

Acclimation of Arabidopsis Leaves Developing at Low Temperatures. Increasing Cytoplasmic Volume Accompanies Increased Activities of Enzymes in the Calvin Cycle and in the Sucrose-Biosynthesis Pathway¹

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Photosynthetic and metabolic acclimation to low growth temperatures were studied in *Arabidopsis* (Heynh.). Plants were grown at 23°C and then shifted to 5°C. We compared the leaves shifted to 5°C for 10 d and the new leaves developed at 5°C with the control leaves on plants that had been left at 23°C. Leaf development at 5°C resulted in the recovery of photosynthesis to rates comparable with those achieved by control leaves at 23°C. There was a shift in the partitioning of carbon from starch and toward sucrose (Suc) in leaves that developed at 5°C. The recovery of photosynthetic capacity and the redirection of carbon to Suc in these leaves were associated with coordinated increases in the activity of several Calvin-cycle enzymes, even larger increases in the activity of key enzymes for Suc biosynthesis, and an increase in the phosphate available for metabolism. Development of leaves at 5°C also led to an increase in cytoplasmic volume and a decrease in vacuolar volume, which may provide an important mechanism for increasing the enzymes and metabolites in cold-acclimated leaves. Understanding the mechanisms underlying such structural changes during leaf development in the cold could result in novel approaches to increasing plant yield.

A rapid shift from warm growth conditions to chilling temperatures (typically between 5°C and 10°C) leads to a pronounced inhibition of photosynthetic carbon fixation. In chilling-sensitive C₃ plants such as tomato and bean, this inhibition is associated with the inactivation of regulatory thioredoxin-activated enzymes of the Calvin cycle (Sassenrath and Ort, 1990; Sassenrath et al., 1991; Holaday et al., 1992; Brüggemann et al., 1994). Because the shift to low temperature is also associated with a strong activation of NADP-malate dehydrogenase in bean (Holaday et al., 1992), it is not due to a chill-induced lack of stromal reduc-

ing equivalents. In tomato the chill-induced reduction in photosynthesis is also associated with irreversible inactivation of Rubisco and loss of Rubisco protein (Brüggemann et al., 1992). Low temperature leads to a selective inhibition of end-product synthesis. This results in an accumulation of phosphorylated metabolites and a short-term phosphate limitation of photosynthesis (Leegood and Furbank, 1986; Sharkey et al., 1986) that may be partly due to the temperature-dependent changes in the affinity of cFBPase for its effectors, especially AMP (Stitt and Grosse, 1988).

Chilling inhibits phloem export, leading to a rapid accumulation of soluble sugars and repressing photosynthetic gene expression (Krapp and Stitt, 1995); Strand et al. (1997) showed this effect recently for *Arabidopsis* during short-term exposure to low temperatures. The inhibition of carbon fixation by low temperature may be followed by photoinhibition (Somersalo and Krause, 1989; Hurry and Huner, 1992). Chilling also interrupts the circadian-regulated transcription of several nuclear-encoded photosynthetic genes in both chilling-sensitive tomato (Martino-Catt and Ort, 1992) and chilling-tolerant *Arabidopsis* (Kreps and Simon, 1997). These data show that short-term exposure of chilling-sensitive and -tolerant species to low temperatures results in feedback-mediated down-regulation of photosynthesis and photosynthetic gene expression, perturbations to circadian-regulated processes, and, in some instances, irreversible damage to photosynthetic proteins.

In contrast, when leaves of cold-hardy herbaceous plants develop at low growth temperatures (5°C), they show a remarkable recovery of photosynthetic capacity. Even in the short term, low temperatures lead to an increase in the activation state of the Calvin-cycle enzymes in chilling-tolerant C₃ plants such as spinach, winter rye, and winter oilseed rape (Holaday et al., 1992; Hurry et al., 1994, 1995b), which is the opposite of the response in cold-sensitive species (see above). When *Arabidopsis* leaves develop at low temperature, they no longer show the suppression of

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Abbreviations: cFBPase, cytosolic Fru-1,6-bisphosphatase; GAPDH, NADP-glyceraldehyde-P dehydrogenase; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; SPS, Suc-6-P synthase.

transcript levels for photosynthetic proteins that is found after shifting warm-grown leaves to low temperatures (Strand et al., 1997). As a result, leaves that have developed at low temperature contain higher activities of selected enzymes of the photosynthetic carbon-reduction cycle (Hurry et al., 1994, 1995b). This reversal of the sink regulation of photosynthetic gene expression (Jang and Sheen, 1994; Jang et al., 1997) occurs even though these leaves produce and maintain large pools of soluble sugars.

Leaves of *Arabidopsis* that develop at 5°C greatly increase their expression of SPS and cFBPase in the Suc-synthesis pathway in the cytosol but not in the starch-synthesis pathway in the plastid (Strand et al., 1997). Marked increases in the SPS activity have also been reported for spinach (Guy et al., 1992; Holaday et al., 1992), for winter cultivars of oilseed rape and wheat (Hurry et al., 1995b), and rye (Hurry et al., 1994) after prolonged exposure to cold. The increased expression and activity of Suc-synthesis enzymes correlates with an accumulation of sugars in leaves at low temperature. It has been proposed that high sugar levels might be important for cryoprotection (Santarius, 1982; Anchordoguy et al., 1987; Carpenter and Crowe, 1988). However, there is no direct evidence that the increased sugar levels are due to increased synthesis and not just to a passive response to the inhibition of growth and phloem transport. It is also not clear whether the increased activities of the enzymes leading to Suc merely counteract the direct inhibitory effect of low temperature (see above) or whether they actually lead to increased Suc synthesis.

