CO₂ Assimilation, Carbohydrate Metabolism, Xanthophyll Cycle, and the Antioxidant System of ‘Honeycrisp’ Apple Leaves with Zonal Chlorosis

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ABSTRACT. To determine the cause of a characteristic zonal chlorosis of ‘Honeycrisp’ apple (*Malus × domestica* Borkh.) leaves, we compared CO₂ assimilation, carbohydrate metabolism, the xanthophyll cycle and the antioxidant system between chlorotic leaves and normal leaves. Chlorotic leaves accumulated higher levels of nonstructural carbohydrates, particularly starch, sorbitol, sucrose, and fructose at both dusk and predawn, and no difference was found in total nonstructural carbohydrates between predawn and dusk. This indicates that carbon export was inhibited in chlorotic leaves. CO₂ assimilation and the key enzymes in the Calvin cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase, NADP-glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, stromal fructose-1,6-bisphosphatase, and the key enzymes in starch and sorbitol synthesis, ADP-glucose pyrophosphorylase, cytosolic fructose-1,6-bisphosphatase, and aldose 6-phosphate reductase were significantly lower in chlorotic leaves than in normal leaves. However, sucrose phosphate synthase activity was higher in chlorotic leaves. In response to a reduced demand for photosynthetic electron transport, thermal dissipation of excitation energy (measured as nonphotochemical quenching of chlorophyll fluorescence) was enhanced in chlorotic leaves under full sun, lowering the efficiency of excitation energy transfer to PSII reaction centers. This was accompanied by a corresponding increase in both xanthophyll cycle pool size (on a chlorophyll basis) and conversion of violaxanthin to antheraxanthin and zeaxanthin. The antioxidant system, including superoxide dismutase and ascorbate peroxidase and the ascorbate pool and glutathione pool, was up-regulated in chlorotic leaves in response to the increased generation of reactive oxygen species via photoreduction of oxygen. These findings support the hypothesis that phloem loading and/or transport is partially or completely blocked in chlorotic leaves, and that excessive accumulation of nonstructural carbohydrates may cause feedback suppression of CO₂ assimilation via direct interference with chloroplast function and/or indirect repression of photosynthetic enzymes.

‘Honeycrisp’ is a new apple cultivar that is being extensively planted in the cooler apple-producing areas due to its unique fruit quality. A persistent problem in ‘Honeycrisp’ is a leaf disorder that often develops in late June or early July when shoot growth slows down or stops (Rosenberger et al., 2001). The initial symptom of zonal chlorosis appears randomly in part (toward the edges) of a leaf confined by secondary or tertiary veins, and then gradually spreads to other parts of the leaf. The chlorotic area becomes thicker, leathery, and brittle, and turns brown later in the season. The symptoms occur on almost every tree, but trees bearing a light crop have a larger number of leaves developing symptoms compared with those with a heavy crop (Robinson and Watkins, 2003; Schupp, 2003). It is not known what causes this disorder and how it affects tree carbon supply to sink organs.

The symptoms of this disorder are similar to the damage that potato leafhoppers [*Empoasca fabae* (Harris)] cause on apple leaves (Rosenberger et al., 2001). However, trees that are protected

from potato leafhoppers still develop the disorder (Schupp et al., 2001). The symptoms of this disorder resemble those observed when carbohydrates accumulate in leaves of ‘Golden Delicious’ apple trees after deblossoming (Schupp et al., 1992), in citrus (*Citrus reticulata* Blanco) leaves after branch girdling and fruit removal (Schaffer et al., 1986), or in transgenic tobacco (*Nicotiana tabacum* L.) plants overexpressing yeast invertase in the cell wall (Stitt et al., 1991). The concurrence of initial chlorosis symptoms with cessation of shoot growth and the severity of the disorder in relation to cropload have led us to hypothesize that phloem loading or transport of carbohydrates is partially blocked in the leaf minor veins, which leads to feedback repression of the key enzymes in CO₂ assimilation via accumulation of nonstructural carbohydrates in the leaves. If this hypothesis is correct, the chlorotic leaves would be expected to accumulate higher levels of nonstructural carbohydrates and have lower CO₂ assimilation and lower activities of the key enzymes in the Calvin cycle. Considering that C3 photosynthesis has a structure of a convergent metabolic pathway in terms of light and CO₂ inputs (Woodrow and Berry, 1988), feedback repression of CO₂ assimilation should be also reflected in the light harvesting and processing branch of the photosynthetic system as higher levels of xanthophyll cycle-dependent thermal dissipation of excitation energy and up-regulation of the antioxidant system, both of which protect the leaves from the excess absorbed light under high light. As the first step to characterizing the disorder, we compared chlorotic leaves with normal leaves in terms of CO₂ assimilation, carbohydrate metabolism, xanthophyll cycle-dependent thermal dissipation and the antioxidant system.

