CARBON AND NITROGEN METABOLISM IN TRANSGENIC APPLE LEAVES WITH DECREASED SORBITOL SYNTHESIS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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MAY 2012

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Cornell University 2012

Sorbitol serves as a main photosynthetic end-product and a primary translocated form of carbon in apple and many other tree fruit species of the Rosaceae family. Sorbitol synthesis shares the same hexose phosphate pool with sucrose synthesis in the cytosol. Previous work showed that the expression of aldose-6-phosphate reductase (A6PR, the key enzyme in sorbitol synthesis) in 'Greensleeves' apple was decreased via antisense inhibition, A6PR activity in mature leaves was decreased to approximately 15-30% of the untransformed control. The present worh showed that a consequence of this inhibition was that sorbitol synthesis was significantly decreased. Both glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) accumulated in the cytosol at the expense of inorganic phosphate (Pi), leading to up-regulation of starch synthesis without altering CO_2 assimilation. Downstream metabolic responses to accumulation of G6P and F6P as well as the decreased pool of Pi in the cytosol were investigated in this study. It was found that transgenic plants had higher activities of several key enzymes in glycolysis, anaplerotic pathway and tricarboxylic acid cycle, higher respiration rate, and higher levels of organic acids and amino acids in mature leaves than the untransformed control, indicating that both organic acid metabolism and nitrogen metabolism were up-regulated in the transgenic plants. This up-regulation was mimicked, to a certain extent, by feeding detached leaves of the untransformed control with 10 mM mannose (a Pi sequester), suggesting that the decreased level of Pi in the cytosol due to the accumulation of hexose phosphates was also involved in the responses of organic acid metabolism and nitrogen metabolism in the transgenic plants. When

grown under N deficiency, the transgenic plants had higher activities of several key enzymes and higher contents of several organic acids and amino acids in organic acid metabolism and nitrogen metabolism. This enabled the transgenic plants to synthesize more proteins (enzymes), thereby maintaining a higher photosynthesis per unit leaf area relative to the untransformed control under N deficiency. As a result of having a more active organic acid and nitrogen metabolism, the transgenic plants are more tolerant of N deficiency.

BIOGRAPHICAL SKETCH

Fangfang grew up at the foot of Mount Tai in Shandong Province of China. She received her B. S. and M.S. in horticulture from Shandong Agricultural University in 2003 and 2006. In January 2007, Fangfang left her hometown, came to Cornell, her dream university, and started her Ph.D in fruit crops in Dr. Lailiang Cheng's Lab. Over the last five years, she has accomplished a lot, in both her research and her personal development. She is very proud that she has established a GC/MS protocol to profile a large number of primary metabolites in apple and has helped many other graduate students use the protocol in their research. After obtaining her Ph.D degree, she plans to do a couple years of postdoc to further her skills for a career in plant research.

This dissertation is dedicated to my family, especially to my most beloved mother Junzhu Wang and my deceased grandmother Suyun Sun. I can never repay your endless love.

献给我的家人，特别献给我最亲爱的妈妈王均竹和姥姥孙素云。
谢谢你们给予我的爱。

ACKNOWLEDGMENTS

I would never have been able to finish my dissertation without the guidance of my committee, Dr. Lailiang Cheng, Dr. Timothy L. Setter, and Dr. David Walter Wolfe. Here I would like to express my deepest gratitude to my mentor, Dr. Lailiang Cheng, for providing me the opportunity to pursue a Ph.D degree in my dream school, for his excellent guidance, full support and great kindness over the past five years.

I would like to thank Department of Horticulture and Ithaca Pomology Endowment for financial support during my Ph.D studies. I also would like to thank all other members of the Cheng Lab, especially Pengmin Li, Vinay Pagay, Huicong Wang, Zhenchang Liang, Richard Raba, Cankui Zhang and Rui Zhou for their help, advice and friendship. I want to thank Dr. David Zimerman, Cornell Pomology Ph.D. (1954), for generously donating the Agilent GC/MS system to our Lab, which made my work possible. To my dear friends Yi Yang and Xiang Ma, your company and support meant a lot to me, and for that I want to thank you.

Last but not the least, I would like to thank my family far away in China for their love and support throughout the years. I particularly want to thank my mother, Junzhu Wang. It is her love that sustained me in every step of my Ph.D studies at Cornell.

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2-OG	2-oxoglutarate
3-PGA	3-phosphoglycerate
6PGDH	6-phosphogluconate dehydrogenase
A6PR	Aldolase-6-phosphate reductase
ADP	Adenosine-5’-diphosphate
ADPG	Adenosine-5’-diphosphoglucose
AGPase	ADP-glucose pyrophosphorylase
Ala	Alanine
Arg	Arginine
AS	Asparagine synthetase
Asn	Asparagine
Asp	Asparate
Asp-AT	Aspartate aminotransferase
ATP	Adenosine-5’-triphosphate
BSA	Albumin from bovine serum
CoA	Coenzyme A
DHAP	Dihydroxyacetone phosphate
DPE2	Glycosyl transferase
DTNB	5,5’-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F1,6BP	Fructose-1,6-bisphosphate
F2,6BP	Fructose-2,6-bisphosphate
F6P	Fructose-6-phosphate
FBPase	Fructose-1,6-bisphosphate phosphatase
Fd-GOGAT	Ferredoxin-glutamate synthase
G3P	Glyceraldehyde-3-phosphate
G3PDH	Glyreraldehyde-3-phosphate dehydrogenase
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GABA	γ-aminobutyric acid
GAPDH	NADP-glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GOGAT	Glutamate synthase

GPT	Glutamate pyruvate transaminase
GS	Glutamine synthetase
HAC	Acetic acid
HCl	Hydrochloric acid
His	Histidine
HK	Hexokinase
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
Ile	Isoleucine
KAC	Potassium acetate
LDH	Lactate dehydrogenase
Leu	Leucine
Lys	Lysine
MDH	Malate dehydrogenase
ME	Malic enzyme
Met	Methionine
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NR	Nitrate reductase
NUE	Nitrogen use efficiency
OAA	Oxaloacetate
OPPP	Oxidative pentose phosphate pathway
Orn	Ornithine
PAHBAH	p-hydroxybenzoic acid hydrazide
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPP	Phosphoenolpyruvate phosphatase
PGM	Phosphoglucomutase
PFD	Photon flux denaity
PFK	Phosphofructokinase
PGDH	3-phosphoglycerate dehydrogenase
PGI	Phosphoglucose isomerase
Phe	Phenylalanine
PK	Pyruvate kinase
PPDK	Pyruvate, phosphate dikinase
PPi	Pyrophosphate
PRK	Ribulose-5-phosphate kinase
Pro	Proline

PVPP	Polyvinylpolypyrrolidone
R5P	Ribose-5-phosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
RuBP	Ribulose-1,5-bisphosphate
Ser	Serine
SLW	Specific leaf weight
SPS	Sucrose-6-phosphate synthase
SPP	Sucrose-phosphate phosphatase
TCA	Trichloroacetic acid
Thr	Threonine
TPI	Triose phosphate isomerase
TPT	Triose phosphate translocator
Tyr	Tyrosine
UDPG	Uridine-5’-diphosphoglucose
UTP	Uridine-5’-triphosphate
Val	Valine

CHAPTER 1

LITERATURE REVIEW: PRIMARY METABOLISM IN SOURCE LEAVES

Primary metabolism, the cornerstone of plant life, provides energy and substrates for sustaining cellular function, and intermediates for secondary metabolism. Generally speaking, primary metabolic pathways comprise the following chemical processes, photosynthesis, glycolysis, tricaboxylic acid cycle (TCA cycle), synthesis of amino acids, proteins, enzymes, structural materials, duplication of genetic materials, and reproduction of cells, etc. After synthesizing in source leaves, photosynthetic end products are, on one hand, exported to sink tissues to support the sink growth and development; on the other hand, stored or utilized by source leaves themselves to support the cellular activities there. In this chapter, the metabolism of photosynthetic end products is summarized to highlight the uniqueness of sorbitol in apple source leaves; then several primary metabolic pathways involved in carbon and nitrogen metabolism, such as glycolysis, TCA cycle, anaplerotic pathway, oxidative pentose phosphate pathway (OPPP), nitrate reduction, and amino acid biosynthesis are reviewed mainly in terms of their regulatory mechanisms; and the coordination between carbon metabolism and nitrogen metabolism is described to set the stage for the research described in this dissertation.

1. Photosynthetic carbon metabolism in source leaves

End product synthesis has characteristics of a divergent metabolic pathway: triose phosphates formed in the photosynthetic carbon reduction cycle either remain in the chloroplast for starch synthesis, or are transported across the chloroplast envelope to the cytosol for sucrose synthesis. In sorbitol-synthesizing species, sorbitol synthesis shares a common hexose phosphate

pool with sucrose synthesis in the cytosol. Both sucrose and sorbitol are loaded into phloem and transported to sink tissues (Fig. 1-1).

