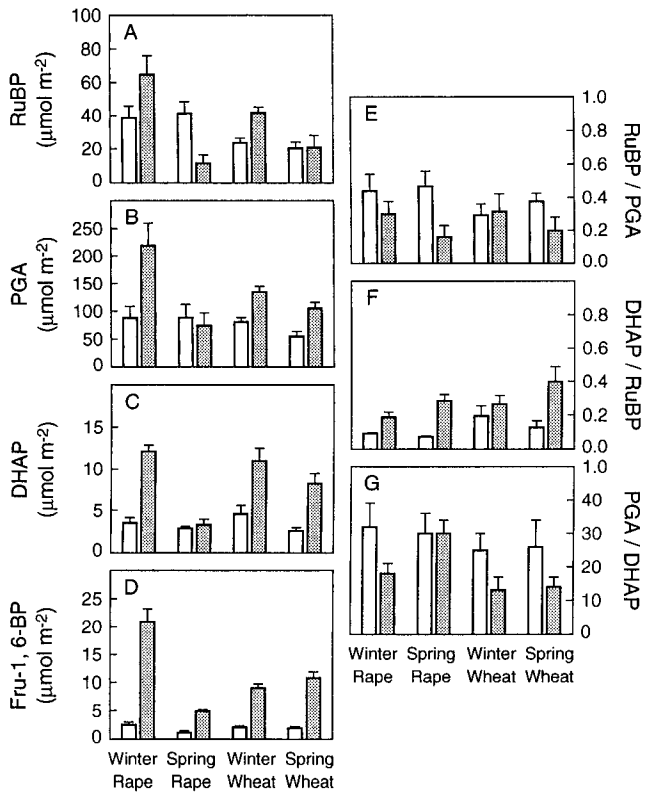

Figure 4. Pool sizes of photosynthetic carbon reduction cycle intermediates extracted from NH (open bars) and CH (shaded bars) leaves of winter and spring wheat and rape. Three hours into the light period leaves (6 cm^2) were frozen in the light at the temperature of liquid N_2 using a freeze clamp. Each bar represents the mean (±SD) of three leaves.

for winter rye following cold acclimation, and these data point to a long-term regulation of Pi status and cycling in cold-acclimated leaves.

Starch and Soluble Sugar Content

Samples for carbohydrate analysis were collected 3 h into the light period. Under NH conditions, both wheat cultivars preferentially accumulated Suc (Fig. 6A), whereas both rape cultivars accumulated starch and Suc equally (Fig. 6, A and D). Following cold hardening, winter wheat and winter rape increased Suc approximately 2- and 3-fold, respectively (Fig. 6A), and increased starch 1.6- and 1.7-fold, respectively (Fig. 6D). Spring wheat increased Suc 1.5-fold and increased starch 2-fold following cold hardening. However, CH spring rape showed no increase in Suc content and showed a 3-fold decrease in starch content (Fig. 6, A and D). This decrease in starch content for CH spring rape correlated with the measured reductions in photosynthesis (Fig. 2) and growth (Table I) for this cultivar.

Winter rape and winter wheat leaves also increased the pool sizes of Glc (4- and 2-fold, respectively) and Fru (6- and 2-fold, respectively) following cold hardening (Fig. 6, B and C). However, there were no changes in hexose content in spring wheat, and spring rape decreased the content of these simple sugars 2-fold (Fig. 6, B and C). The cumulative effect of these changes is that the soluble/insoluble carbohydrate ratios increased in both winter cultivars following cold hardening (3- and 1.3-fold for winter rape and winter wheat, respectively) (Fig. 6E). The large increase in winter rape was caused primarily by increases in Glc and Fru content. The increase in winter wheat was due primarily to the accumulation of Suc. There was also a small relative increase in soluble carbohydrates in spring rape due to the large decrease in starch content. However, spring wheat showed a relative decrease in soluble carbohydrates following cold hardening due to the accumulation of more starch, although the soluble/insoluble ratio remained high (Fig. 6E).

DISCUSSION

Winter and spring wheat and winter rape were shown to have similar growth rates following long-term growth at low temperature. These cultivars all had calculated Q_{10} values for total dry matter accumulation of between 2.1 (winter wheat) and 2.7 (winter rape). This suggests that the reductions in the growth rates at 5°C compared with 24°C were largely a consequence of thermodynamic limitations. This conclusion is supported by the similar growth coefficients under nonhardening and cold hardening conditions when growth was calculated based on K_T rather than real time (Table I). However, similar growth coefficients for biomass accumulation do not necessarily equate to similar allocation of resources (Chapin et al., 1990). Under cold-hardening conditions, spring wheats are taller and produce more leaf area than winter cultivars, with the consequence that the unit leaf rate (total dry matter per unit leaf area) is