Another marked short-term effect found after shifting warm-grown plants to low temperatures is the accumulation of large pools of phosphorylated intermediates (Labate and Leegood, 1988; Hurry et al., 1994; Strand et al., 1997), thought to reflect the inhibition of end-product synthesis at low temperatures (see above). It has been suggested that this accumulation of phosphorylated intermediates contributes to the feedback down-regulation of photosynthesis, because the cytosolic and chloroplastic pools of Pi become depleted to the point where photophosphorylation is Pi limited (Herold, 1980; Mächler et al., 1984; Leegood and Furbank, 1986; Labate and Leegood, 1988; Stitt and Grosse, 1988). Although this may be true for leaves exposed to short-term (minutes to hours) drops in temperature, it is unclear whether Pi limitation persists in the long term (days) in plants acclimating to low temperature. The subcellular compartmentation of Pi is tightly regulated, and 85% to 95% of the Pi may be located in the vacuole in well-fertilized plants (Bieleski and Ferguson, 1983). Prolonged exposure to low-temperature-induced Pi deficiency might therefore be buffered by the release of Pi from the vacuole (Woodrow et al., 1984; Mimura et al., 1990), as occurs when choline feeding depletes Pi in the cytosol (Bligny et al., 1990). Reports that cold-acclimated plants show damping of oscillations in O₂ evolution under conditions of light- and CO₂-saturated photosynthesis (Hurry et al., 1993) indicate a repositing of the cytosolic Pi pool after cold development of leaves. This conclusion is supported by data showing an increase, rather than a decrease, in the ATP/ADP ratio in cold-developed leaves (Sobczyk

et al., 1985; Hurry et al., 1995c; Strand et al., 1997). The role of Pi in acclimation to the cold and in the shift from starch to soluble carbohydrate accumulation therefore remains unclear.

Studies of *Arabidopsis* and other cold-tolerant herbaceous plants indicate that growth at low temperature leads to a developmental reprogramming of carbon metabolism that involves changes in gene expression and enzyme activity and possibly also changes of phosphate compartmentation. The resulting recovery of the photosynthetic rate and the preferential accumulation of soluble sugars could be an essential element for acclimation to low growth temperatures. Most previous studies have concentrated on a single or a small number of enzymes, however, and have not demonstrated that the changes in expression and activity actually lead to significant increases in the rate of photosynthesis and Suc synthesis. Furthermore, investigations of metabolism have not yet been integrated with other effects of low temperature on leaf development. Older studies of winter rye leaf development at low growth temperatures reported increases in cell size and cytoplasmic content, smaller vacuoles, and multivacuolated cells (Huner et al., 1981, 1984; Griffith et al., 1985). Ristic and Ashworth (1993) have shown that *Arabidopsis* parenchyma cells also undergo ultrastructural changes after transfer to low temperatures in continuous light, leading to multiple invaginations of the plasma membrane, and the accumulation of microvesicles associated with the plasma membrane, tonoplast, chloroplast envelope, and mitochondrial outer membrane.

In this study we investigated whether (a) the recovery of photosynthesis in the cold is associated with coordinate changes in the activity of several Calvin-cycle enzymes; (b) these changes plus the up-regulation of the cytosolic pathway for Suc synthesis reported previously for *Arabidopsis* after growth and development at low temperature (Strand et al., 1997) result in an increased flux into Suc; and (c) the Pi status in the cytoplasm is also adjusted during acclimation to low growth temperatures. Finally, we assessed whether there is a link between metabolic adaptations and anatomical changes after leaf development at low temperatures.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Heynh.) ecotype Colombia seeds were sown in 5-cm square plastic pots containing a mixture of peat moss:washed river sand:perlite (2:1:1, v/v) and germinated under controlled-environment conditions: 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, supplied by a mixture of incandescent and cool-white fluorescent lights; a day/night temperature regime of 23°C/18°C; and an 8-h photoperiod. Approximately 21 d after sowing, seedlings were transplanted to a final density of one seedling per pot and given one-half-strength nutrient solution twice weekly and water as required (Hurry et al., 1994). Approximately 28 d later, when the leaves had developed into fully mature source leaves, the plants were shifted to 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, a day/night temperature regime of 5°C/5°C, and an

8-h photoperiod. Leaves were sampled after 10 d at 5°C, and again approximately 40 d after the shift, when a new rosette of leaves had fully developed.

Chlorophyll and Protein Content and Specific Leaf Weight

Chlorophyll was determined in 80% buffered acetone (Porra et al., 1989) and total protein according to the method of Bradford (1976). We determined the specific leaf fresh weight, specific leaf dry weight, and water content by drying leaf discs to a constant weight at 80°C.

Enzyme Activities

Two hours into the photoperiod, leaf material was frozen in light at the temperature of liquid N₂ and ground to a fine powder. To assay the activity of SPS and cFBPase, 200 to 300 mg of powdered, frozen leaf tissue was homogenized in 2 volumes of buffer (50 mM Mops-KOH, pH 7.4, 12 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 mM ϵ -aminocaproic acid, 1 mM DTT, 0.1% [v/v] Triton X-100, and 10 mg of polyvinylpyrrolidone) and then centrifuged at 11,000g for 5 min. The supernatant was then collected and 500 μ L was spin-desalted on a mini-column filled with 5 mL of Sephadex G-25 (Pharmacia), pre-equilibrated with 50 mM Mops-KOH, pH 7.4, 12 mM MgCl₂, and 1 mM DTT. We immediately assayed the SPS activity (Hill et al., 1996) and quantified the Suc produced using the anthrone reagent (Huber et al., 1989). The remaining desalted extract was frozen in liquid N₂ and later used to assay for cFBPase activity (Hurry et al., 1995a).

To assay the activity of the Calvin-cycle enzymes, 25 mg of frozen tissue was homogenized in 500 μ L of extraction buffer (50 Hepes-KOH, pH 7.4, 12 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 mM ϵ -aminocaproic acid, 10 μ M leupeptin, 2 mM DTT, 0.1% [v/v] Triton X-100, 10 mg of polyvinylpyrrolidone, 5 mM KHCO₃ [for Rubisco only], 1 mM Fru-1,6-bisP [for stromal FBPase only], and 10% [v/v] glycerol) and then centrifuged at 11,000g for 5 min. We collected the supernatant and immediately assayed enzymatic activity (Haake et al., 1998).