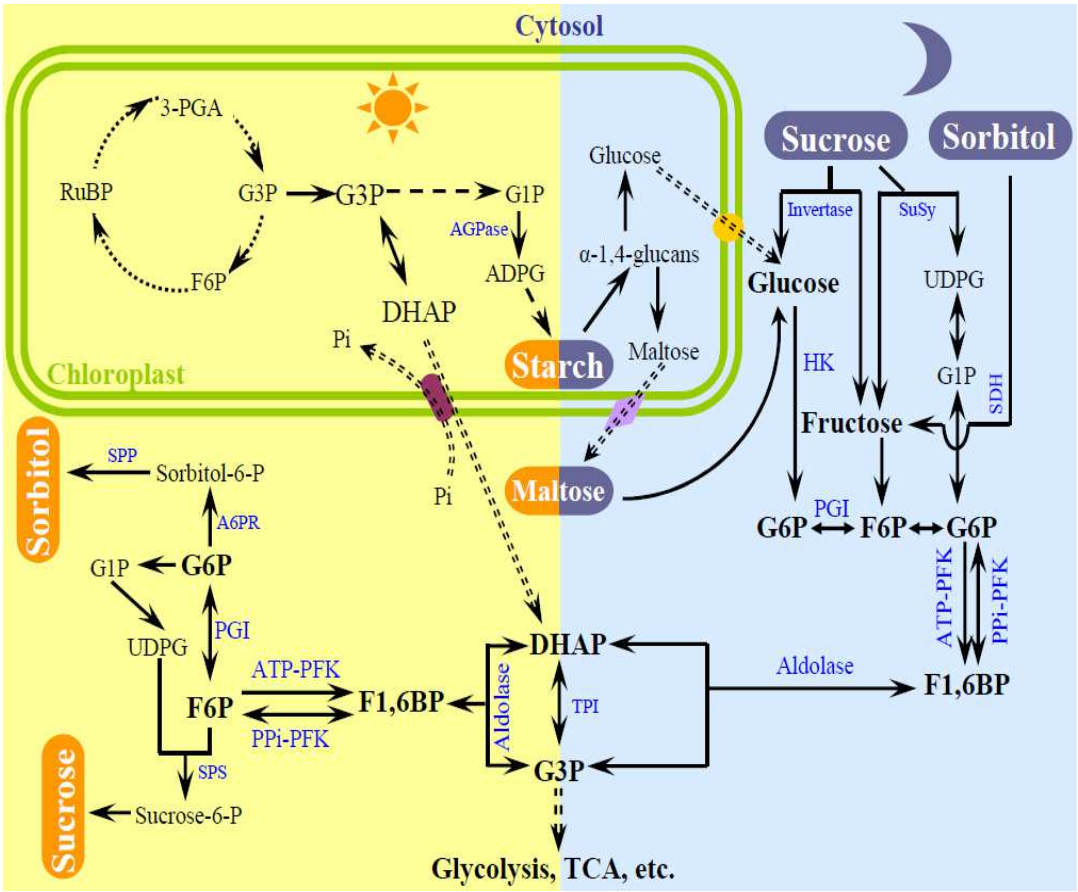

Figure 1-1. Photosynthetic end product metabolism in source leaves at both daytime and nighttime. Abbreviations of metabolites and enzymes are as follows: 3-PGA, 3-phosphoglycerate; ADPG, adenosine-5'-diphosphoglucose; DHAP, dihydroxyacetone phosphate; F1,6BP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G1P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; A6PR, aldolase-6-phosphate reductase; AGPase, ADP-glucose pyrophosphorylase; ATP-PFK, ATP-phosphofructokinase; FBPase, fructose- 1,6-bisphosphate phosphatase; PGI, phosphoglucose isomerase; PPi-PFK, PPi-phosphofructokinase; RuBP, ribulose-1,5-bisphosphate; SDH, sorbitol dehydrogenase; SPP, sucrose-phosphate phosphatase; SuSy, sucrose synthase; TPI, triose phosphate isomerase.

1.1. Sucrose synthesis

Triose phosphates produced in the chloroplast are transported across the chloroplast envelope to the cytosol by triose phosphate translocator (TPT) in counter exchange for inorganic phosphate (P_i) from the cytosol (Flügge and Heldt, 1991). The biochemical regulation of sucrose synthesis has been well characterized (Daie, 1993; Huber and Huber, 1996). The key regulatory steps of sucrose biosynthesis are the conversion of fructose-1,6-bisphosphate (F1,6BP) to fructose-6-phosphate (F6P) catalyzed by fructose-1,6-bisphosphatase (FBPase), and the formation of sucrose-6-phsphate from UDP-glucose and F6P catalyzed by sucrose phosphate synthase (SPS) (Daie, 1993; Huber et al., 1985). Both FBPase and SPS are allosteric enzymes. FBPase activity is inhibited by the signal metabolite fructose-2,6-bisphosphate (F2,6BP) (Stitt, 1990). SPS activity is subject to allosteric regulation by metabolites (G6P and Pi) (Doehlert and Huber, 1983) and post-translational modification via reversible protein phosphorylation (Huber and Huber, 1992), but species differ considerably in this regard (Huber et al., 1989; Huber and Huber, 1992). It appears that apple leaf SPS is only weakly regulated by G6P and Pi (Zhou et al., 2002) and there is no evidence of covalent modification in vivo in response to light/dark signal (Zhou et al., 2001).

Sucrose synthesis is coordinated with photosynthetic CO_2 fixation by a feedforward mechanism. When triose phosphate content rises above a threshold level as photosynthesis increases, cytosolic FBPase activity is stimulated by a decrease in the content of F2,6BP (Stitt, 1990), and SPS is activated by dephosphorylation of SPS protein and an increase in $G6P/P_i$ (Huber and Huber, 1992). The activation of both enzymes allows sucrose synthesis coordinated with rising photosynthesis (Stitt and Heldt, 1985; Stitt, 1996).

The roles of TPT (Riesmeier et al., 1993; Häusler et al., 1998), cytosolic FBPase (Sharkey et al., 1988, 1992; Zrenner et al., 1996), and SPS (Krause, 1994; Worrell et al., 1991; Signora et

al., 1998) in sucrose synthesis in vivo have been studied by using antisense suppression, mutants and overexpression of the respective proteins. Biochemical and physiological characterization of these transgenic plants and mutants have revealed that, 1) sucrose synthesis is inhibited, resulting in elevated 3-PGA/Pi ratio. As a result, starch synthesis is up-regulated; 2) CO_2 assimilation at ambient CO_2 is not affected whereas CO_2-saturated photosynthesis is generally decreased by reduced sucrose synthesis; 3) starch degradation and subsequent carbon export is increased (Heineke et al., 1994; Häusler et al., 1998) although the underlying biochemical mechanism remains unclear; and 4) When sucrose synthesis is increased by overexpression of SPS in potato (Galtier et al., 1993, 1995) and Arabidopsis (Signora et al., 1998), starch synthesis is decreased. Photosynthesis at ambient CO_2 is not significantly increased by elevated SPS activity. When grown at elevated CO_2 conditions, however, transgenic plants with increased SPS activity have higher photosynthesis compared with the untransformed controls, indicating a reduced limitation imposed by end product synthesis (Micallef et al., 1995; Signora et al., 1998).

1.2. Sorbitol synthesis

Sorbitol is a primary end-product of photosynthesis and a major phloem-translocated carbohydrate in many agriculturally important tree fruit species of the Rosaceae family, such as apple, pear, peach, cherry, apricot, plum, and almond (Bieleski, 1982; Loescher, 1987). In these species, sorbitol accounts for 50 to 90% of the newly fixed carbon and the carbon exported from leaves (Bieleski, 1982; Bieleski and Redgwell, 1985; Loescher, 1987; Escobar-Gutierrez and Gaudillere, 1996, 1997). On a global scale, sugar alcohols (polyols) are estimated to account for about 30% of primary carbon production (Bieleski, 1982).

In source leaves, sorbitol is synthesized through aldose-6-phosphate reductase (A6PR), which converts G6P to sorbitol-6-phosphate, followed by dephosphorylation catalyzed by

sorbitol-6-phosphatase (Fig. 2; Negm and Loescher, 1981; Grant and ap Rees, 1981; Kanayama and Yamaki, 1993; Zhou et al., 2003). The reaction catalyzed by A6PR is thought to be the key regulatory step in sorbitol synthesis.

When sorbitol is transported to sink tissues, it is converted to fructose by sorbitol dehydrogenase (SDH) (Loescher et al., 1982), which is encoded by a multiple-gene family (Park et al., 2002; Nosarszewski et al., 2004). As a leaf undergoes developmental changes from sink to source, there is a striking increase in A6PR activity and a corresponding decrease in SDH activity (Loescher et al., 1982; Merlo and Passera, 1991). Since SDH activity is very low in source leaves, leaf sorbitol level is mainly determined by the rates of synthesis and export.

Sorbitol is implicated in responses of plants to abiotic and biotic stresses. As a compatible solute, sorbitol plays a role in osmotic adjustment of apple (Wang and Stutte, 1992) and cherry (Ranney et al., 1991). Accumulation of sorbitol could also provide cold hardiness (Raese et al., 1978; Whetter and Taper, 1966). In vitro studies on sorbitol have revealed its unique ability to stabilize protein structure (Wimmer et al., 1997). Sorbitol also displays the ability to scavenge hydroxyl radicals (Smirnoff and Cumbes, 1989). In celery, synthesis and accumulation of mannitol, another sugar alcohol, increased in response to salinity (Everard et al., 1994). Transgenic tobacco and Arabidopsis plants engineered to synthesize mannitol showed an increased tolerance to salinity (Tarczynski et al., 1993; Gifang and Loescher, 2003). Sorbitol increases the mobility of boron in the phloem and enhances the tolerance of boron deficiency by forming a boron-sorbitol complex (Brown and Hu, 1996; Hu et al., 1997; Brown et al., 1999; Bellaloui et al., 1999). Sorbitol accumulation may also play a role in resistance to fire blight (Erwinia amylovora) infection (Suleman and Steiner, 1994).