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Materials and Methods

PLANT MATERIALS. Four-year-old 'Honeycrisp' apple trees on Malling 9 rootstocks were used in this study. The trees were grown at a spacing of 1.2 × 4.2 m in the field at Cornell Orchards in Ithaca, N. Y. They received standard horticultural practices, and disease and insect control. The cropload of trees was hand-thinned to four fruit/cm² trunk cross-sectional area when the diameter of the largest fruit was 10 mm. On 6 July (2 weeks after the initial appearance of leaf disorder symptoms), recent fully expanded normal and chlorotic leaves were chosen at random on single-tree replicates to measure gas exchange, enzyme activities, metabolites, carbohydrates, chlorophyll (Chl) fluorescence, pigments, and antioxidant enzymes and antioxidant metabolites.

GAS EXCHANGE MEASUREMENTS. Carbon dioxide assimilation and stomatal conductance were measured with a CIRAS-1 portable photosynthesis system (PP systems, Herts, U.K.) using a standard broadleaf cuvette on both normal leaves and the chlorotic area of symptomatic leaves, at noon under ambient CO₂ (360 μmol·mol⁻¹), a photon flux density (PFD) of 1650 ± 30 μmol·m⁻²·s⁻¹, air temperature of 22.0 ± 0.5 °C, and ambient water vapor pressure of 1.6 ± 0.2 kPa.

EXTRACTION AND ASSAY OF KEY ENZYMES IN THE CALVIN CYCLE AND CARBOHYDRATE METABOLISM. Leaf disks (1 cm² in size) were taken from both normal leaves and chlorotic areas of symptomatic leaves at noon under full sun (PFD of 1650 μmol·m⁻²·s⁻¹), frozen in liquid N₂, and stored at -80 °C until assay.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC4.1.1.39), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC1.2.1.12), phosphoribulokinase (PRK, EC2.7.1.19), both stromal and cytosolic fructose-1,6-bisphosphatase (FBPase, EC3.1.3.11), and sucrose phosphate synthase (SPS, EC2.4.1.14) were extracted according to Chen and Cheng (2003a). Two or three frozen leaf disks were ground with a precooled mortar and pestle in 1.5 mL extraction buffer containing 50 mM Hepes-KOH (pH 7.5), 10 mM MgCl₂, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DDT), 1% (v/v) Triton X-100, 5% (w/v) insoluble polyvinylpyrrolidone (PVPP), 1% (w/v) bovine serum albumin (BSA), and 10% (v/v) glycerol. The extract was centrifuged at 13,000 g_n for 5 min in an Eppendorf microcentrifuge at 2 °C, and the supernatant was used immediately for enzyme assays.

Total Rubisco activity was measured after incubating the leaf extract in the assay solution for 15 min at room temperature as described previously (Cheng and Fuchigami, 2000).

GAPDH activity was determined in a mixture (1 mL) of 100 mM Tricine-KOH (pH 8.0), 4 mM 3-phosphoglycerate (PGA), 5 mM ATP, 10 mM MgCl₂, 0.2 mM NADPH, and 20 units 3-phosphoglyceric phosphokinase (PCK, EC2.7.2.3). The reaction was initiated by adding the enzyme extract (Leegood, 1990).

PRK activity was assayed in a mixture (1 mL) of 100 mM Tricine-KOH (pH 8.0), 0.5 mM ribose 5-phosphate (R5P), 1 mM ATP, 10 mM MgCl₂, 50 mM KCl, 5 mM phosphoenolpyruvate (PEP), 0.4 mM NADH, seven units pyruvate kinase (EC2.7.1.40), 10 units lactate dehydrogenase (LDH, EC1.1.1.27), and one unit R5P isomerase (EC5.1.3.6). The reaction was initiated by adding the enzyme extract (Leegood, 1990).

Stromal FBPase was assayed in a mixture (1 mL) of 50 mM Tris-HCl (pH 8.2), 10 mM MgCl₂, 1 mM EDTA, 0.1 mM fructose 1,6-bisphosphate (FBP), 0.5 mM NADP, four units of phosphoglucoisomerase (PGI, EC5.3.1.9), and two units of glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49). The reaction

was initiated by adding the enzyme extract (Holaday et al., 1992; Leegood, 1990).

Cytosolic FBPase was assayed according to Holaday et al. (1992) with some modifications. The enzyme was assayed in 1 mL reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 2 mM MgCl₂, 0.1 mM FBP, 0.5 mM NADP, four units of PGI and two units of G6PDH. The reaction was initiated by adding the enzyme extract.