Photosynthetic Carbon Flux

We cut leaf discs from the leaves 2 h into the photoperiod and determined photosynthetic carbon flux at both 23°C and 5°C by incubating the leaf discs in a cuvette (model LD-2, Hansatech, Kings Lynn, Norfolk, UK) at a saturating irradiance of 650 μ mol m⁻² s⁻¹ at 5% CO₂, containing 4 μ Ci ¹⁴C₂O₂. The pulse times were 20 min at 23°C and 40 min at 5°C. We analyzed the incorporation of ¹⁴C into the soluble fractions and starch as described in Kruckenberg et al. (1989). The short incubation was to avoid complications due to photoinhibition.

Soluble Sugars and Starch

At various times during the photoperiod, leaf material was frozen in the light in liquid N₂. We measured Suc, Glc,

Fru, and starch in the soluble and residual fractions of ethanol-water extracts. Samples were ground to powder in liquid N₂ and extracted in 80% ethanol containing 4 mM Hepes-KOH, pH 7.5, at 80°C for 30 min. Samples were then centrifuged for 15 min at 11,000g; the supernatant was decanted and stored on ice; the pellet was resuspended in 80% ethanol-Hepes, pH 7.5, and put on the heat block again for 30 min. We repeated this hot extraction twice, once with 50% ethanol-Hepes, pH 7.5, and once with only 4 mM Hepes, pH 7.5; the supernatants were then combined and assayed for soluble sugars (Stitt et al., 1989).

For starch extraction, we added 0.5 mL of distilled water to resuspend the pellet and autoclaved the samples for 3 h. For starch cleavage we added a 50- μ L aliquot of the autoclaved suspension to 450 μ L of 50 mM sodium-acetate incubation buffer, pH 4.8, containing 28 units of amyloglucosidase and 36 units of α -amylase, and we incubated the samples at 37°C for 16 h. We then centrifuged the samples for 15 min at 11,000g and assayed the supernatant for Glc (Stitt et al., 1989).

Pi Determination

Two hours into the photoperiod, we collected the leaf samples by freezing them in the light at the temperature of liquid N₂. The frozen material was ground to a fine powder at the same temperature and extracted in 3% (v/v) HClO₄. We centrifuged the samples for 5 min at 11,000g and used the supernatant to assay total leaf Pi (Tausky and Shorr, 1953). The "metabolic" Pi pool was estimated by feeding 200 mM Glc into the leaf through the petiole at 23°C with 150 μ mol photons m⁻² s⁻¹ for 30 min (Morcuende et al., 1998). We measured the increases in Glc-6-P and Fru-6-P spectrophotometrically (Stitt et al., 1989) and then determined their chlorophyll content as pheophytin (Vernon, 1960).

Transmission Electron Microscopy

We harvested the control 23°C leaves, the 23°C/5°C leaves, and the 5°C leaves 2 h into the photoperiod and submerged them in 20 mM phosphate buffer, pH 7.0, containing 0.3 M Suc (for the 23°C and 23°C/5°C leaves) or 0.6 M Suc (for the 5°C leaves). We cut samples (about 1 mm²) with a new scalpel blade and fixed them with 4% glutaraldehyde in the buffer/Suc solutions for 2 h at room temperature and then postfixed them in 2% osmium tetroxide in the same buffers for another 1 h at room temperature. The samples were rinsed twice in 20 mM phosphate buffer, pH 7.0, and then dehydrated using 20- to 30-min steps in a graded series of acetone (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and two changes of 100%). We transferred the dehydrated samples to a mixture of Spurr's embedding medium (Spurr, 1969) and 100% acetone (1:1, v/v) for 12 h at room temperature, with the sample vials clipped onto a rotating mixing wheel. We replaced the acetone/Spurr's medium mixture with pure Spurr's medium and incubated the samples in the mixer for 48 h at room temperature. We transferred the samples to 65°C for 8 to 10 h to complete the embedding. Silver-gold thin sections of the tissue were

cut with a diamond knife on an ultramicrotome (model MT2-B, Sorvall) and collected on either 400-mesh nickel grids or carbon-coated Formvar 200-mesh hexose high-transmission grids. The sections were stained with 2% aqueous uranyl acetate and lead citrate before we viewed them through an electron microscope (model CM10, Philips, Eindhoven, The Netherlands), operating at 80 kV.

RESULTS

Calvin-Cycle Enzymes

Arabidopsis plants were grown at 23°C for approximately 49 d and then transferred to 5°C. We examined Calvin-cycle enzyme activities in leaves from plants at 23°C; after 10 d at 5°C; in leaves that had already matured before the plants were transferred to low temperature (23°C/5°C); and in new leaves after about 40 d of development at 5°C. In Figure 1 enzyme activities are shown in relation to chlorophyll. Chlorophyll increased by 35% on a fresh-weight basis and by 80% on a leaf-area basis in leaves that had developed at low temperature (see below). The following increases in enzyme activities in cold-acclimated leaves would therefore be even larger if they had been expressed on a fresh-weight or leaf-area basis.

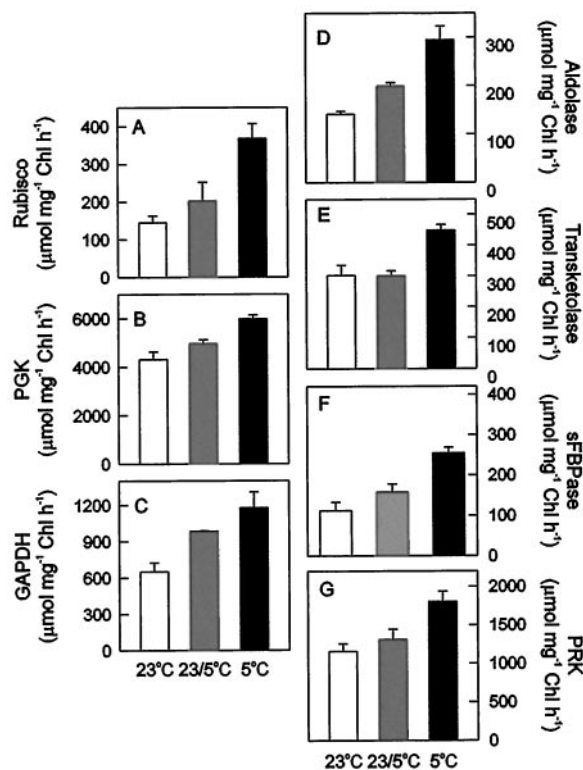


Figure 1. Activity of the Calvin-cycle enzymes, Rubisco, PGK, GAPDH, aldolase, transketolase, stromal FBPAse (sFBPase), and PRK, per unit of chlorophyll (Chl) in 23°C leaves (open bars), 23°C/5°C leaves (shaded bars), and 5°C leaves (black bars). Leaves were collected 2 h into the photoperiod and frozen at the temperature of liquid N₂. Each value represents the mean ± SD of three different leaves from three different plants.