1.3. Starch synthesis and degradation

In leaves, starch is synthesized in the chloroplast during the day. The key enzyme in starch synthesis is ADP-glucose pyrophosphorylase (AGPase). AGPase is subject to allosteric regulation, being activated by 3-phosphoglycerate (PGA) and inhibited by Pi (Preiss, 1988; Stitt, 1991). When sucrose synthesis decreases, phosphorylated intermediates accumulate, and P_i content in the cytosol falls. This causes an increase in PGA content and a decrease in P_i content in the chloroplast, leading to up-regulation of starch synthesis (Neuhaus et al., 1989; Preiss and Sivak, 1996; Stitt, 1991). AGPase is also regulated via redox modification in response to light and sucrose levels in leaves (Hendriks et al., 2003). In cases where 3-PGA/Pi ratio is not altered, redox regulation seems to explain the changes in AGPase activity and starch synthesis (Tiessen et al., 2002).

Antisense inhibition of AGPase expression in potato plants (Müller-Röber et al., 1992; Leidreiter et al., 1995) resulted in lower starch in leaves. Leaf soluble sugars remained unchanged. However, a higher proportion of the assimilated carbon is transported from leaves to sink tissues during the light period. As a result, photosynthesis is not affected at ambient CO_2. When grown at elevated CO_2, transgenic plants with decreased AGPase activity have lower photosynthesis than the control (Ludewig et al., 1998). This indicates that the capacity for starch synthesis limits photosynthesis at elevated CO_2 in these transgenic plants. When potato plants are double-transformed with antisense constructs of TPT and AGPase (Hattenbach et al., 1997), photosynthesis at ambient CO_2 remains unchanged in transgenic plants with 50% reduction in AGPase, but it is significantly lower in those with 95% reduction in AGPase. Both lines have lower CO_2 saturated photosynthesis than control plants. Starch deficient mutants of Arabidopsis have lower CO_2 assimilation at both ambient CO_2 and at saturated CO_2 compared to controls

(Sun et al., 1999). This indicates that starch synthesis plays an important role in ameliorating any potential reduction in photosynthesis caused by feedback regulation.

Transitory starch in source leaves provides carbon for sinks at night (Caspar et al., 1985; Schulze et al., 1991). Significant progress has been made recently in understanding the pathway of starch degradation and carbon export out of chloroplasts at night. Starch granules are first attacked by a previously unknown enzyme called glucan, water dikinase, which phosphoylates the C6 or C3 positions of the glucosyl residues of amylopectin (Ritte et al., 2002). It appears that the presence of the phosphate residue is required for starch degradation to proceed (Lorberth et al., 1998; Yu et al., 2001). The breakdown of α-1,6-linkage in amylopectin is accomplished by debranching enzymes. The resulting linear glucans are primarily converted to maltose by β-amylase (Scheidig et al., 2002) and, to a much less extent, to glucose by the action of disproportionating enzyme on maltotriose (D-enzyme, Critchley et al., 2001). Most of the carbon from starch degradation is exported as maltose via the maltose transporter (MEX1) with a smaller fraction as glucose to the cytosol (Schleucher et al., 1998; Niittylä et al., 2004; Weise et al., 2004). Mutants of Arabidopsis (mex1-1 and -2) lacking a functional MEX1 accumulate high levels of both maltose and starch and have yellow leaves (Niittylä et al., 2004). The maltose exported to the cytosol is metabolized by a cytosolic glucanotransferase, which catalyzes the transfer of a glucosyl residue onto a polyglucan acceptor (Chia et al., 2004; Lu and Sharkey, 2004), releasing the second glucose. The nature of the acceptor is currently unknown, but it's likely that a cytosolic maltodextrin phosphorylase is involved, resulting in formation of glucose-1-phosphate (G1P), as maltodextrin phosphorylase does in E. coli (Lu and Sharkey, 2004). Both the glucose exported from chloroplast and the glucose released from maltose by cytosolic glucanotransferase are converted to G6P by hexokinase (Veramendi et al., 1999). Finally both G6P and G1P are used for sucrose synthesis.

2. Organic acid metabolism and nitrogen metabolism in source leaves

Although the pathways of primary metabolism have been well established, their regulatory metabolisms are still not fully understood. Considerable efforts have been made on model plants to figure out a universal principle in regulating plant central metabolism but always appended with flexibilities, exceptions or adaptations to various external and internal factors (Fernie et al., 2004). Multiple entry points come from diverse respiratory substrates, such as sucrose, sorbitol, starch, glucose, hexoses, hexose phosphates, lipids, proteins, and amino acids. Respiratory isozymes exist in the cytosol, plastid, mitochondria or nucleus and this multiple compartment complicates the functions of these pathways. Alternative pathways, non-cyclic TCA cycle, and non-phosphorylating miETC pathways all interact with the predominant routes to develop the metabolic flexibility (Plaxton and Podestá, 2006; Van Dongena et al., 2011). With the metabolism of photosynthetic end products reviewed above, the following parts will be focused on the major respiratory pathways in carbon metabolism and interrelated nitrogen metabolism (Fig. 1-2).

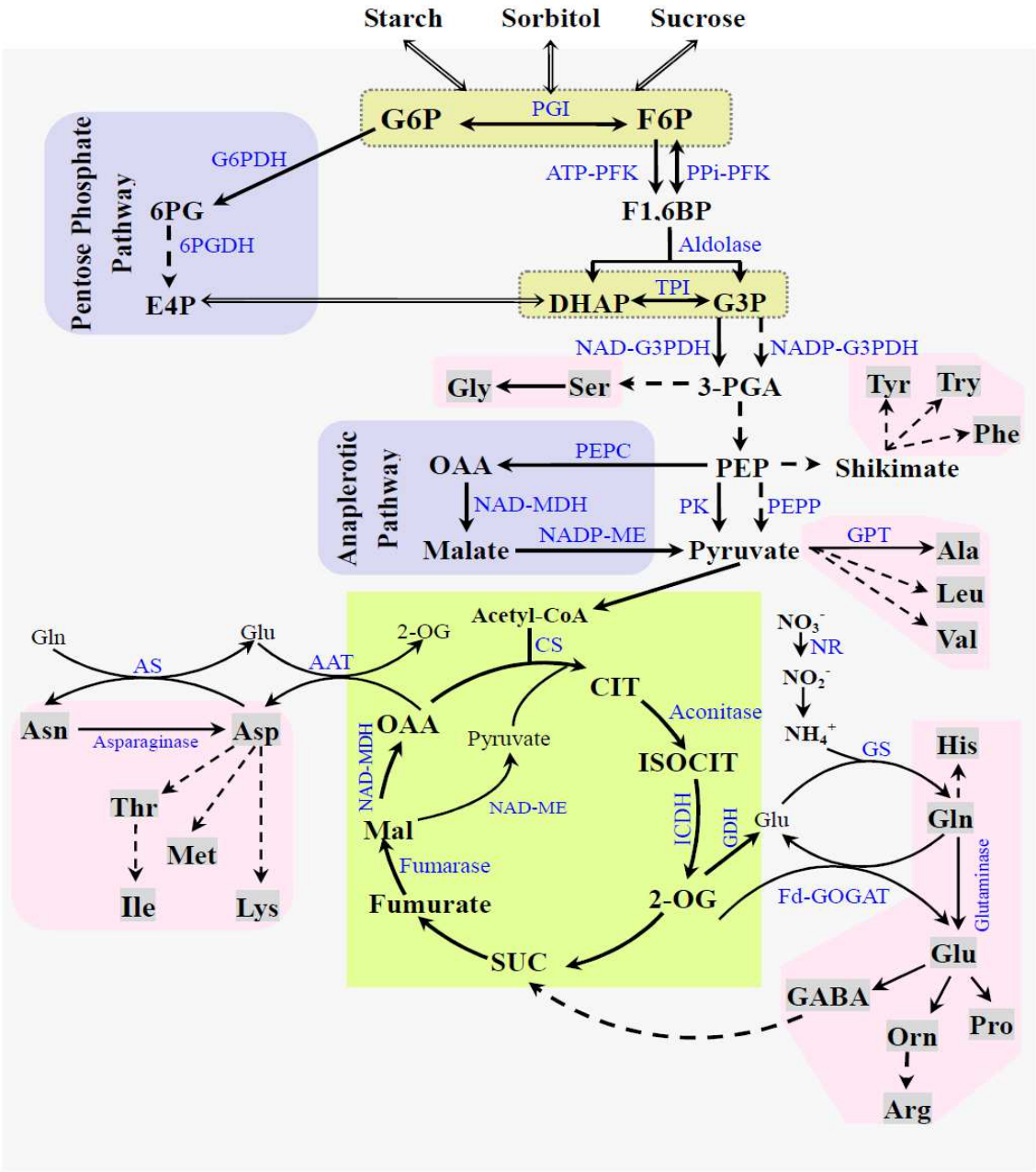

Figure 1-2. Primary carbon and nitrogen metabolism in source leaves. Abbreviations of enzymes are as follows:AAT, aspartate aminotransferase; AS, asparagine synthetase; ATP-PFK, ATP-phosphofructokinase; CS, citrate synthase; FBPase, fructose-1,6-bisphosphate phosphatase; Fd-GOGAT, Ferredoxin -glutamate synthase; G3PDH, glyreraldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GPT, glutamate pyruvate transaminase; GS, glutamine synthetase; HK, Hexokinase; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; NR, nitrate reductase; PEPC, phosphoenolpyruvate carboxylase; PEPP, phosphoenolpyruvate phosphatase; PGI, phosphoglucose isomerase; PK, pyruvate kinase; PPi-PFK, PPi-phosphofructokinase; TPI, triose phosphate isomerase.