SPS was assayed according to Grof et al. (1998) with some modifications. Sixty microliters of enzyme extract were incubated for 15 min at 30 °C with 100 mM Hepes-KOH (pH 7.5), 100 mM KCl, 6 mM EDTA, 30 mM uridine 5'-diphosphoglucose (UDPG), 10 mM fructose-6-phosphate (F6P), and 40 mM glucose-6-phosphate (G6P) in a total volume of 100 μL. At the end of the incubation period, the reaction was stopped by adding 100 μL ice-cold 1.2 N HClO₄ and held on ice for another 15 min. The reaction mixture was neutralized by adding 60 μL of 2 M KHCO₃, held on ice for 15 min, then centrifuged at 13,000 g_n for 1 min. An aliquot (130 μL) of the supernatant was assayed for uridine 5'-diphosphate (UDP) by coupling to oxidation of NADH with LDH and pyruvate kinase. The reaction mixture (1 mL) contained 50 mM Hepes-NaOH (pH 7.0), 5 mM MgCl₂, 0.3 mM NADH, 0.8 mM PEP, 14 units LDH, and four units pyruvate kinase. The reaction was started by adding pyruvate kinase (Stitt et al., 1988). Controls without F6P and G6P were carried through for all the samples.

ADP-glucose pyrophosphorylase (AGPase, EC2.7.7.27) was extracted and assayed according to Chen and Cheng (2003a) without including reduced glutathione (GSH) in the extraction buffer.

Aldose-6-phosphate reductase (A6PR, EC1.1.1.200) was extracted according to Negm and Loescher (1981) with some modifications. Three frozen leaf disks were ground with a precooled mortar and pestle in 1.5 mL extraction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM DTT, 0.3% (v/v) Triton X-100, 5% insoluble PVPP, and 6% (v/v) glycerol. The extract was then centrifuged at 13,000 g_n for 5 min in an Eppendorf microcentrifuge, and the supernatant was used immediately for the assay. A6PR was assayed in the direction of sorbitol-6-phosphate synthesis by following the oxidation NADPH at 340 nm. The reaction mixture (1 mL) contained 0.1 M Tris-HCl (pH 9.0), 0.11 mM NADPH, 50 mM G6P, and 25 μL of the extract. Reaction was initiated by adding the enzyme extract.

EXTRACTION AND DETERMINATION OF G6P, F6P, AND PGA. Both normal leaves and chlorotic leaves were taken at noon under full sun (PFD of 1650 μmol·m⁻²·s⁻¹), frozen in liquid N₂, and stored at -80 °C until analysis. Metabolites were extracted and measured according to Chen et al. (2002) with some modifications (Chen and Cheng, 2003).

EXTRACTION AND MEASUREMENT OF LEAF NONSTRUCTURAL CARBOHYDRATES. Three leaf disks (total of 3 cm²) were taken right after sunset (dusk) and at predawn from the same leaf, frozen in liquid N₂ and stored at -80 °C until assay. Sorbitol, sucrose, glucose, and fructose were extracted with 80% (v/v) ethanol at 80 °C and determined by a Dionex DX-500 series chromatograph system (Dionex, Sunnyvale, Calif.) as previously described (Cheng and Fuchigami, 2002). The tissue residue after soluble sugar extraction was retained for determination of starch. After digesting the residue with amyloglucosidase (EC3.2.1.3), starch was determined enzymatically as glucose equivalents using G6PDH and hexokinase (EC2.7.1.1) (Chen et al., 2002).

MEASUREMENTS OF CHL FLUORESCENCE. Chl fluorescence was

measured with a pulse-modulated fluorometer FMS2 (Hansatech Instrument, Norfolk, U.K.) on both normal leaves and the chlorotic area of symptomatic leaves at noon right after gas exchange measurements and also at predawn, under natural conditions.

The maximum PSII efficiency of dark-adapted leaves was calculated as: $F_v/F_m = (F_m - F_o)/F_m$ (van Kooten and Snel, 1990). Thermal dissipation of excitation energy was estimated from nonphotochemical quenching (NPQ) as: $F_m'/F_m - 1$ (Stern-Volmer quenching; Bilger and Björkman, 1990). The photochemical quenching coefficient qP was calculated as: $(F_m' - F_s)/(F_m' - F_o)$. The efficiency of excitation transfer to open PSII centers under natural light exposure was $F_v'/F_m' = (F_m' - F_o)/F_m'$. PSII operating efficiency was $(F_m' - F_s)/F_m'$ (Genty et al., 1989).

ANALYSIS OF LEAF PIGMENTS. After Chl fluorescence measurements, one disk (1 cm²) was punched from the leaf and frozen in liquid N₂. Frozen leaf disks were stored at -80 °C until analysis. Extraction and analysis of the pigments by HPLC was as previously described (Cheng, 2003).