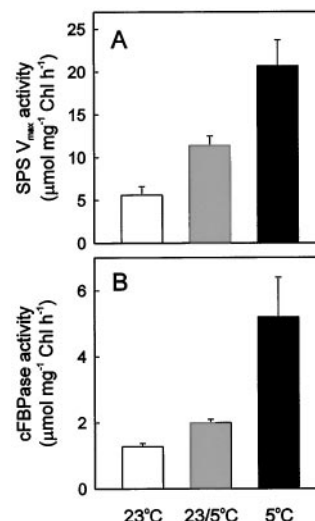


Figure 2. Activity per unit of chlorophyll (Chl) of two cytosolic enzymes, SPS (A) and cFBPase (B), for 23°C leaves (open bars), 23°C/5°C leaves (shaded bars), and 5°C leaves (black bars). Leaves were collected 2 h into the photoperiod and frozen at the temperature of liquid N₂. Each value represents the mean ± SD of three different leaves from three different plants.

Of the seven Calvin-cycle enzymes measured, only Rubisco (Fig. 1A), GAPDH (Fig. 1C), aldolase (Fig. 1D), and possibly stromal FBPAse (Fig. 1F) showed substantial increases in total extractable activity in the 23°C/5°C leaves. In contrast, 5°C leaves showed significant increases in the extractable activity of all seven enzymes, with Rubisco (2.5-fold), aldolase (2.1-fold), and, to a lesser degree, GAPDH (1.8-fold) and stromal FBPAse (1.9-fold) showing the largest increases. The remaining enzymes, PGK (Fig. 1B), transketolase (Fig. 1E), and PRK (Fig. 1G), all showed approximately 1.5-fold increases in activity per unit of chlorophyll when 5°C leaves were compared with control 23°C leaves. Although increases in Rubisco and stromal FBPAse activity at 5°C have been reported previously for spinach (Holaday et al., 1992), winter rye (Hurry et al., 1994), and winter oilseed rape (Hurry et al., 1995b), our data demonstrate that the activities of all of the Calvin-cycle enzymes, including nonregulated enzymes such as aldolase, increased significantly in the cold.

Cytosolic Enzyme Activities

We previously documented a gradual up-regulation of the transcripts for SPS and cFBPase, the two cytosolic enzymes that are needed for Suc synthesis when Arabidopsis plants are shifted to 5°C (Strand et al., 1997). The present experiments show that there is an accompanying increase in activity, with both cFBPase and SPS activity increasing by 3- to 4-fold in 5°C leaves (Fig. 2). The increases were larger in 5°C leaves than in 23°C/5°C leaves. The increases were also larger than for any of the Calvin-cycle enzymes (compare Figs. 1 and 2).

Calvin-Cycle Enzyme Activities Increased Because of a General Increase of Protein

Compared with the control 23°C leaves, the 23°C/5°C leaves showed a slight decrease in water content (see also Lång et al., 1994), a slight increase in the specific leaf fresh weight and the specific leaf dry weight, a small (12%) increase in leaf protein, and no change in chlorophyll (Table I). In contrast, leaf development at 5°C resulted in a marked reduction of leaf water content from 91% to 78%, a 40% increase in the specific leaf fresh weight, a 3-fold increase in the specific leaf dry weight, a 2.5-fold increase in total leaf protein, and a 35% increase in chlorophyll, relative to the control 23°C leaves (Table I). When chlorophyll and protein were expressed on a leaf-area basis, 5°C leaves contained 80% more chlorophyll than 23°C leaves (0.054 compared with 0.029 mg chlorophyll cm⁻²) and about 4-fold more protein (0.57 compared with 0.15 mg protein cm⁻²), calculated from Table I. The increase in protein is a major contributor to the increase in specific leaf dry weight.

We recalculated the enzyme activities shown in Figures 1 and 2 on a protein basis (Table II). There were only minor changes in the activities of Rubisco, PGK, GAPDH, aldolase, stromal FBPase, transketolase, and PRK among 23°C leaves, 23°C/5°C leaves, and 5°C leaves when the activity was related to total leaf protein. The increased activity of the Calvin-cycle enzymes on a chlorophyll, fresh-weight, or leaf-area basis was therefore mainly due to the general increase in leaf protein.

SPS activity still showed a 1.8-fold increase in activity on a protein basis, which was already evident in the 23°C/5°C leaves (Table II). cFBPase showed a 1.2-fold increase in the 23°C/5°C leaves, but the biggest increase was in the 5°C leaves (1.7-fold). The increased activity of the Suc-biosynthesis enzymes at low temperatures is therefore attributable to two processes: (a) a selective increase in the expression and activity of the enzymes for Suc synthesis that also occurs when preformed leaves are transferred to low temperature and (b) a further increase, linked to a general increase in protein, in the leaves that developed at low temperature.

Photosynthetic Carbon Flux

Although higher enzyme activities were observed in the 5°C leaves under optimal assay conditions at 25°C, this does not provide direct evidence for enhanced carbon fixation at low temperature. To provide this evidence, photosynthetic carbon flux was investigated as ¹⁴C incorporation

Table II. Changes in Calvin-cycle and Suc-biosynthesis enzyme activities expressed on a protein basis in 23°C leaves, 23°C/5°C leaves, and 5°C leaves

These activities were recalculated from the data shown in Figures 1 and 2 and Table I.