2.1. Glycolysis

Glycolysis, which occurs in the cytosol and plastids, is the oxidation of glucose to pyruvate. Glycolysis provides substrates and generates ATP and reducing equivalents for TCA cycle and amino acid biosynthesis, secondary metabolism, and other cellular activities. As mentioned above, sucrose, sorbitol, starch, hexoses, hexose phosphates and triose phosphates can all serve as a carbon source for glycolysis. From a classic point of view, any metabolic pathway is regulated by the total activity or the amount of enzymes involved (Fig. 1-2), which provides 'coarse' control to the pathway. However, transgenic plants with altered amount of glycolytic enzymes, such as hexokinase (HK), phosphoglucomutase (PGM), phosphofructokinase (PFK), and glyreraldehyde-3-phosphate dehydrogenase (G3PDH) only brought tiny changes on respiration rate (Fernie et al., 2002; Veramendi et al., 2002; Davies, 2005; Hajirezaei et al., 2006). Van Dongena et al (2011) pointed out that, modifying the activities of individual glycolytic enzymes could not be considered as a key controlling mechanism of plant respiration. More efforts should be focused on the regulatory mechanisms at the transcriptional and posttranslational level (Tang et al., 2003).

The 'fine' metabolic control of glycolysis primarily involves the phosphorylated level of F6P and phosphoenolpyruvate (PEP) turnover from the bottom up (Plaxton and Podestá, 2006). The concept of 'bottom-up' regulation of plant respiration has been well established, i.e. downstream intermediates can feedback to the upstream steps to simulate or inhibit enzymes there (Beaudry et al., 1989; Hatzfeld and Stitt, 1991; Plaxton, 1996) to avoid a futile accumulation of intermediates as well as a waste of energy while meeting the demand for both substrates and energy. For example, the cellular level of ADP can regulate plant respiration from the downstream electron transport chain, up to TCA cycle, and finally to glycolysis. In all organisms, the conversion of F6P to F1,6BP catalyzed by PFKs and FBPase is considered to be a

major regulatory step in glycolysis, and plant-specific PPi-PFK brings more flexibilities into the system. Both ATP-PFK and PPi-PFK are subject to allosteric inhibition of PEP whereas the latter is activated by another key regulatory metabolite, F2,6BP. Besides PEP and F2,6BP, other metabolite effectors also function in the allosteric regulation of glycolysis, such as G6P, F6P, F1,6BP, Pi, maltate, glutamate (Glu) and aspartate (Asp). PEP turnover is highly active in plants. The conversion of PEP to pyruvate is primarily catalyzed by pyruvate kinase (PK) whereas the phosphoenolpyruvate carboxylase-malate dehydrogenase-malic enzyme (PEPC-MDH-ME) bypass of PK is activated under Pi deficiency (Moraes and Plaxton, 2000; Le Roux et al., 2006). PEP is also the substrate of shikimate pathway, which leads to the synthesis of flavonoids, lignin and other secondary metabolites.

According to Van Dongena et al (2011), PK, catalyzing the terminal step in glycolysis from PEP to pyruvate, possibly participates in the regulation of glycolysis. Pyruvate feeding increased the O_2 consumption rate in pea roots and the availability of pyruvate was a key regulator for the mitochondrial electron transport chain (Zabalza et al., 2009). The activation of PK dependeds on the contents of its two substrates ADP and PEP in addition to the presence of Mg^{2+} (Podestá and Plaxton, 1992; Ruiz et al., 1999). And ATP inhibition of PK was found in many organisms, such as tomato (Besford and Maw, 1975) and some seeds (Duggleby and Dennis, 1973; Nakayama et al., 1976). The activity of PK can also be regulated by the cellular need for carbon skeletons, ammonia assimilation and some amino acids. Cytosolic PK of green algae S. minutum was inhibited by Glu produced in the chloroplast (Lin et al., 1989). The first isoform of PK in spinach leaves was inhibited by citrate whereas the second isoform was activated by Asp and inhibited by Glu (Baysdorfer and Bassham, 1984).

2.2. Bypasses of glycolysis

The presence of more than one enzyme catalyzing a metabolic step contributes to the complexity and flexibility of plant metabolism. Besides the PEPC-MDH-ME bypass of PK, by which glycolysis can maintain the generation of pyruvate from PEP under Pi deficiency, PPi-PFK, non-phosphorylating G3PDH and phosphoenolpyruvate phosphatase (PEPP) can also bypass the regular glycolytic steps. Pi-starved plants are a good system for studying these bypasses because most of them are operating in these plants to circumvent adenine nucleotide or Pi-dependent steps. The review of Plaxton and Podestá (2006) provides a clear summarization of alternative pathways of cytosolic glycolysis in Pi-starved cells.

The PEPC-MDH-ME bypass is emphasized because it can not only circumvent the reaction catalyzed by PK under Pi depletion (Moraes and Plaxton, 2000; Miyao and Fukayama, 2003; Le Roux et al., 2006), but also replenish intermediates into TCA cycle to supply the biosynthesis of amino acids with carbon skeletons; PEPC can also regulate cellular pH and electroneutrality (Doubnerová and Ryšlavá, 2011). PEPC is a cytosolic enzyme, whose activity is regulated by both allosteric effectors and covalent modification: G6P activates whereas Asp inhibits its activity; phosphorylation of a Ser residue of PEPC by a Ca^{2+}-dependent PEPC kinase also activates this enzyme (Vidal and Chollet, 1997; Izui et al., 2003) and PEPC kinase is also inhibited by its allosteric effectors, malate and Glu. According to Borsani et al (2009), the activity of PEPC was increased during the post-harvest ripening of peach fruit and PEPC was a key component in controlling organic acid accumulation. Under excessive N supply, more N was partitioned into PEPC than rubisco, making PEPC a reservoir for redundant N in field-grown maize leaves (Uribelarrea et al., 2009). NADP-ME is another C4 photosynthetic enzyme which is believed to play important physiological roles in C_3 plants especially in stress, as antioxidative enzyme (Valderrama et al., 2006), or by responding to abiotic stresses via increasing its activity

to generate NADPH and pyruvate (Liu et al., 2007; Fahnenstich et al., 2007), and regulating stomatal closure by altering organic acid metabolism (Doubnerová and Ryšlavá, 2011). Different from NADP-ME, which is functioning in the cytosol and chloroplasts, NAD-ME is found in mitochondria. Besides the similar roles that NADP-ME plays, NAD-ME as well as NAD-MDH facilitates the mitochondrion to respire malate, but compared with the flux from PEP to pyruvate, the flux from malate to pyruvate is relatively subordinate (Plaxton and Podestá, 2006). Tronconi et al (2008) found that, the expression of Arabidopsis NAD-ME during the night period was higher than the day period; NAD-ME during the night period could convert excess mitochondrial malate to pyruvate and played a central role in coordinating carbon and amino acid synthesis in nocturnal metabolism. Doubnerová and Ryšlavá (2011) suggest that, when stomata are closed, PEPC recaptures CO_2 generated by NADP-ME; the generated NADPH was vital for amino acid biosynthesis, and PEP could be regenerated by pyruvate, phosphate dikinase (PPDK) at the expense of ATP consumption.

2.3. Oxidative pentose phosphate pathway

The oxidative pentose phosphate pathway (OPPP) is another route available for sugar oxidation in both the cytosol and plastids, with the one in plastids being predominant. This pathway comprises an irreversible section and a freely reversible section. In the irreversible section, G6P is oxidized to ribulose-5-phosphate via Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), with NADPH being generated; in the reversible section, ribulose-5-phosphate is converted to F6P and then G3P, which can regenerate G6P via glytolytic enzymes. OPPP functions at a very low rate in the chloroplast of photosynthetic cells. However, in photosynthetic cells during the dark period or non-photosynthetic cells, OPPP is the primary source for the generation of reducing equivalents

(NADPH) for N assimilation and lipid biosynthesis; the generated NADPH can also be involved in O_2 reduction. Intermediates of this pathway supply other metabolic pathways with substrates: ribulose-5-phosphate is required by DNA and RNA synthesis; erythrose-4-phosphate is needed in shikimate pathway for aromatic amino acids, lignin, and flavonoids synthesis (Kruger and Von Schaewen, 2003). OPPP might also be involved in the sugar-sensing pathway, regulating root nitrogen and sulfur acquisition (Lejay et al., 2008). G6PDH was found to be involved in the regulation of reduced glutathione levels and H_2O_2 accumulation in reed callus under salt stress (Wang et al., 2008). The disruption in cytosolic G6PDH resulted in a metabolic change in Arabidopsis that, more carbon skeletons were partitioned into storage compounds, such as seed oil content and mass (Wakao et al., 2008).

Generally speaking, the first reaction catalyzed by G6PDH is the key regulatory step in OPPP and both plastid and cytosolic G6PDH activity are inhibited by a high $NADPH/NADP^+$ ratio. However, the regulatory mechanism of cytosolic G6PDH is much more complicated. Hauschild and Von Schaewen (2003) developed a model about the regulation of cytosolic G6PDH in potato leaves. Via sugar signaling to the nucleus, higher hexoses content increased mRNA level of cytosolic G6PDH, which led to higher enzyme activities.