ASSAY OF SUPEROXIDE ANION GENERATION. Generation of superoxide anion was determined based on its ability to reduce nitroblue tetrazolium (NBT) (Doke, 1983). Fresh leaf disks (total of 5 cm²) taken under full sun (PFD of 1650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at midday were immersed immediately in 3 mL mixture of 10 mM Na₂HPO₄-KH₂PO₄ (pH 7.8), 0.05% (w/v) NBT, and 10 mM NaN₃ for 1 h. Then the mixture was heated to 85 °C for 15 min and cooled rapidly. Absorbance at 580 nm was recorded and the activity of the disks to reduce NBT was expressed as increased $\text{OD}_{580}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

EXTRACTION AND DETERMINATION OF HYDROGEN PEROXIDE (H₂O₂). Leaf disks (total of 5 cm²) were taken under full sun (PFD of 1650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at midday, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. H₂O₂ was extracted and determined according to Patterson et al. (1984) with some modifications. The frozen leaf disks were ground in liquid nitrogen with a mortar and pestle and extracted with 2 mL 5% (w/v) trichloroacetic acid (TCA) and 70 mg charcoal (washed with HCl), then centrifuged at 16,000 g_n for 10 min. The supernatant was neutralized to pH 8.5 with 17 M NH₄OH, then centrifuged at 12,000 g_n for 2 min. The supernatant was used immediately for assay. Each extract was divided into two aliquots of 0.6 mL. Four units of catalase (CAT, EC1.11.1.6) were added to one aliquot (blank). Both the blank and the other aliquot without catalase added were kept at 20 °C for 10 min, and then 0.6 mL colorimetric reagent was added. The colorimetric reagent was made daily by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-(2-pyridylazo) resorcinol monosodium salt. The assay mixture (1.2 mL) was incubated at 45 °C for 60 min before absorbance at 508 nm was recorded.

LIPID PEROXIDATION. Three leaf disks (1 cm² in size) were taken at noon under full sun (PFD of 1650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), frozen in liquid N₂, and stored at -80 °C until assay.

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA). MDA content in leaves was assayed according to Hodges et al. (1999), which excludes the influence of interfering compounds in the assay.

EXTRACTION AND ASSAY OF ANTIOXIDANT ENZYMES. Leaf disks (1 cm² in size) were taken at noon under full sun (PFD of 1650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), frozen in liquid N₂, and stored at -80 °C until assay.

Superoxide dismutase (SOD, EC1.15.1.1), monodehydroascorbate reductase (MDAR, EC1.6.5.4), dehydroascorbate reductase (DHAR, 1.8.5.1), glutathione reductase (GR, EC1.6.4.2), and

CAT were extracted according to Chen and Cheng (2003b). Ascorbate peroxidase (APX, EC1.11.1.11) was extracted according to Schwanz and Polle (2001) with some modifications. Two frozen leaf disks were ground with a precooled mortar and pestle in 2.5 mL extract buffer containing 100 mM K₂HPO₄-KH₂PO₄ (pH 7.8), 1 mM DTT, 2 mM EDTA, 5 mM ascorbate (AsA), 1 % (v/v) Triton X-100, 5 mM MgCl₂, and 4% insoluble PVPP. The extract was then centrifuged at 16,000 g_n for 5 min in an Eppendorf microfuge, and the supernatant was gel-filtered through a PD-10 column (Amersham Biosciences, Piscataway, N.J.), which had been equilibrated with 100 mM K₂HPO₄-KH₂PO₄ (pH 7.0) containing 1 mM AsA.

SOD activity was assayed spectrophotometrically at 550 nm by the cytochrome c method (McCord and Fridovich, 1969). One unit of SOD activity is defined as the amount necessary to produce a 50% inhibition of cytochrome c reduction. APX, CAT, MDAR, DHAR, and GR activities were measured as described by Chen and Cheng (2003b).

EXTRACTION AND ANALYSIS OF ANTIOXIDANT METABOLITES. Leaf disks were taken under full sun (PFD of 1650 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at midday, frozen in liquid N₂, and stored at -80 °C until assay.

Two leaf disks (total of 2 cm²) were ground in 1 mL ice-cold 7% (w/v) sulfosalicylic acid. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to Griffith (1980).