Enzyme	23°C	23°C/5°C	5°C
	$\mu\text{mol mg}^{-1} \text{protein h}^{-1}$		
Rubisco	29.4	36.5	35.3
PGK	875.5	893.7	574.0
GAPDH	132.0	177.1	113.1
Aldolase	28.6	35.8	28.3
Stromal FBPase	25.8	26.5	23.4
Transketolase	61.5	54.4	43.2
PRK	234.7	235.8	173.2
cFBPase	0.30	0.36	0.50
SPS	1.14	2.05	1.99

by leaf discs provided with CO₂ from labeled bicarbonate solutions in a leaf disc electrode. We determined the flux at both 23°C and 5°C, using incubation times of 20 and 40 min, respectively.

When photosynthesis was measured at 23°C, no differences were found in ¹⁴C incorporation between the control 23°C and the 23°C/5°C leaves; the 5°C leaves showed a 1.5-fold higher rate of ¹⁴CO₂ incorporation at 23°C (Fig. 3A). When similar measurements were made at 5°C, the control 23°C leaves had very low rates of ¹⁴C incorporation, the 23°C/5°C leaves showed a slight enhancement of ¹⁴C incorporation, and the 5°C leaves showed a dramatic 5-fold increase in photosynthetic carbon fixation (Fig. 3B). The rate of ¹⁴C incorporation in the 5°C leaves at 5°C was similar to that in the 23°C or the 23°C/5°C leaves at 23°C. These data demonstrate that the enhancements in enzymatic capacity translated into an almost complete recovery of photosynthetic carbon flux at the lower growth temperature.

These experiments also provided an opportunity to assess whether leaf development at 5°C alters the partitioning of newly fixed carbon. When photosynthesis was carried out at 23°C, the control 23°C leaves allocated slightly more than 50% of their newly fixed carbon into starch (Fig. 3A). This proportion remained at a similarly high level (approximately 45%) in the 23°C/5°C leaves but decreased to less than 30% in the 5°C leaves (Fig. 3B). This was not simply an effect of treatment temperature because the reduced partitioning into starch on a percentage basis was similar irrespective of when the 5°C-labeling experiment was carried out. Of the three soluble fractions, the newly fixed carbon was primarily partitioned into the neutral fraction, representing soluble carbohydrates. In the control

Table I. Physical characteristics of 23°C leaves, 23°C/5°C leaves, and 5°C leaves

Values represent the means \pm SD for three leaves collected from three different plants.

Parameter	23°C	23°C/5°C	5°C
Water content (%)	91 \pm 0.4	86 \pm 1.0	78 \pm 0.2
Specific leaf fresh wt (mg cm ⁻²)	22.8 \pm 0.5	24.3 \pm 0.6	31.9 \pm 0.5
Specific leaf dry wt (mg cm ⁻²)	2.1 \pm 0.1	3.3 \pm 0.1	7.1 \pm 0.3
Protein (mg g ⁻¹ fresh wt)	6.4 \pm 0.5	7.2 \pm 0.4	17.8 \pm 0.4
Chlorophyll (mg g ⁻¹ fresh wt)	1.3 \pm 0.2	1.3 \pm 0.1	1.7 \pm 0.1

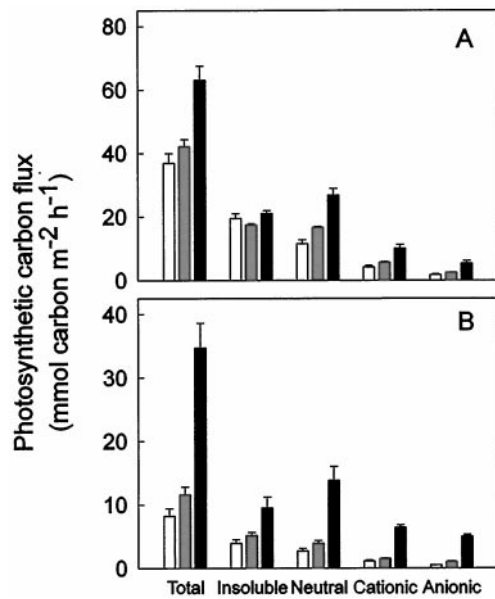


Figure 3. Photosynthetic carbon flux measured as ¹⁴C incorporation after illumination in a leaf disc electrode for 20 and 40 min at 23°C (A) and 5°C (B), respectively. The carbon flux was measured for 23°C leaves (open bars), 23°C/5°C leaves (shaded bars), and 5°C leaves (black bars). Leaves were collected 2 h into the photoperiod. Each bar represents the mean \pm SD of at least three different incubations. Insoluble, Starch; Neutral, sugars; Cationic, amino acids; Anionic, organic acids and phosphorylated intermediates.

(23°C) leaves, approximately 30% of the labeled carbon went into the neutral fraction, whereas in the 5°C leaves it increased to 40% (Fig. 3). There was also a small relative increase in partitioning into the cationic (amino acid) fraction in the 5°C leaves (18% versus 11% for the 23°C leaves) and a relatively larger increase of flux into the anionic fraction (phosphorylated intermediates and organic acids) in the 5°C leaves (14% versus 5% for the 23°C leaves). Therefore, the primary effect of leaf development at 5°C is to restore a high overall rate of photosynthesis, but there is also a shift of emphasis in partitioning, away from starch and toward soluble compounds including sugars.

Starch and Soluble Sugar Content

To provide more evidence for the increased synthesis of sugars in low-temperature-acclimated leaves, we investigated the diurnal turnover of leaf carbohydrates. Under warm growth conditions *Arabidopsis* leaves preferentially accumulated large amounts of starch during the light period (Fig. 4A) and contained a relatively small pool of Suc (Fig. 4B) and minimal pools of the reducing sugars Glc (Fig. 4C) and Fru (Fig. 4D). After 10 d at 5°C, the 23°C/5°C leaves contained large, relatively stable pools of all three soluble carbohydrates, particularly Glc and Fru. They also maintained a large and stable starch pool, which was not remobilized during the night. These data indicate that, although the 23°C/5°C leaves contained high carbohydrate pools at 5°C, there was actually little synthesis during the day and little remobilization from these pools during the

long (16-h), dark period. This correlates with the lack of any substantive recovery of photosynthesis in these leaves at 5°C (Fig. 3) and with the continued suppression of photosynthetic gene expression (Strand et al., 1997). The high levels of sugars and starch in these 23°C/5°C leaves may be due at least partly to decreased export, but this remains to be investigated.