2.4. TCA cycle

Conventional TCA cycle occurs in the matrix of mitochondria, by which pyruvate and malate generated in the cytosol are converted to CO_2 and H_2O, providing energy and reducing power (Fig. 1-2). Similar to the regulation of glycolysis, changes in TCA enzyme activities, such as citrate synthase, aconitase, or MDH only slightly affect cellular respiration rate. Although TCA cycle is largely regulated by the mitochondrial electron transport chain itself (Van Dongen et al., 2011), evidences indicate that the early steps in TCA cycle that generate 2-oxoglutarate (2-

OG) are parts of TCA cycle regulation and maintaining the level of 2-OG is important for regulating N and amino acid metabolism (Hodges, 2002; Stitt and Fernie, 2003; Fernie et al., 2004). In addition to the conventional TCA cycle, some specific non-cyclic TCA fluxes also exist in plants which can adjust the carboxylic acid metabolism to be more suitable for the biosynthesis of amino acids (Sweetlove et al., 2010). In the illuminated spinach leaves, the mitochondria took malate as a respiratory substrate and the TCA cycle enzymes catalyzed a non-cyclic flux converting OAA to citrate via malate and pyruvate. The generated citrate was then exported to the cytosol supporting ammonium assimilation (Hanning and Heldt, 1993). The operation of TCA cycle was found to vary between daytime and nighttime in cocklebur leaves: TCA cycle ran as a conventional cycle at nighttime whereas as two weakly connected branched pathways at daytime with the stored citrate and intermediates produced by PEPC as precursors (Tcherkez et al., 2009). And the synthesized or stored citrate was an important precursor for Glu and GABA synthesis (Hanning and Heldt, 1993).

2.5. Nitrogen metabolism

Nitrogen (N) is vital for plants to fulfill fundamental cellular functions as the primary constituent of nucleoside phosphates, phospholipids, proteins, nucleic acids and secondary metabolites. N exists in inorganic or organic forms in leaves. Inorganic N includes nitrate and ammonium; organic N mainly includes amino acids and proteins. Proteins account for a large proportion of leaf total N (Chapin and Kedrowski, 1983) and 70-80% of these proteins perform their functions in chloroplasts in C_3 plants (Morita and Kono, 1975). For example, rubisco, the key enzyme in plant photosynthesis, usually accounts for 20-30% of leaf total N in C_3 plants (Evans, 1989).

Both ammonium and nitrate are taken up by plants roots (Näsholm et al., 2009) from soil

via high or low affinity transporters. The fates of nitrate in plant cells are various (Crawford and Glass, 1998). In addition to efflux back to the apoplasm and storage in the vacuole and transport from roots to shoots, nitrate is rapidly converted to ammonium by NR (Srivastavab, 1980) and NiR (Joy and Hageman, 1966) and this so-called nitrate assimilation occurs in both roots and shoots. Ammonium in plant cells also comes from photorespiration and amino acid cycling. Amino acids are then synthesized in both roots and shoots mainly via glutamine synthetase (GS) and glutamate synthase (GOGAT) by incorporating ammonium from different sources into carbon skeletons where organic acids function as precursors or ammonium acceptors, which tightly coordinates nitrogen metabolism and carbon metabolism in plants (Dutilleul et al., 2005). Both nitrate assimilation and ammonium assimilation are transcriptionally and post-translationally regulated by various metabolic and cellular activities in plants and this complex mechanism primarily centers on plant carbon-nitrogen interactions and enzymes in both N and C metabolism are coordinately regulated, in response to the diurnal balance between N reduction and ammonium assimilation (Stitt et al., 2002).

3. Hypotheses

Previous work in our lab showed that, when 'Greensleeves' apple was transformed with a cDNA of A6PR in the antisense orientation, A6PR activity in mature leaves of the transgenic plants decreased to approximately 30 to 15% of the untransformed control. The antisense plants had lower contents of sorbitol but higher contents of sucrose and starch in mature leaves at both dusk and predawn. $^{14}CO_2$ pulse-chase labeling at ambient CO_2 demonstrated that partitioning of the newly fixed carbon to starch was significantly increased whereas that to sucrose remained unchanged in the antisense lines with decreased sorbitol synthesis. Contents of G6P and F6P were significantly higher in the antisense plants than in the control, but 3-phosphoglycerate

content was lower in the antisense plants with 15% of the control A6PR activity. F2,6BP content increased in the antisense plants, but not to the extent expected from the increase in F6P comparing sucrose-synthesizing species. There was no significant difference in CO_2 assimilation in response to photon flux density or intercellular CO_2 content. It was concluded that cytosolic FBPase activity in vivo was down-regulated and starch synthesis was up-regulated in response to decreased sorbitol synthesis. As a result, CO_2 assimilation in source leaves was sustained at both ambient CO_2 and saturating CO_2. Detailed characterization of the response of shoot tips to decreased sorbitol availability and increased sucrose availability indicate that sorbitol dehydrogenase is down-regulated whereas sucrose synthase is up-regulated in shoot tips of the transgenic apple trees with decreased sorbitol synthesis, leading to homeostasis of vegetative growth. Sorbitol and sucrose act as signal molecules to modulate the expression and activities of sorbitol dehydrogenase and sucrose synthase, both of which play an important role in determining the sink strength of apple shoot tips (Cheng et al., 2005; Zhou et al., 2006). Antisense plants were also expected to have decreased level of inorganic phosphate because the accumulation of hexose phosphates in the cytosol would tie up a large proportion of Pi.

Considering 1) the direct effects of antisense inhibition of A6PR are an accumulation in hexose phosphates (G6P and F6P) and a decrease in inorganic phosphate in the cytosol, 2) hexose phosphates are the direct substrates of primary metabolism, and 3) Pi itself is also involved in regulating primary metabolism (Plaxton, 1996; Duff et al., 1989), we hypothesized that 1) both organic acid metabolism and amino acid metabolism are up-regulated in the transgenic plants and 2) as a result of this up-regulation of organic acid metabolism and amino acid metabolism the transgenic plants are more tolerant of nitrogen deficiency. My dissertation research will be focused on testing these two hypotheses by comparing the transgenic plants with

the untransformed control under adequate N supply and N deficiency conditions in terms of metabolites and activities of key enzymes in primary metabolism.

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CHAPTER 2

ORGANIC ACID METABOLISM AND AMINO ACID METABOLISM IN TRANSGENIC APPLE LEAVES WITH DECREASED SORBITOL SYNTHESIS

Abstract

In apple and many other tree fruit species of the Rosaceae family, sorbitol serves as a main photosynthetic end-product, and a primary translocated form of carbon. Sorbitol synthesis shares the same hexose-phosphate pool with sucrose synthesis in the cytosol. The conversion from glucose-6-phosphate (G6P) to sorbitol-6-phosphate catalyzed by aldose-6-phosphate reductase (A6PR) is the limiting step in sorbitol synthesis. It has been shown that, when the expression of A6PR in 'Greensleeves' apple was decreased via antisense inhibition, A6PR activity in mature leaves was decreased to approximately 15-30% of the control, and consequently sorbitol synthesis was significantly decreased. As a result, both G6P and fructose 6-phosphate (F6P) accumulated in the cytosol at the expense of decrease in inorganic phosphate (Pi) content, leading to up-regulation of starch synthesis without altering CO_2 assimilation. Since G6P and F6P also play a pivotal role in glycolysis, and the down-stream tricarboxylic acid (TCA) cycle and amino acid synthesis, it was hypothesized that both organic acid metabolism and nitrogen metabolism are up-regulated in the antisense plants with decreased sorbitol synthesis. In this study, the activities of key enzymes in glycolysis, TCA cycle and amino acid biosynthesis, and the contents of organic acids and free amino acids in leaves of the antisense plants were compared with the untransformed control at both noon and midnight. The leaves of the antisense plants were found to had higher activities of several key enzymes in glycolysis, anaplerotic pathway and tricarboxylic acid cycle, higher respiration rate, and higher levels of organic acids

and amino acids in mature leaves than the untransformed control, which included higher activities of ATP-phosphofructokinase (ATP-PFK), PPi-phosphofructokinase (PPi-PFK), NAD-glyceraldehyde3-phosphate dehydrogenase (NAD-G3PDH), non-phosphorylating NADP-glyceraldehyde3-phosphate dehydrogenase (NADP-G3PDH), aldolase, enolase, phosphoenolpyruvate carboxylase (PEPC), pyruvate kinase (PK), NAD-malic enzyme (NAD-ME), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), and Ferridoxin-glutamate synthase (Fd-GOGAT), higher contents of phosphoenolpyruvate (PEP), pyruvate, succinate, citrate and oxaloacetate (OAA), and higher contents of glutamate, glutamine, aspartate, asparagine, serine, threonine, γ-aminobutyric acid, etc. These results indicate that both organic acid metabolism and amino acid biosynthesis are enhanced in the antisense plants. Moreover, when detached leaves of the untransformed control were fed with 10 mM mannose (a Pi sequester) to lower the Pi pool as a mimic of what happened in the antisense plants, organic acid metabolism showed a similar trend as found in the antisense plants, suggesting that the smaller pool of Pi also contributes to the responses of organic acid metabolism observed in the antisense plants to decreased sorbitol synthesis.

Introduction

Sorbitol is the dominant photo-assimilate synthesized along with sucrose and starch in apple source leaves. Sorbitol synthesis shares a common pool of hexose phosphates with sucrose synthesis in the cytosol, with the reduction of G6P to sorbtiol-6-phosphate, a reaction catalyzed by A6PR, being the key regulatory step (Negm and Loescher, 1981; Loescher et al., 1982). After unloading to sink tissues, sorbitol is converted to fructose via NAD-sorbitol dehydrogenase (NAD-SDH) and the resulting fructose enters downstream metabolic pathways (Yamaguchi et al., 1994; Oura et al., 2000). Sorbitol plays important roles in drought tolerance (Ranney et al., 1991;

Wang and Stutte, 1992; Lo Bianco et al., 2000), cold hardiness (Raese et al., 1978) and phloem mobility of boron (Blevins and Lukaszewski, 1998; Brown et al., 1999).