AsA and dehydroascorbate (DAsA) were measured according to Logan et al. (1998). Briefly, one leaf disk (1 cm²) was ground in 1 mL ice-cold of 6% (v/v) HClO₄. The extract was centrifuged at 10,000 g_n for 10 min at 2 °C. The supernatant was immediately used for the measurements. One hundred microliters of extract was neutralized with 30 μL 1.5 M Na₂CO₃ to raise the pH to 1~2. AsA was assayed spectrophotometrically at 265 nm in 200 mM sodium acetate buffer (pH 5.6), before and after 15 min incubation with 1.5 units AsA oxidase (EC1.10.3.3). For total ascorbate, 100 μL extract was neutralized with 30 μL 1.82 M Na₂CO₃ to raise the pH to 6~7 and incubated for 30 min at room temperature with equal volume (130 μL) of 20 mM GSH in 100 mM Tricine-KOH (pH 8.5). Total ascorbate was assayed as above. DAsA was calculated as the difference between total ascorbate and AsA.

Results

CO₂ ASSIMILATION. CO₂ assimilation was significantly lower in chlorotic leaves than in normal leaves (Fig. 1A). Stomatal conductance showed the

Fig. 1. CO₂ assimilation (A), stomatal conductance (B), and intercellular CO₂ concentration (C) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

same trend (Fig. 1B) as CO_2 assimilation. However, no significant difference was found in the calculated intercellular CO_2 concentrations between the two leaf types (Fig. 1C).

ACTIVITIES OF KEY ENZYMES IN THE CALVIN CYCLE AND CARBOHYDRATE METABOLISM. Activities of key enzymes in the Calvin cycle, Rubisco, GAPDH, PRK, stromal FBPase (Fig. 2A–C, F), and those in end-product synthesis, AGPase, A6PR, cytosolic FBPase (Fig. 2D, E, and G) were significantly lower in chlorotic leaves than in normal leaves. However, SPS activity was higher in chlorotic leaves than in normal leaves (Fig. 2H).

G6P, F6P, AND PGA. Leaf G6P, F6P, and PGA contents were lower in chlorotic leaves than in normal leaves (Fig. 3A, B, and D). No difference was found in the ratio of G6P:F6P between chlorotic leaves and normal leaves (Fig. 3C).

CONCENTRATIONS OF NONSTRUCTURAL CARBOHYDRATES AT DUSK AND PREDAWN. Concentrations of sorbitol, fructose, sucrose, starch, and total nonstructural carbohydrates at both dusk and predawn were higher in chlorotic leaves than in normal leaves (Fig. 4A, C–F). There was no difference in glucose concentration between chlorotic leaves and normal leaves at dusk or predawn (Fig. 4B). Concentrations of sorbitol, fructose, sucrose, starch, and total noncarbohydrates in normal leaves were lower at predawn than at dusk whereas the concentration of fructose in chlorotic leaves was higher at predawn than at dusk. Glucose concentration remained unchanged between predawn and dusk in both normal and chlorotic leaves.

CHL FLUORESCENCE VARIABLES. Nonphotochemical quenching (NPQ) of Chl fluorescence was much higher in chlorotic leaves than in normal leaves (Fig. 5A). Consequently, the efficiency of excitation transfer (F_v/F_m') was much lower in the chlorotic leaves than in normal leaves (Fig. 5B). Photochemical quenching coefficient (qP) was also lower in the chlorotic leaves (Fig. 5C). As a result, PSII operating efficiency was much lower in the chlorotic leaves (Fig. 5D). Maximal PSII quantum efficiency (F_v/F_m) of dark-adapted leaves at predawn was slightly lower in chlorotic leaves than in normal leaves (Fig. 5E).

LEAF PIGMENTS. Both Chl content and Chl a:b ratio at both midday and predawn were lower in chlorotic leaves than in normal leaves (Fig. 6A and H). On a leaf area basis, total carotenoids, lutein, β -carotene, and neoxanthin were lower in chlorotic leaves than in normal leaves at both midday and predawn (Fig. 6B, E, F, and G). No difference was found in xanthophyll cycle pool size ($V + A + Z$) at both midday and predawn comparing chlorotic leaves and normal leaves (Fig. 6C). On a Chl basis, however, total carotenoids, $V + A + Z$, lutein, and β -carotene were higher in chlorotic leaves than in normal leaves at both midday and predawn (Fig. 6I, J, L, and M). No difference was found in neoxanthin content at both midday and predawn between chlorotic leaves and normal leaves (Fig. 6N).

The A + Z level expressed on a xanthophyll cycle pool basis (Fig. 6D) or a Chl basis (Fig. 6K) at both midday and predawn was higher in chlorotic leaves than in normal leaves. For a given leaf type, the A + Z level was higher at midday than at predawn; no difference was found in any other pigment or Chl a:b between predawn and midday.