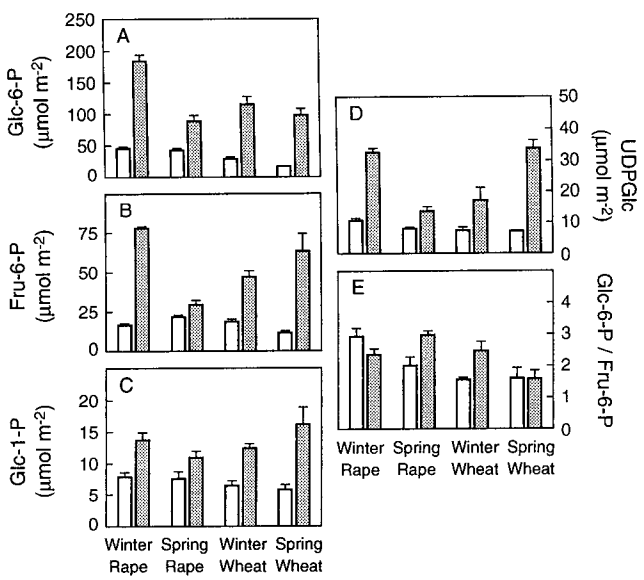

Figure 5. Pool sizes of intermediates of Suc synthesis extracted from NH (open bars) and CH (shaded bars) leaves of winter and spring wheat and rape. Three hours into the light period leaves (6 cm^2) were frozen in the light at the temperature of liquid N_2 using a freeze clamp. Each bar represents the mean (±SD) of three leaves.

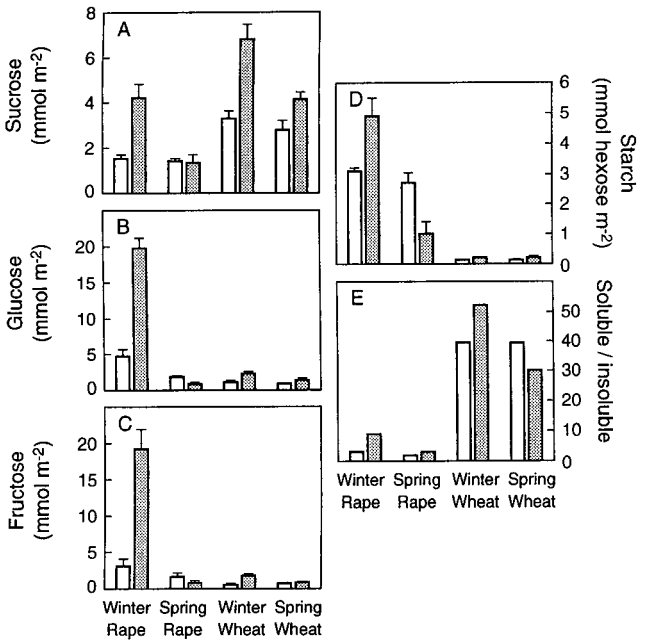

Figure 6. Carbohydrate content of NH (open bars) and CH (shaded bars) leaves of winter and spring wheat and rape. Three hours into the light period leaves (6 cm^2) were frozen in the light at the temperature of liquid N_2 using a freeze clamp. Each bar represents the mean (±SD) of three leaves.

1.5- to 2-fold higher for winter cultivars (Hurry, 1991). Similarly, the NAR of winter wheat was 1.5-fold and that of winter rape was 10-fold higher than the values of the respective spring cultivars (Fig. 1), showing greater apparent productivity of the leaves of winter cultivars. In accordance with the very low NAR, spring rape had a calculated Q_{10} for total dry matter accumulation of 4.8 and was clearly unable to acclimatize to the lower growth temperature.

Correlated with the ability of the two winter cultivars and spring wheat to continue active growth was the ability to sustain high rates of photosynthesis at the low growth temperature. This was associated with increases in extractable activity of enzymes of the photosynthetic carbon reduction cycle and of Suc synthesis and in the ability of CH leaves to sustain large pools of phosphorylated intermediates. In particular, the activity of both sFru-1,6-BPase and cFru-1,6-BPase increased about 2-fold per unit of protein for both winter cultivars following cold hardening, but there were no increases in either spring cultivar (Table II). Spring rape showed a pronounced inability to regenerate RuBP such that the RuBP/PGA ratio was lower in CH leaves. This was not the result of reduced availability of either ATP or NADPH because the PGA/DHAP ratio was similar in NH and CH leaves, indicating a ready flow of fixed carbon through to triose phosphates. Similarly, the DHAP/RuBP ratio and Fru-1,6-bisP pools are higher in CH leaves, showing a relative accumulation of triose- and hexose-phosphates. The Chl *a* fluorescence data for spring rape also supports the conclusion that neither ATP nor NADPH is limiting carbon fixation in spring rape leaves. In spring rape there was a pronounced over-reduction of the Q_A pool at very low irradiances, which is indicative of inhibited electron transport and a slow rate of consumption of NADPH (Fig. 3). Thus, given that the winter rape cultivar increased the activity of sFru-1,6-BPase 6-fold, it may be the failure of the spring cultivar to increase the activity of this enzyme that is one critical failure in cold acclimation. Furthermore, experiments with tomato have also shown that the activation energy for sFru-1,6-BPase decreases following long-term chilling and that this occurs to a greater extent in chilling-tolerant, high-altitude lines (Brüggemann et al., 1994). Similar responses to cold hardening have been reported for Rubisco from winter rye (Huner and Macdowall, 1979) and spinach (Grafflage and Krause, 1993). That there are also possible differences between spring and winter cultivars in the kinetics of these enzymes at the low temperature, in addition to levels of extractable activity, is a possibility that remains to be examined.