When 'Greensleeves' apple was transformed with a cDNA of A6PR in the antisense orientation, A6PR activity in mature leaves of the transgenic plants decreased to approximately 15-30% of the untransformed control (Cheng et al., 2005). The antisense plants had lower contents of sorbitol but higher contents of sucrose and starch in mature leaves at both dusk and predawn. In the antisense lines with decreased sorbitol synthesis, $^{14}CO_2$ pulse-chase labeling at ambient CO_2 demonstrated that partitioning of the newly fixed carbon to starch was significantly increased whereas partitioning to sucrose remained unchanged. Contents of G6P and F6P were much higher in the antisense plants than in the control, but 3-phosphoglycerate (3-PGA) content was lower in the antisense plants with 15% of the control A6PR activity. Fructose-2,6-bisphosphate (F2,6BP) content increased in the antisense plants, but not to the extent expected from the increase in F6P comparing sucrose-synthesizing species. There was no significant difference in CO_2 assimilation in response to photon flux density or intercellular CO_2 content. It was concluded that cytosolic FBPase activity in vivo was down-regulated and starch synthesis was up-regulated in response to decreased sorbitol synthesis. As a result, CO_2 assimilation in source leaves was sustained at both ambient CO_2 and saturating CO_2 (Cheng et al., 2005). As a result of decreased supply of sorbitol and increased supply of sucrose to shoot tips, SDH was down-regulated whereas sucrose synthase (SuSy) was up-regulated, leading to homeostasis of vegetative growth. So, both sorbitol and sucrose acted as signal molecules to modulate the expression and activities of SDH and SuSy, both of which played important roles in determining the sink strength of apple shoot tips (Zhou et al., 2006). These findings suggest that the altered carbon partitioning between sorbitol, sucrose and starch in source leaves affects the carbohydrate metabolism not only in both source leaves but also in sink organs. However, it is not known how

the down-stream processes that utilize carbohydrates in leaves (glycolysis / TCA cycle and amino acid metabolism) respond to the decreased sorbitol synthesis.

The significant accumulation of hexose phosphates resulting from antisense suppression of A6PR expression in the leaves of transgenic plants would directly influence glycolysis, TCA cycle, and amino acid metabolism because hexose phosphates are not only involved in photosynthetic carbon metabolism but also function as the starting point for central metabolism. Hexose phosphates enter glycolysis and TCA cycle to generate organic acids, which are then used for the synthesis of amino acids. Another G6P-consuming route is oxidative pentose phosphate pathway (OPPP). Changes in the contents of hexose phosphates have been found to affect all metabolic pathways listed above. Merwe et al. (2010) reported that, down-regulation of PPi-PFP, the enzyme catalyzing a readily reversible reaction between F6P and FBP, resulted in an eightfold increase in the hexose-phosphate: triose-phosphate ratio in immature internodes of transgenic sugarcane, which in turn drove an increased carbon partitioning to sucrose. In Arabidopsis shrunken seed 1 (sse1)/pex16 mutant seeds, a dramatic reduced rate of fatty acid synthesis caused starch accumulation (Lin et al., 2006). The levels of hexoses, hexose phosphates and most detectable sugars were found to be higher in sse1 and the elevated level of hexose phosphates might have directly caused the increased carbon partitioning to starch. More carbon also flowed into the downstream glycolysis and TCA cycle, and as a result, pyruvate, total TCA cycle intermediates and other organic acids were higher in sse1 mutant seeds. Based on these findings, it is predicted that the downstream organic acid metabolism and amino acid metabolism are stimulated by the accumulation of G6P and F6P in transgenic apple leaves.

The significant accumulation of hexose phosphates could also indirectly affect glycolysis, TCA cycle and amino acid metabolism by lowering the content of inorganic phosphate (Pi). Since the total amount of phosphates (sum of phosphorylated intermediates and inorganic

phosphates) in the cytosol is relatively constant, the significant accumulation of G6P and F6P would tie up a large amount of Pi in the cytosol of the transgenic plants with decreased sorbitol synthesis, consequently lowering the level of Pi even under adequate supply of phosphates. We have found that the level of Pi in leaf tissues of transgenic plants was significantly higher than that of the untransformed control, which indicated that the roots of the transgenic plants sensed this low Pi in the cytosol of leaves and responded to this Pi deficiency by taking up more Pi from the soil. In response to Pi starvation, plants have developed physiological and biochemical mechanisms in addition to morphological changes (Li et al., 2006; Plaxton and Carswell, 1999) to cope with low Pi. These include 1) enhancing the excretion of organic acids, malate and citrate, by PEPC to increase Pi availability in the rhizosphere (Plaxton and Carswell, 1999); 2) increasing the expression of high-affinity Pi transporter genes to allow plants to take up Pi more efficiently (Raghothama, 1999); 3) recycling and scavenging Pi via acid phosphatase, nuclease, phosphodiesterase and other metabolic phosphate recycling enzymes (Plaxton, 2004; Ticconi, 2010); 4) conserving the limited cellular pools of ATP via tonoplast H^+-pumping pyrophosphatase and using metabolic by-passes to circumvent those Pi-demanding steps and turn to use pyrophosphate (PPi) or even produce Pi (activation of PPi-PFK and upregulation of UTP-glucose pyrophosphorylase, NADP-G3PDH and PEP phosphatase (PEPP) (Plaxton, 1996; Duff et al., 1989). Of these physiological and biochemical mechanisms, those that involve glycolysis and TCA cycle are expected to up-regulate organic acid synthesis in response to low Pi in the transgenic apple leaves, leading to enhanced levels of both organic acids and amino acids.

Considering the possible direct and indirect effects of accumulation of hexose phosphates, it is hypothesized that both organic acid metabolism and amino acid metabolism are up-regulated in the transgenic apples plants with decreased sorbitol synthesis. The objective of this study is to

test the hypothesis above to gain insight into how photosynthetic carbon metabolism, organic acid metabolism and amino acid metabolism are coordinated in apple.

Methods and materials

Plant materials and growth conditions

Expression of antisense A6PR in 'Greensleeves' apple was described in detail in Cheng et al. (2005). RNA gel blotting showed that all the transformed lines had significantly lower A6PR transcript levels compared with the untransformed control. Line A27 had about 30% of the control A6PR activity. All the other lines (A04, A05, A10, A14, A18, A41, and A75) had about 15% of the control A6PR activity (Cheng et al., 2005).

One-year-old untransformed 'Greensleeves' plants (as the control) and three transformed lines A27, A04 and A10 grafted onto M26 rootstocks were used in the following experiment. After budbreak, all plants were cut back to 30cm and only one shoot was allowed to grow on each plant. They were fertilized with 15mM N Plantex NPK (20-10-20) with micronutrients twice weekly during the growing season. Fungicides and pesticides were sprayed at regular intervals during the growing season. Fully expanded, mature leaves were used for the biochemical and physiological measurements.

Respiration

Fully expanded, mature leaves of the control and antisense lines were taken at noon and adapted in dark for 30 min at 26°C. Leaf dark respiration was measured via a CIRAS-1 portable photosynthesis system (PP systems, Herts, UK).

Measurements of primary metabolites

Leaf discs were taken from fully expanded, mature leaves of the untransformed control and antisense lines A27, A04 and A10 at both noon (12:30PM-1:30PM) and midnight (12:00AM-1:00AM) on a sunny day at the end of July 2007 (90 days after budbreak), frozen in liquid N_2, and stored at -80°C until assayed.

Extraction and analysis of soluble sugars, sugar alcohols, hexose phosphates and organic acids

Non-structural carbohydrates, such as sugars, sugar alcohols, hexose phosphates, and most organic acids (citrate, 2-oxoglutarate (2-OG), succinate, fumarate, malate, glycerate and shikimate) were extracted, derivatized and analyzed according to the protocol of Lisec et al. (2006) with some modifications. Three leaf discs (4 cm^2 in size; about 100 mg FW) were extracted in 1.4 ml 75% (v/v) methanol with 60µl ribitol (0.6mg/ml) added as the internal standard. After fractionating the non-polar metabolites into chloroform, the non-diluted, aqueous extracts were used to determine low abundance metabolites and the extracts at 1:20 dilution were used to measure high abundance metabolites, such as sorbitol, sucrose and malate. After sequential derivatization with methoxyamine hydrochloride and N-methyl-N- trimethylsilyl-trifluoroacetamide (MSTFA), metabolite profiling was operated at an Agilent 7890A GC /5975C MS (Agilent Technology, Palo Alto, CA, USA). Injection, chromatography and MS parameters were described in detail in Wang et al. (2010). Metabolites were identified by comparing fragmentation patterns against a mass spectral library generated on our GC-MS system and an annotated quadrupole GC-MS spectral library and quantified based on standard curves generated for each metabolite and internal standard.

3-phosphoglycerate (3-PGA), pyruvate, phosphoenolpyruvate (PEP), oxaloacetate (OAA),

isocitrate, fructose-1,6-bisphosphate (F1, 6BP), glyceraldhyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) were extracted according to Chen et al. (2002) with modifications. Five leaf discs (6 cm^2 in size) were pulverized with a liquid N_2 pre-cooled mortar and pestle in 2 ml of 5% (v/v) $HClO_4$ and 100 mg insoluble PVPP ($(C_6H_9NO)_X$, MW 111.1). After centrifugation at 16100 g for 10 min, the supernatant was withdrawn and neutralized with 5M KOH/1M triethanolamine, keeping on ice for assay. 3-PGA was measured according to Chen and Cheng (2003). Pyruvate, PEP, OAA, isocitrate, and F1,6BP ere measured according to Chen et al. (2002) with minor modifications. Pyruvate was assayed in 1ml reaction medium containing 100 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM ADP, 0.1 mM NADH, and 1 unit of LDH (EC 1.1.1.27). PEP was assayed in the same reaction medium of pyruvate assay by adding 1 unit of PK (EC 2.7.1.40). OAA was assayed in a 1ml reaction medium containing 100 mM triethanolamine-HCl (pH 7.6), 0.1 mM NADH, and 1 unit of MDH (EC 1.1.1.37). Isocitrate was assayed in 1ml reaction medium containing 100 mM Tris-HCl (pH 7.6), 3.3 mM $MnSO_4$, 0.15 mM NADP, and 1 unit of ICDH (EC 1.1.1.42). F1,6BP was assayed in 1ml reaction medium containing 100 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADH, 2 units of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 4 units of TPI (EC 5.3.1.1), and 0.5 unit of aldolase (EC 4.1.2.13). G3P and DHAP were assayed according to Copper et al. (1958).