SUPEROXIDE ANION GENERATION, H_2O_2 CONTENT AND LIPID PEROXIDATION. Nitroblue tetrazolium (NBT) reducing activity, an indication of superoxide generation, was higher in chlorotic leaves than in normal leaves (Fig. 7A). Similarly, H_2O_2 content

Fig. 2. Activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, A), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, B), phosphoribulokinase (PRK, C), ADP-glucose pyrophosphorylase (AGPase, D), aldose-6-phosphate reductase (A6PR, E), stromal and cytosolic fructose-1,6-bisphosphatase (FBPase, F and G), and sucrose phosphate synthase (SPS, H) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

Fig. 3. Glucose-6-phosphate (G6P) content (A), fructose-6-phosphate (F6P) content (B), G6P:F6P (C), and 3-phosphoglycerate (PGA) content (D) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

Fig. 4 (above). Sorbitol (A), glucose (B), fructose (C), sucrose (D), starch (E), and total nonstructural carbohydrate (F) contents at dusk and predawn in chlorotic leaves and normal leaves. Specific leaf dry mass for normal leaves and chlorotic leaves at dusk were 106.96 ± 1.83 and 135.79 ± 2.89 g·m⁻², respectively. Black and white bars represent dusk and predawn, respectively. Each bar is mean $\pm$ standard error (n = 6). Significant difference was tested between predawn and dusk for a given leaf type and between chlorotic leaves and normal leaves taken at the same time. Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

Fig. 5 (right). Nonphotochemical quenching, NPQ (A), efficiency of excitation transfer, F_v/F_m (B), photochemical quenching coefficient, qP (C), PSII operating efficiency (D) and maximum PSII efficiency, F_v/F_m (E) of chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error (n = 6). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

Fig. 6 (above). Chlorophyll (A), Chl a : b (H), carotenoids (B and I), xanthophyll cycle pool (C and J), conversion of violaxanthin to antheraxanthin and zeaxanthin (D and K), lutein (E and L), β -carotene (F and M) and neoxanthin (G and N) expressed on the basis of leaf area or Chl at predawn and midday in chlorotic leaves and normal leaves. Black and white bars represent predawn and midday, respectively. Each bar is mean $\pm$ standard error (n = 6). Significant difference was tested between predawn and dusk for a given leaf type and between chlorotic leaves and normal leaves taken at the same time. Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively. In the label for the Y axis: V: violaxanthin, A: antheraxanthin, Z: zeaxanthin.

Fig. 7. Nitroblue tetrazolium (NTB) reducing activity (A), H_2O_2 content (B), and malondialdehyde (MDA) content (C) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

was higher in chlorotic leaves than in normal leaves (Fig. 7B). However, no difference was found in MDA level (indicative of oxidative lipid metabolism) between chlorotic leaves and normal leaves (Fig. 7C).

ANTIOXIDANT ENZYMES AND METABOLITES. On a leaf area basis, SOD and APX activities were higher in chlorotic leaves than in normal leaves (Fig. 8 A and B), but no difference was found in MDAR, DHAR or GR activities between chlorotic leaves and normal leaves (Fig. 8C–E). In contrast, CAT activity was lower in chlorotic leaves than in normal leaves. On a leaf Chl basis, SOD, APX, MDAR, DHAR, and GR activities were all higher in chlorotic leaves than in normal leaves and no difference was found in CAT activities between chlorotic leaves and normal leaves (data not shown).

Contents of AsA, DASA, GSH, and GSSG were higher in chlorotic leaves than in normal leaves (Fig. 9A–B, D–E), whereas the ratios of both AsA:DASA and GSH:GSSG were lower in chlorotic leaves than in normal leaves (Fig. 9 C and F).

Discussion

Compared with normal leaves, chlorotic leaves accumulated higher levels of soluble sugars, especially sorbitol and sucrose (Fig. 4 A and D). Since the activity of A6PR, a key enzyme for sorbitol synthesis (Negm and Loescher, 1981), was lower in chlorotic leaves than in normal leaves (Fig. 2E) and there was no difference in sorbitol content in chlorotic leaves between predawn and dusk (Fig. 4A), the elevated sorbitol level in chlorotic leaves was apparently the result of decreased sorbitol export. Similarly, the higher sucrose content in chlorotic leaves (Fig. 4D) was also caused by the decreased export of sucrose. However, in contrast to the key enzymes in the Calvin cycle and other important enzymes in carbohydrate metabolism, the activity of SPS, a key enzyme for sucrose synthesis, was higher in chlorotic leaves than in normal leaves (Fig. 2H).

Fig. 8. Activities of superoxide dismutase (SOD, A), ascorbate peroxidase (APX, B), monodehydroascorbate reductase (MDAR, C), dehydroascorbate reductase (DHAR, D), glutathione reductase (GR, E), and catalase (CAT, F) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

Fig. 9. Contents of ascorbate (AsA, A), dehydroascorbate (DASA, B), reduced glutathione (GSH, D) and oxidized glutathione (GSSG, E), and ratios of AsA : DASA and GSH : GSSG (C and F) in chlorotic leaves and normal leaves. Each bar is mean $\pm$ standard error ($n = 6$). Different capital or small letters above the bars indicate significant difference at $P < 0.01$ or 0.05 , respectively.