In contrast, the 5°C leaves contained a large Suc pool that showed marked diurnal changes. Large amounts of Suc accumulated throughout the day and a substantial amount was mobilized after the end of the day. A significant pool was nevertheless maintained throughout the dark period. The cold-developed 5°C leaves also contained substantial pools of reducing sugars, although it is noteworthy that they were smaller than in the 23°C/5°C leaves and that they were relatively stable throughout the photoperiod. The starch pool in the 5°C leaves built up during the day, although to a lesser extent than in the control 23°C leaves, and was remobilized during the dark period. These data support the conclusions drawn from Figure 3 that development at 5°C enabled *Arabidopsis* leaves to recover significant photosynthetic capacity and simultaneously allowed a marked shift toward partitioning into a large and active Suc pool.

Total Pi Pools and Available Pi

We determined the amount of Pi readily available for metabolism and the total Pi pools in the cytoplasm in samples collected 2 h into the photoperiod. Total Pi in the whole-leaf extracts did not change substantially in either the 23°C/5°C leaves or the 5°C leaves (Fig. 5A). The values reported here correspond with previous reports of *Arabidopsis* leaves grown under warm conditions (Poirier et al., 1991). We determined the metabolically accessible Pi pool by providing the leaves with 200 mM Glc for 30 min and

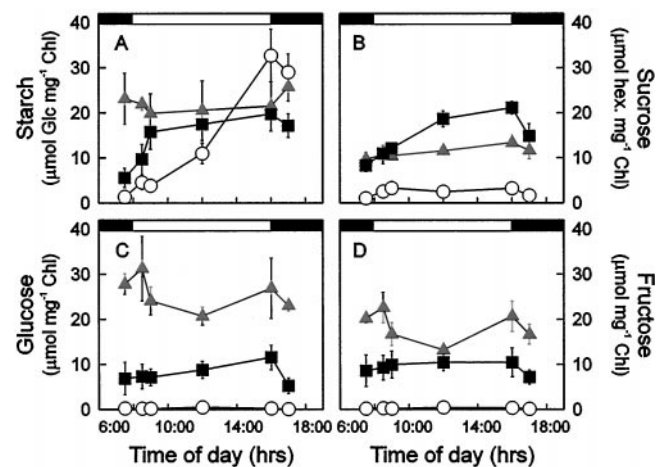


Figure 4. Carbohydrate content for 23°C leaves (○), 23°C/5°C leaves (△), and 5°C leaves (■). Samples were frozen in the light at various times during the photoperiod at the temperature of liquid N₂. Each point represents the mean \pm SD of three different leaves from three different plants. The bar at the top of the graphs indicates the times when the lights were off (black bars) or on (white bars). Chl, Chlorophyll; hex, hexose.

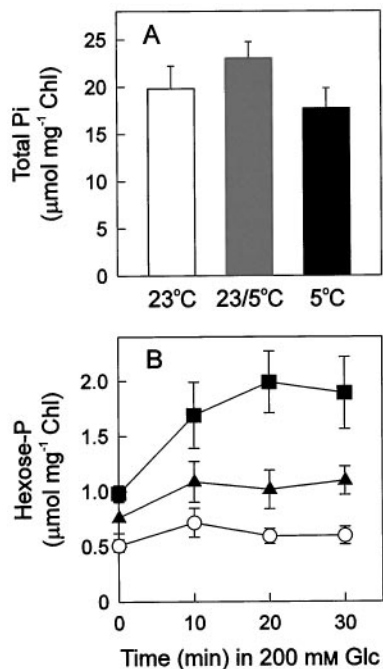


Figure 5. Total Pi content (A) and available Pi pools (B) in Arabidopsis leaves. Leaf samples for the determination of total Pi were collected 2 h into the photoperiod by freezing in light at the temperature of liquid N₂. To determine available Pi, 200 mM Glc was fed through the petiole in ambient light for 30 min. The increase in hexose-P was then determined. The figures show the results for 23°C leaves (open bars, ○), 23°C/5°C leaves (shaded bars, △), and 5°C leaves (black bars, ■). Each value represents the mean ± SD of at least three different leaves. Chl, Chlorophyll.

then measuring the overall level of phosphorylated hexoses (Morcuende et al., 1998). This approach provided only a qualitative picture of the changes in Pi in the cytoplasm, because (a) Pi is also incorporated into other compounds and (b) even in this time frame we cannot exclude the possibility that some Pi was drawn back from the vacuole. The level of hexose phosphates found after a short pulse

with 200 mM Glc increased 1.3-fold in the 23°C/5°C leaves and 3-fold in the 5°C leaves compared with the 23°C leaves. Thus, even though phosphorylated intermediates increased at low temperatures (Strand et al., 1997), Pi availability increased rather than decreased. This indicates that Pi was not likely to inhibit photophosphorylation and photosynthesis after long-term exposure to low temperature, in contrast to the effects of short-term exposure (see the introduction).

Leaf Anatomy

The control 23°C leaves of Arabidopsis contained mesophyll cells that exhibited a typical, large central vacuole surrounded by a thin layer of cytoplasm (arrow) (Fig. 6A). Figure 6B illustrates that control leaves also exhibited a normal tonoplast membrane and chloroplast morphology. Shifting plants to low temperature led to minimal changes in the amounts of cytoplasm observed in cross-sections (Fig. 6C, arrows) relative to controls, although enhanced staining of the tonoplast membranes was observed (Fig. 6D). In contrast, mesophyll cells in 5°C leaves showed an increase in both cytoplasmic volume and density (Fig. 6E, arrows). The increase in cytoplasmic volume was accompanied by a decrease in the relative contribution of the vacuole to total cell volume. Development at low temperature did not appear to affect either the staining of the tonoplast membranes or the chloroplast morphology (Fig. 6F).