Extraction and analysis of amino acids

Amino acid analysis was performed at a HP1100 Liquid Chromatograph equipped with an Agilent 1200 fluorescence detector (Agilent Technology, Palo Alto, CA, USA). Contents of twenty free amino acids glutamate (Glu), glutamine (Gln), proline (Pro), arginine (Arg), γ-aminobutyric acid (GABA), ornithine (Orn), histidine (His), asparate (Asp), asparagine (Asn), threonine (Thr), lysine (Lys), methionine (Met), isoleucine (Ile), alanine (Ala), valine (Val),

leucine (Leu), serine (Ser), glycine (Gly), tyrosine (Tyr), and phenylalanine (Phe) were measured in this study. Extraction, derivatization of free amino acids and chromatography parameters were described in detail in Wang et al. (2010). Amino acids were indentified and quantified according to Cohen and Michaud (1993) with modifications.

Assay of enzymes involved in primary metabolism

Enzymes in carbon and nitrogen metabolism were extracted according to the protocols of Häusler et al. (2000), Häusler et al. (2001) and Chen and Cheng (2003) with modifications. Enzymes used in assays were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the unit of enzymes was in standard form (IU). Contents and combinations of reagents used in assays were optimized according to standard enzyme assay procedures described in Bergmeyer et al. (1983) and preliminary assays performed in our lab.

Assay of enzymes in carbon metabolism

ATP-phosphofructokinase (ATP-PFK), PPi-phosphofructokinase (PPi-PFP), NAD-glyreraldehyde-3-phosphate dehydrogenase (NAD-G3PDH) and non-phosphorylating NADP-G3PDH were extracted from five leaf discs (6 cm^2 in size) with 1.5 mL extraction mixture containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% (w/v) insoluble PVPP, and a pinch of sand. The homogenate was centrifuged at 16,000 g for 10 min and the resultant supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK). ATP-PFK and PPi-PFP were measured according to Moorhead and Plaxton (1988) and Botha et al. (1992) with modifications. ATP-PFK was assayed in a reaction mixture containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM NADH, 10 mM F6P, 25 mM NaH_2PO_4, 1mM ATP, 5 units of aldolase (EC 4.1.2.13), 5 units of TPI (EC 5.3.1.1), and 5 units

of G3PDH (EC 1.1.1.8). PPi-PFP was assayed in a reaction mixture containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM NADH, 10 mM fructose-6-phosphate, 1 mM PPi, 0.01 mM F2,6BP, 5 units of aldolase (EC 4.1.2.13), 5 units of TPI (EC 5.3.1.1), and 5 units of G3PDH (EC 1.1.1.8). NAD-G3PDH was assayed in a reaction mixture containing 50 mM triethanolamine-HCl (pH 8.5), 4 mM NAD, 10 mM sodium arsenate, 1 mM F1,6BP, and 6 units of alsolase (EC 4.1.2.13). Non-phosphorylating NADP-G3PDH was assayed in a reaction mixture containing 50 mM tricine-NaOH (pH 8.5), 0.4 mM NADP, 1 mM F1, 6BP, and 6 units of alsolase (EC 4.1.2.13).

Aldolase, enolase, pyruvate kinase (PK), PEP phosphatase (PEPP), phosphoglucose isomerase (PGI), hexokinase (HK), PEP carboxylase (PEPC), NAD-malate dehydrogenase (NAD-MDH), NAD-malic enzyme (NAD-ME), NADP-ME, glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), citrate synthase, aconitase, NADP-isocitrate dehydrogenase (NADP-ICDH), and fumarase were extracted from three leaf discs (4 cm^2 in size) with 1.5ml of extraction mixture containing 100 mM Hepes-KOH (pH 7.5), 30% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and 0.3% (v/v) Triton X-100, 4% (w/v) insoluble PPVP, and a pinch of sand. After centrifugation at 16000g for 10 min at 4 °C, the supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK). Aldolase was assayed in a 1ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM FBP, 0.2 mM NADH, 10 units of TPI (EC 5.3.1.1) and 1 unit of α-glycerophosphate dehydrogenase (EC 1.1.99.5). Enolase was assayed in a 1ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM 2-PGA, 0.2 mM NADH, 2 units of LDH (EC 1.1.1.27) and 2 units of PK (EC 2.7.1.40). PK was assayed in a reaction mixture containing 100mM Hepes-KOH (pH 7.0), 2 mM PEP, 2 mM ADP, 10 mM $MgCl_2$, 0.2 mM NADH, 0.2 mM ammonium molybdate, 0.2 mg/ml BSA, 2 mM DTT, and 2 units of LDH (EC

1.1.1.27). PEPP was assayed in the same mixture as PK in the absence of ADP and ammonium molybdate. PGI was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM NAD, 2 mM F6P and 2 units NAD-G6PDH (EC 1.1.1.49). HK was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 1 mM NAD, 1 mM ATP, 30 mM glucose and 2 units NAD-G6PDH (EC 1.1.1.49). PEPC was assayed in a reaction mixture containing 50 mM Tris-HCl (pH 9.2), 10 mM $MgSO_4$, 4 mM PEP, 10 mM $NaHCO_3$, 0.2 mM NADH and 5 units MDH (EC 1.1.1.37). NAD-MDH was assayed in a reaction mixture containing 50 mM Bicine-KOH (pH 8.0), 1 mM DDT, 0.2 mM NADH, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM OAA and 0.01% BSA. NAD-ME was assayed in a reaction mixture containing 100 mM imidazole-HCl (pH 7.0), 10 mM MgSO4, 30 μM coenzyme A (CoA), 2 mM NAD, and 20 mM malate. NADP-ME was assayed in the same reaction mixture with NAD-ME except that 0.25mM NADP instead of NAD. G6PDH was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgSO_4$, 5 mM KCl, 0.5 mM NADP and 3 mM G6P. 6PGDH was assayed in the same reaction mixture with G6PDH only except that 3mM 6-phosphogluconate was used instead of G6P. Citrate synthase was assayed in a 1ml reaction mixture containing 50 mM Tris-HCl (pH 7.8), 0.1 mM DTNB, 0.2 mM acetyl-CoA, and 0.5 mM OAA (Chen et al., 2009). Aconitase was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgSO_4$, 5 mM $MnCl_2$, 2 mM DTT, 5 mM NADP, 10 mM cis-aconitate, and 2 units of NADP-ICDH (EC 1.1.1.42). NADP-ICDH activity was measured in a reaction mixture containing 100 mM KH_2PO_4-KOH (pH 7.5), 5 mM $MgCl_2$, 0.25 mM NADP, and 250 mM isocitrate. Fumarase was assayed in a reaction mixture containing 100 mM KH_2PO_4 (pH 7.3), 50 mM L-malate followed by measuring the formation of fumarate at 240 nm.

Assay of enzymes involved in N and amino acid metabolism

Nitrate reductase (NR), glutamine synthetase (GS), aspartate aminotransferase (Asp-AT), glutamate pyruvate transaminase (GPT), asparaginase, glutaminase, glutamate dehydrogenase (GDH), and 3-PGA dehydrogenase (PGDH) were extracted from five leaf discs (6 cm^2) with 2 ml of extraction medium containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.3% (v/v) Triton X-100, and 5% (w/v) PVPP. The homogenate was centrifuged at 16,000g for 10 min and the supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK). NR was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM KNO_3, and 0.25 mM NADH, and incubated at 30°C for 30 min for the determination of nitrite production according to Hageman et al. (1980). The reaction was stopped by adding 0.5 ml 1% sulfanidamide (in 3M HCl), followed by addition of 0.5 ml 0.2% 1-naphthylamine and held for 15min. The mixture was then centrifuged at 13000 g for 5min. A standard curve was made using authentic KNO_2 and the absorbance was measured at 540 nm. GS was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 20 mM $MgSO_4$, 10 mM NH_2OH, 5 mM ATP, 30 mM Glu, incubating at 30 °C for 20 min, followed by addition of 0.7 ml $FeCl_3$ reagent (2.5% $FeCl_3$, 5% (w/v) TCA in 1.5 M HCl). The resulting precipitate was spun down at 16100 g for 5 min and the absorbance was measured at 540nm (González et al., 1995). A standard curve was made using authentic r-glutamyl hydroxamate. Asp-AT was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 25 mM Asp, 0.2 mM NADH, 25 mM 2-OG and 5 units MDH (EC 1.1.1.37) according to Murray and Kennedy (1980). GPT was assayed in a 1ml reaction mixture containing 100 mM Tris-HCl (pH 7.5), 25 mM Ala, 0.2 mM NADH, 10 mM 2-OG and 5 units LDH (EC 1.1.1.27). Asparaginase was assayed in a reaction system containing 62.5 mM KH_2PO_4-KOH (pH 7.5), 6.25 mM 2-OG, 12 mM Asn, 0.2 mM NADH and 1 unit of GDH (EC 1.1.1.47) according to Murray and Kennedy (1980). Glutaminase was assayed in the

same reaction system with asparaginase except that 12mM Gln was used instead of asparagine. GDH was assayed in a reaction system contained 100 mM Tris-HCl (pH 7.0), 10 mM 2-OG, 50 mM ammonium chloride, and 0.1mM NADH according to Lutts et al. (1999). PGDH was assayed in a reaction mixture containing 200 mM Tris-HCl (pH 9.0), 25 mM EDTA, 5 mM 3-PGA, 2.5 mM DTT, and 0.5mM NAD according to Ho et al. (1999).