The exact reason why SPS activity was increased in chlorotic leaves remains unclear. Transgenic tobacco plants overexpressing yeast invertase also had higher SPS activity, whereas activities of the Calvin cycle enzymes decreased (Stitt et al., 1991). It was suggested that high SPS activity may help to maintain a certain flux of sucrose synthesis while hexoses are recycled. Although the chlorotic leaves had higher fructose content at both dusk and predawn (Fig. 4C), no difference was found in glucose content (Fig. 4B). Measurement of acid invertase activity indicated that there was no difference between chlorotic leaves and normal leaves (data not shown). As high sucrose levels lead to deactivation of SPS (Stitt et al., 1988), increasing maximum SPS activity may help to compensate for the deactivation of SPS to maintain a certain level of sucrose synthesis in chlorotic leaves.

Starch levels in chlorotic leaves were much higher than in normal leaves at dusk and predawn (Fig. 4E) and no difference was observed in starch content between dusk and predawn. This indicates that phloem loading or transport in chlorotic leaves was partially or completely inhibited. When phloem transport is blocked by petiole girdling (Krapp and Stitt, 1995; Krapp et al., 1993) or overexpression of yeast invertase (Stitt et al., 1991), soluble sugars accumulate to a high level, leading to decrease of sucrose synthesis in the cytosol. As a result, hexose phosphates accumulate in the cytosol, resulting in down-regulation of the carbon flux through cytosolic FBPase via fructose 2,6-bisphosphate, and up-regulation of starch synthesis in the chloroplast. However, it seems that the elevated starch level in chlorotic 'Honeycrisp' leaves resulted from decreased export of carbon from starch degradation, not from increased starch synthesis, because G6P, F6P, and PGA all decreased rather than increased (Fig. 3 A, B, and D). In addition, AGPase activity decreased in chlorotic leaves, which contrasts with previous findings that AGPase transcript levels or activities increase in response to petiole or phloem girdling (Krapp and Stitt, 1995; Zhou and Quebdeaux, 2003). It is possible that metabolic up-regulation of starch synthesis might have operated during the very early stages of 'Honeycrisp' leaf disorder development as it may help to assimilate and store more carbon in a short term. In the long term, down-regulation of AGPase is necessary to avoid rupture of the chloroplasts if phloem transport remains blocked.

The observed lower rate of CO₂ assimilation in chlorotic leaves (Fig. 1A) was due to nonstomatal limitation as intercellular CO₂ concentration in chlorotic leaves was the same as in normal leaves (Fig. 1C). It is very likely that one or a combination of the following two mechanisms caused the decrease in CO₂ assimilation in chlorotic leaves. First, excessive accumulation of starch may directly interfere with the function of chloroplasts. An apparent correlation between starch accumulation and inhibition of photosynthesis has been observed in soybean (*Glycine max* L.) (Nafziger and Koller, 1976) and cotton (*Gossypium hirsutum* L.) (Sasek et al., 1985). Cave et al. (1981) found that *Trifolium subterraneum* L. plants grown at elevated CO₂ had higher starch content and that this excessive starch accumulation physically distorted the structure of chloroplast, leading to lower CO₂ assimilation and lower Chl content. Deblossoming 'Golden Delicious' apple trees (Schupp et al., 1992) and removing major sinks of citrus trees by both defruiting and branch girdling (Schaffer et al., 1986) resulted in excessive starch accumulation in chloroplasts and leaf chlorosis. The starch level observed in chlorotic leaves in our study may be high enough to cause damage to the membrane and the enzyme system of the chloroplast. However, this mechanism cannot explain why photosynthesis of starch-

less mutant of tobacco (*Nicotiana sylvestris* L.) also decreased to a similar extent as that of the wild type in response to petiole girdling (Goldschmidt and Huber, 1992). Secondly, high levels of soluble sugars may indirectly repress the expression of the genes that encode photosynthetic enzymes. In maize (*Zea mays* L.) protoplasm transient expression system (Sheen, 1990), autotrophic *Chenopodium* L. cell-suspension cultures, and girdled leaves of tobacco and potato (*Solanum tuberosum* L.) (Krapp et al., 1993) and spinach (*Spinacia oleracea* L.) (Krapp and Stitt, 1995), high levels of soluble carbohydrates led to down-regulation of expression of several key genes in photosynthesis, including Rubisco. The finding that chlorotic leaves had higher levels of soluble sugars and lower activities of Rubisco and other Calvin cycle enzymes compared with normal leaves (Fig. 2A–C and F) is consistent with feedback repression of photosynthetic genes. In transgenic tobacco plants overexpressing yeast invertase, accumulation of nonstructural carbohydrates was accompanied by a decrease of Rubisco and other Calvin cycle enzymes, resulting in an inhibition of photosynthesis (Stitt et al., 1991).