Previous studies using transgenic tobacco have shown that, under conditions that lead to carbohydrate accumulation in leaves, photosynthesis is inhibited by the loss of photosynthetic carbon reduction cycle enzymes (Stitt et al., 1990; von Schaewen et al., 1990) and expression of both Rubisco small subunit and Chl *a/b*-binding protein mRNA decrease (Krapp et al., 1993). Similar results have been shown for spinach leaves fed Glc via the transpiration stream (Krapp et al., 1991) and subjected to cold girdling (Krapp and Stitt, 1995). However, recent studies with transgenic tomato expressing elevated levels of SPS and accumulating high Suc levels, a situation analogous to that of cold-acclimated leaves of winter cultivars, showed that these plants increased photosynthesis and maintained equivalent levels of extractable Rubisco activity compared with control plants (Galtier et al., 1993). Transgenic potato expressing elevated invertase or pyrophosphatase activity (Jelitto et al., 1992; Sonnewald, 1992) also accumulated high concentrations of soluble carbohydrates without evidence of a significant feedback effect on photosynthesis. Rather, potato initiated many side shoots and increased tuber numbers. Tobacco similarly transformed to express elevated invertase or pyrophosphatase activity increased soluble carbohydrate content but showed a strong down-regulation of photosynthetic activity and of photosynthetic enzymes (Sonnewald et al., 1991; Jelitto et al., 1992). These findings have led to the proposal that the regulatory role of soluble carbohydrates may vary with species depending on their genetic capacity to modulate sink strengths (Stitt and Schulze, 1994).

In tobacco, feedback regulation of photosynthesis was associated with a characteristic patchy loss of Chl as the leaves aged and went through the transition from sink to source leaves (von Schaewen et al., 1990). In spring rape, cold hardening was also associated with the patchy loss of Chl and in reduced photosynthetic capacity. This occurred not only in leaves that first expanded at the warm temperature and were then transferred to the low growth temperature but also in those that developed at the low temperature. In contrast, winter rape leaves that developed at CH temperatures increased Chl and soluble carbohydrate content, and increased extractable enzyme activity and photo-

synthetic capacity. It has recently been reported that both Rubisco small subunit and Chl *a/b*-binding protein expression decrease in leaves of winter rape moved to cold-hardening conditions but increase in young leaves expanding at the low temperature (Singh and Johnson-Flanagan, 1994). These data indicate a developmental requirement to the cold acclimation of hardy cultivars that may be associated with overcoming the regulatory effects of high levels of soluble carbohydrates. This developmental capacity may be lacking in the spring cultivars and it may be related to a lack of a capacity to modulate resource allocation in response to low growth temperature. As yet few data are available to show what happens to gene expression in cereals during cold hardening. However, the data presented for wheat, and previous studies with winter rye (Hurry et al., 1994, 1995a), suggest that the expression of genes coding for several photosynthetic enzymes and Chl *a/b*-binding proteins would also be enhanced during cold hardening and that there may be differences between winter and spring cultivars.

These experiments have shown that following long-term cold hardening of winter and spring cultivars of wheat and rape, winter cultivars had higher net assimilation rates and higher photosynthetic rates than the respective spring cultivars. This is accomplished by increasing the extractable activity and activation states of photosynthetic and carbon metabolism enzymes and by increasing the capacity of the leaf to generate and sustain large pools of phosphorylated intermediates. For spring rape the loss of seedling vigor during long-term growth at low temperature was sufficient to lead to occasional seedling death. This increase in photosynthetic capacity and in photosynthetic enzyme content in winter cultivars occurs despite increases in soluble carbohydrate content. This result is in contrast to earlier reports from tobacco and spinach that showed that the accumulation of large cytosolic pools of soluble carbohydrate led to the down-regulation of photosynthetic enzymes (Stitt et al., 1990; von Schaewen et al., 1990; Krapp et al., 1991, 1993; Krapp and Stitt, 1995). The data reported here point to differences in the regulation of photosynthetic enzymes between both species and winter and spring cultivars in response to cold hardening and soluble carbohydrate accumulation. They also suggest that during selection for cold hardiness, photosynthetic tolerance to high soluble carbohydrate pools has been co-selected, enabling these cultivars to sustain large pools of sugars with possible cryoprotective functions.

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