This interpretation of the electron micrographs is supported by the large decrease in the water content of the leaves and by the observation that this decrease was accompanied by a marked increase of protein (Table I) but not of starch (Fig. 4).

DISCUSSION

Increased Photosynthetic Capacity and Reprogramming of Allocation in Cold-Acclimated Leaves

In the short-term, a rapid drop in leaf temperature leads to an inhibition of photosynthesis and Suc biosynthesis. For

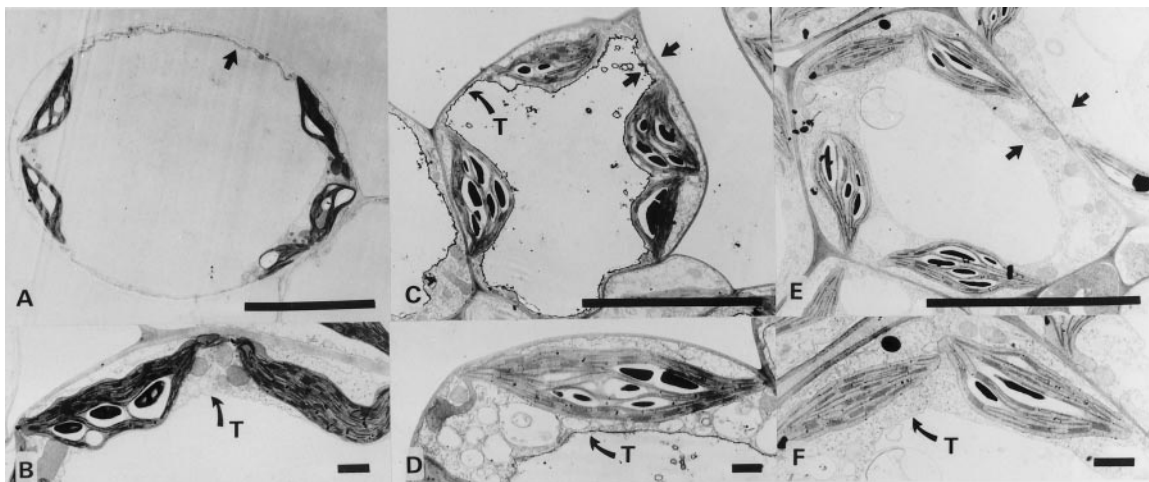


Figure 6. Electron micrographs of Arabidopsis mesophyll cells. A and B, 23°C leaves; C and D, 23°C/5°C leaves; E and F, 5°C leaves. Bars in A, C, and E = 1 μm; bars in B, D, and F = 0.1 μm. T, Tonoplast membrane.

herbaceous plants a reversal of this inhibition is likely to constitute an essential response for their acclimation to low temperatures and consequently for their winter survival.

Arabidopsis leaves that developed at 5°C showed a 5-fold increase in carbon fixation compared with the control 23°C leaves measured at 5°C. When the fluxes were compared at warm temperature (23°C), the 5°C leaves also possessed a higher capacity for photosynthetic carbon fixation than did the control leaves. Indeed, the fully acclimated 5°C leaves were able to maintain a flux of fixed carbon at 5°C that was similar to that maintained by the control leaves at 23°C. This increased capacity for photosynthetic carbon flux required a reprogramming of carbon metabolism (Strand et al., 1997). As a result, the Arabidopsis 5°C leaves were able to produce and accumulate large pools of soluble sugars without any associated suppression of photosynthetic gene expression or metabolism. As we discuss below, the metabolic changes resulting from leaf development at 5°C included changes in Pi allocation and in the activities of Calvin-cycle enzymes and enzymes for Suc synthesis.

Coordinate Increases in the Activities of Regulated and Nonregulated Calvin-Cycle Enzymes Stimulated Photosynthetic Carbon Fixation

Each of the seven Calvin-cycle enzymes that we investigated showed an increase in total activity in the 5°C leaves. The increase was not due to a specific increase of the activity of individual enzymes; rather, there was a general increase in the total leaf protein that resulted in an increase in the activities of all of the Calvin-cycle enzymes. These increases in Calvin-cycle enzyme activity and total leaf protein were very small in the 23°C/5°C leaves and much larger in the 5°C leaves. Strand et al. (1997) found that transcript levels for *rubS* were low in mature leaves after shifting to low temperature, whereas in leaves that developed at low temperature, the transcript levels recovered and resembled those in control leaves at 23°C. This is in agreement with the finding that the 5°C leaves showed a much more marked acclimation of enzyme activities than did the 23°C/5°C leaves and that acclimation was not only due to a specific increase in the expression of photosynthetic enzymes.

There were differences in the extent to which individual Calvin-cycle enzyme activities increased following acclimation to a lower growth temperature. Whether these differential responses resulted from variable sensitivities of the enzymes to low temperature or from other factors remains to be determined. The most pronounced increases in activity in the 5°C leaves were found for Rubisco (2.5-fold) and for the "nonregulated" enzyme, aldolase (2.1-fold). Rubisco activity has frequently been investigated and shown to increase in the cold in several species of monocots and dicots (Treharne and Eagles, 1970; Chabot et al., 1972; Holaday et al., 1992; Hurry et al., 1994, 1995b). Nonregulated enzymes such as aldolase, on the other hand, have been considered to be expressed in excess and to be unimportant for regulation processes such as acclimation. This view clearly requires re-evaluation; Haake et al. (1998)

showed that antisense down-regulation of aldolase in tobacco leaves resulted in a pronounced reduction in carbon fluxes in the Calvin cycle.

Coordinate Increase of the Activities of Enzymes for Suc Synthesis and Changes to Pi Compartmentation Stimulated Suc Synthesis

Previous studies have shown that there is a strong up-regulation of the transcripts and extractable activity for enzymes of the cytosolic pathway for sugar biosynthesis at low temperature, relative to the pathway for starch synthesis (Strand et al., 1997). The present study shows that SPS and cFBPase activity also increased more than Calvin-cycle enzyme activities. The increase probably contains two components: a specific increase in the activity of the enzymes for Suc biosynthesis that also occurs when leaves are shifted to 23°C and a nonspecific increase that is linked to a general increase in leaf protein and is found only in leaves that have developed at 5°C. In agreement with this idea, Strand et al. (1997) found that transcript levels for SPS and cFBPase rose when mature leaves were shifted to 5°C and in leaves that developed in the cold.