Because chlorotic leaves had a much lower photosynthetic capacity, they only used a small fraction of the absorbed light in electron transport (Fig. 5D). As a result, more excess excitation energy existed in chlorotic leaves than normal leaves under high PFD at midday. Correspondingly, thermal dissipation of excitation energy, measured as NPQ, increased in chlorotic leaves under high light (Fig. 5A), lowering the efficiency with which excitation energy was transferred to PSII reaction centers, F_v'/F_m' (Fig. 5B).

Thermal dissipation of excitation energy is dependent on the accumulation of de-epoxidation products (A+Z) of the xanthophyll cycle (Demmig-Adams and Adams, 1996; Niyogi et al., 1998). Both the xanthophyll cycle pool size on a leaf Chl basis (Fig. 6J) and the conversion of V to A and Z at midday (Fig. 6D) increased in chlorotic leaves. These results indicate that in addition to decreasing light absorption by downsizing light-harvesting Chl antennae, both xanthophyll cycle pool size on a Chl basis and the conversion of V to A and Z were up-regulated to dissipate the excess absorbed PFD in chlorotic leaves. Because A and Z are also capable of de-exciting ¹O₂ (Baroli et al., 2003; Havaux and Niyogi, 1999), the increase in both xanthophyll pool size and the conversion of V to A and Z in chlorotic leaves may also help to quench ¹O₂ as production of ¹O₂ may increase in chlorotic leaves under high light due to increased closure of PSII (Fig. 5C).

Of the linear electron transport, a proportion is directly transferred to oxygen. Although the rate of photoreduction of oxygen was not directly measured in this study, the higher NBT reducing activity (Fig. 7A), higher H₂O₂ content (Fig. 7B), and the increase of down-stream antioxidant enzymes, including SOD and APX (Fig. 8 A and B) and antioxidant metabolites (Fig. 9 A, B, D, and E) indicate that photoreduction of oxygen was enhanced in chlorotic leaves to dissipate extra electrons, and the antioxidant system was up-regulated to cope with the reactive oxygen species generated. The up-regulation of the antioxidant system also helps to maintain the linear electron flow from PSII to PSI (Asada, 1999), which generates a ΔpH across the thylakoid membrane (Asada, 1999; Niyogi, 1999) to support the xanthophyll-cycle/ΔpH dependent thermal energy dissipation in the antenna pigment complexes of PSII. Among the antioxidant enzymes, CAT was the only one that showed a decreased activity in the chlorotic leaves (Fig. 8F). This may be related to the fact that CAT is sensitive to photoinactivation in vivo and in vitro (Feierabend and Engel, 1986; Willekens et al., 1995). It may also reflect a lower rate

of photorespiration in chlorotic leaves as Rubisco activity was significantly reduced (Fig. 2A).

AsA and GSH account for most of the total ascorbate pool and glutathione pool, respectively, in plants under normal conditions (Foyer, 1997; Smirnoff and Pallanca, 1996). The ratios of AsA to DAsA and of GSH to GSSG decreased under oxidative stress (Gossett et al., 1994; Law et al., 1983). Although the ratios of both AsA:DAsA and GSH:GSSG were lower in chlorotic leaves than in normal leaves (Fig. 9 C and F), AsA and GSH still accounted for 91% and 90% of the total ascorbate pool and glutathione pool, respectively, in chlorotic leaves. Chlorotic leaves also had a predawn value of F_v/F_m 0.75 (Fig. 5E) and no difference was found in MDA content between chlorotic leaves and normal leaves (Fig. 7C). This indicates that up-regulation of both xanthophyll-dependent thermal dissipation and the antioxidant system provided considerable protection to chlorotic leaves against photo-oxidative damage.

In conclusion, 'Honeycrisp' leaves with zonal chlorosis accumulate high levels of nonstructural carbohydrates. The excessive accumulation of nonstructural carbohydrates may have led to feedback suppression of CO₂ assimilation, possibly via direct interference with chloroplast function and/or indirect repression of photosynthetic enzymes. In response to a low demand for photosynthetic electron transport, both photoprotective mechanisms, the xanthophyll cycle-dependent thermal dissipation and the antioxidant system are up-regulated to cope with the excess photons and electrons under high light. Taken all together, the data are most consistent with the hypothesis that phloem loading and/or transport is partially or completely blocked in chlorotic leaves. Further research is needed to elucidate the molecular mechanism.

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