It has been suggested that the low rates of Pi release in end-product synthesis limit photophosphorylation at low temperatures (Leegood and Furbank, 1986; Sharkey et al., 1986). The increased activities of these cytosolic enzymes involved in Suc biosynthesis could counteract this limitation. Our results also indicate that changes in Pi compartmentation occurred during low-temperature acclimation. Although the total pool of Pi in the leaf cells did not change after cold exposure, the availability of Pi increased, indicating that more Pi was available for metabolism in the cytoplasm, especially in leaves that developed at low temperature. This may explain why Pi did not become limiting for photophosphorylation and photosynthesis, even though phosphorylated intermediates were present at much higher levels in the cold. This conclusion is supported by earlier studies of the effect of leaf development at low temperature on ATP-to-ADP ratios and oscillations in CO₂-saturated O₂ evolution and room-temperature chlorophyll fluorescence (Hurry et al., 1993, 1995c). Further studies may reveal how low temperatures trigger the release of Pi from the vacuole.

Previous investigators reported a significant increase in the soluble sugar-to-starch ratio in several species after leaf development in the cold (Hurry et al., 1995b; Strand et al., 1997). However, analyses of carbohydrate pools at a single time do not reveal whether the changes were due to increased synthesis or to inhibition of export. In this report we show that cold development of leaves led to a shift in carbon partitioning toward soluble sugar synthesis. Whereas in the control 23°C leaves, 50% of the incorporated [¹⁴C]CO₂ was incorporated into starch and only 30% into sugars; in the 5°C leaves, only 30% of the labeled carbon was found in starch and as much as 40% was converted to sugars. The diurnal changes in leaf carbohydrate levels provided independent evidence for a change in allocation. The 5°C leaves accumulated less starch during

the photoperiod than did the control 23°C leaves and instead maintained a large Suc pool that showed significant turnover during the diurnal period. Suc was not fully mobilized during the night, which is consistent with the hypothesis that, in addition to being important for the export of photosynthate, Suc may play an osmoregulatory or cryoprotective role in leaves in the cold (Anchordoguy et al., 1987; Carpenter and Crowe, 1988). In contrast, fully developed source leaves that had been shifted to 5°C (the 23°C/5°C leaves) accumulated large pools of soluble carbohydrates, including very large pools of free hexoses. These leaves also contained a large amount of starch that was not remobilized during the night. The accumulation of carbohydrates in the 23°C/5°C leaves therefore appears to be due at least partly to limited export and mobilization.

Development under low-temperature growth conditions allows plants to acclimate carbon metabolism by shifting partitioning toward soluble-sugar synthesis. Up-regulation of the cytosolic pathway for Suc biosynthesis could be of great importance for the recovery of photosynthesis and the maintenance of the flux of carbon at low temperatures, as well as for the capacity to grow and develop new leaves in the cold. The cFBPase and SPS enzymes play key roles in the cold-acclimation process and molecular modifications of these enzymes will be of great interest in the future.

Changes in Cytoplasmic and Vacuolar Volume Underlay the 2- to 3-Fold Increases in Protein and Enzyme Activity in Cold-Acclimated Leaves

The protein content increased 2.5-fold in the 5°C leaves and was responsible for the increased activities of Rubisco and other Calvin-cycle enzymes; and it played a major role in the increase of cFBPase and SPS activity. To understand the mechanisms underlying acclimation to low temperatures, we need to investigate how the increased protein was physically accommodated in the leaf cells.

A marked decrease of water in leaves that developed at low temperatures accompanied the changes in enzyme activities and protein. Electron micrographs showed that these changes were due to an increase in the relative volume of the cytoplasm and a decrease in the relative volume of the vacuole. In the 5°C leaves the cytoplasm occupied a larger volume of the leaf cell and appeared denser than in the 23°C leaves. The vacuoles were clearly smaller in the 23°C/5°C leaves and in the 5°C leaves, but these structural changes were most pronounced in the 5°C leaves. Similar increases in cytoplasmic volume have been reported previously for tree species (Siminovitch et al., 1967) and winter rye (Huner et al., 1981; Griffith et al., 1985), indicating that the changes observed for *Arabidopsis* in the present study represent a typical response to cold acclimation. However, the functional significance of these changes in cell structure has not previously been recognized.

The anatomical changes observed in cold-developed leaves interact with their metabolic adaptation. On one hand, it appears likely that the increase in the cytoplasmic volume allows the cells to accommodate the 2- to 3-fold increase in leaf protein and therefore plays an integral role in the acclimation of metabolism to low temperature, lead-

ing to an increased capacity for photosynthesis and sugar synthesis. But on the other hand, the increased cytoplasm may give rise to an increased need for solutes, such as Suc, that are typically restricted to the cytoplasm. Higher levels of sugars, especially Suc, may be necessary in the cytoplasm not only to maintain export and provide cryoprotection but possibly also to help mechanize a selective increase in the cytoplasmic volume. Investigations of plants with altered levels of sugars will test these ideas. Insight into the underlying mechanisms of these structural changes in the cold could result in novel approaches to increasing plant yield.

In conclusion, we have shown that leaf development at 5°C results in the recovery of photosynthetic carbon fixation and a shift in the partitioning of carbon away from starch and toward Suc. This photosynthetic recovery is supported by coordinated increases in the activity of several Calvin-cycle enzymes and by marked increases in the activity of key cytosolic enzymes for Suc synthesis. Furthermore, despite the large accumulation of hexose phosphates in the cold, Pi is not limited and probably even increases in the cytosol. Finally, we show a link between anatomical changes in leaf development at 5°C and metabolic adjustments to the cold. An increase in the volume of the cytoplasm may provide an important mechanism for increasing the enzymes and metabolites in cold-acclimated leaves. It correlates with an increased flux through the cytosol and Suc synthesis and possibly with an increased need for solutes in the cytosol.

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