Asparagine synthetase (AS) was extracted and assayed according to Bellucci et al. (2004). Three leaf discs (4 cm^2 in size) were extracted with 2 ml extraction buffer containing 200 mM Hepes-KOH buffer (pH 7.5), 10 mM DTT, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol (v/v), and 10% PVPP (w/v). After centrifugation at 15,000 g for 20 min at 4 ºC, the enzymatic activity of the Gln-dependent AS was determined by measuring the generation of glutamate via HPLC using the same method for free amino acid quantification described above. The 1 ml substrate mixture contained 200 mM KH_2PO_4-KOH (pH 7.5), 2 mM Gln, 10 mM ATP, 17 mM magnesium acetate, 2 mM L-Asp, and 400 μl of enzyme extract. The mixture was incubated at 37 ºC for 15 min and the assay was stopped by adding 1 ml of 80% ethanol followed by centrifugation at 5000 g for 5 min. Controls were assayed in the same conditions without Asp and ATP.

Fd-glutamate synthase (Fd-GOGAT) activity was assayed according to (Lea et al., 1990). The reaction mixture consisted of 200 mM KH_2PO_4-KOH (pH 7.5), 10 mM Gln, 10 mM 2-OG, 15 mM methyl viologen (the electron donor), and 1 mM amino-oxyacetic acid (transaminase inhibitor). After incubation at 30 ºC for 5 min, the reaction was started by the addition of reductant solution (47 mg $Na_2S_2O_4$, 50 mg $NaHCO_3$ in 1 ml of water). After 30 min of incubation at 30 ºC, the reaction was stopped by adding 1 ml of 100% ethanol followed by vigorous vortex. Fd-GOGAT activity was determined by measuring the Glu generation via HPLC using the same method for free amino acid quantification described above.

Feeding detached leaves with sucrose and mannose

Both sucrose and inorganic Pi have been reported to be involved in regulating primary metabolism in leaves (Koch, 2004; Rolland et al., 2006; Morcuende et al., 1998; Plaxton, 1996). To help determine which factor is primarily responsible for the metabolic changes observed in transgenic plants, feeding of exogenous sucrose and mannose (a Pi sequester) to the mature leaves of the untransformed control was used to mimic the higher sucrose content and the expected lower Pi content in the antisense plants. The reason mannose feeding decreases cytosolic Pi content is that mannose can be easily converted to mannose-6-phosphate in cytosol, which can tie up a significant amount of Pi (Weiner et al., 1992). On a sunny day in August 2007 (approximately 100 days after budbreak), mature leaves were detached from the untransformed control plants at the bottom of the petiole using a razor blade and re-cut under water immediately, and then brought to the lab. The detached leaves were fed with water (as the control), 50 mM sucrose or 10 mM mannose for 8 hr in a fume hood at a photon flux density of approximately 100 μmol m^{-2} s^{-1} and a temperature of 23°C. Each treatment was replicated five times in a completely randomized design. Leaf discs were taken at the end of the 8 hr feeding period, frozen in liquid nitrogen and stored at -80°C for analysis of metabolites and enzymes described above.

Results

Non-structural carbohydrates

Previous work in our lab (Cheng et al., 2005) showed that, compared with the untransformed control, both the partitioning of newly fixed carbon to starch and the contents of starch at dusk and predawn were significantly higher in antisense lines A27, A04 and A10. In this study, it was found that the content of sorbitol in antisense lines was significantly decreased

whereas the content of sucrose was significantly increased in three antisense lines at both noon and midnight (Fig. 2-1a, b). There was no significant difference between the control and antisense lines in glucose and fructose contents at noon whereas only A10 had a statistically lower level of fructose. However, the three antisense lines were found to have significantly decreased contents of glucose and fructose at midnight (Fig. 2-1c, d) and significantly decreased contents of galactose at both noon and midnight (Fig. 2-1e). Antisense lines A04 and A10 had significantly higher levels of maltose at both noon and midnight (Fig. 2-1f). Contents of myo-inositol were significantly increased in three antisense lines at both noon and midnight (Fig. 2-1g). Contents of ribose were significantly higher in three antisense lines at noon and in A10 at midnight (Fig. 2-1h).

Hexose phosphates and triose phosphates

Compared with the untransformed control, three antisense lines had significantly higher contents of both G6P and F6P at noon and this increase in hexose phosphate contents was kept in A04 and A10 at midnight (Fig. 2-2a, b). The contents of F1, 6BP were not changed in antisense lines at noon but slightly decreased in A10 at midnight (Fig. 2-2c). No significant differences were detected in G3P content at noon or midnight between the antisense lines and control (Fig. 2-2d). No significant difference was found in DHAP content between antisense lines and the control at noon (Fig. 2-2e); antisense lines A04 and A10 were found to have slightly higher contents of DHAP at midnight.

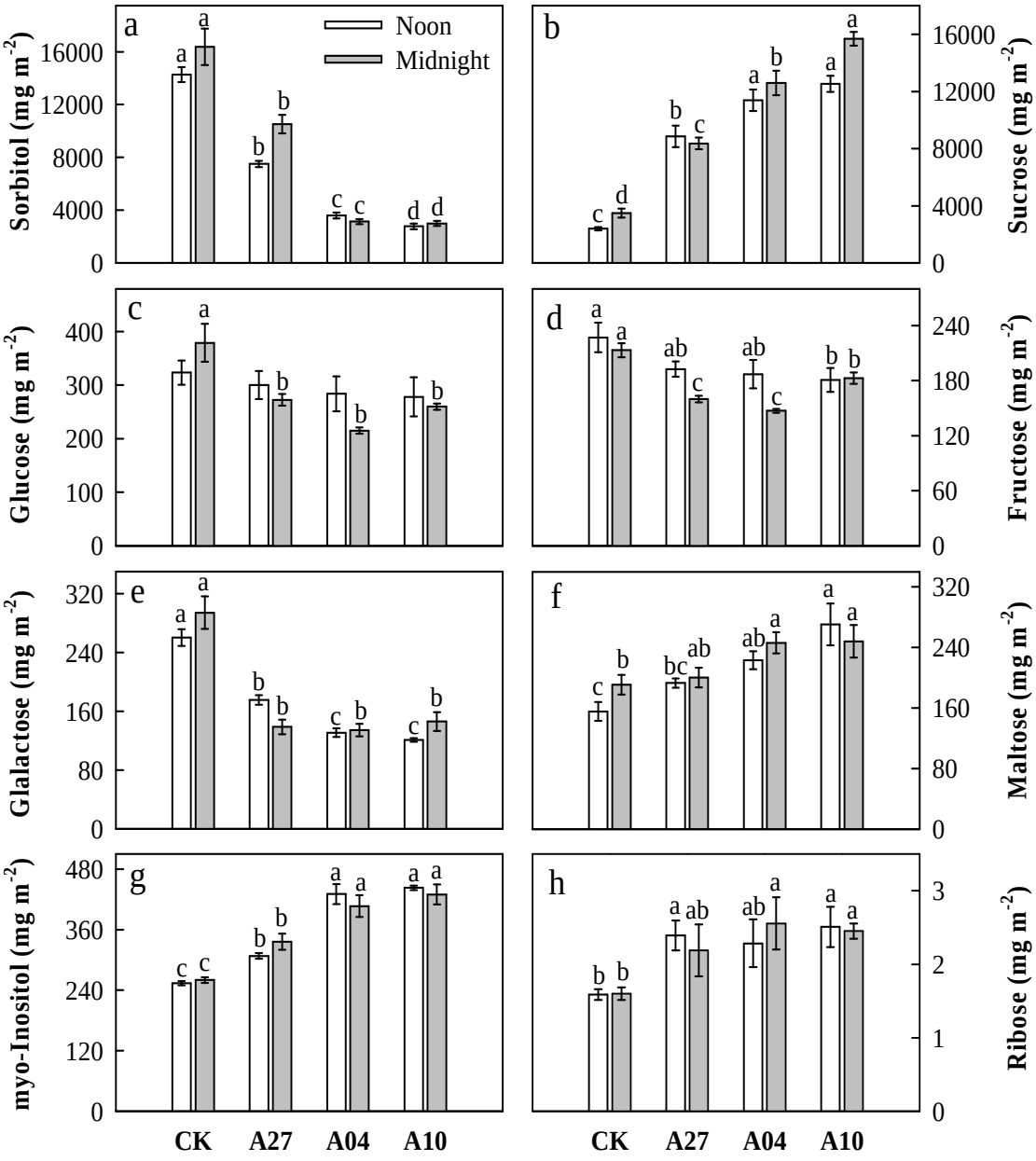

Figure 2-1. Contents of sorbitol (a), sucrose (b), glucose (c), fructose (d), galactose (e), maltose (f), myo-inositol (g), and ribose (h) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. CK: Untransformed control; Line A27 had about 30% of the control A6PR activity whereas both A04 and A10 had about 15% of the control A6PR activity. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

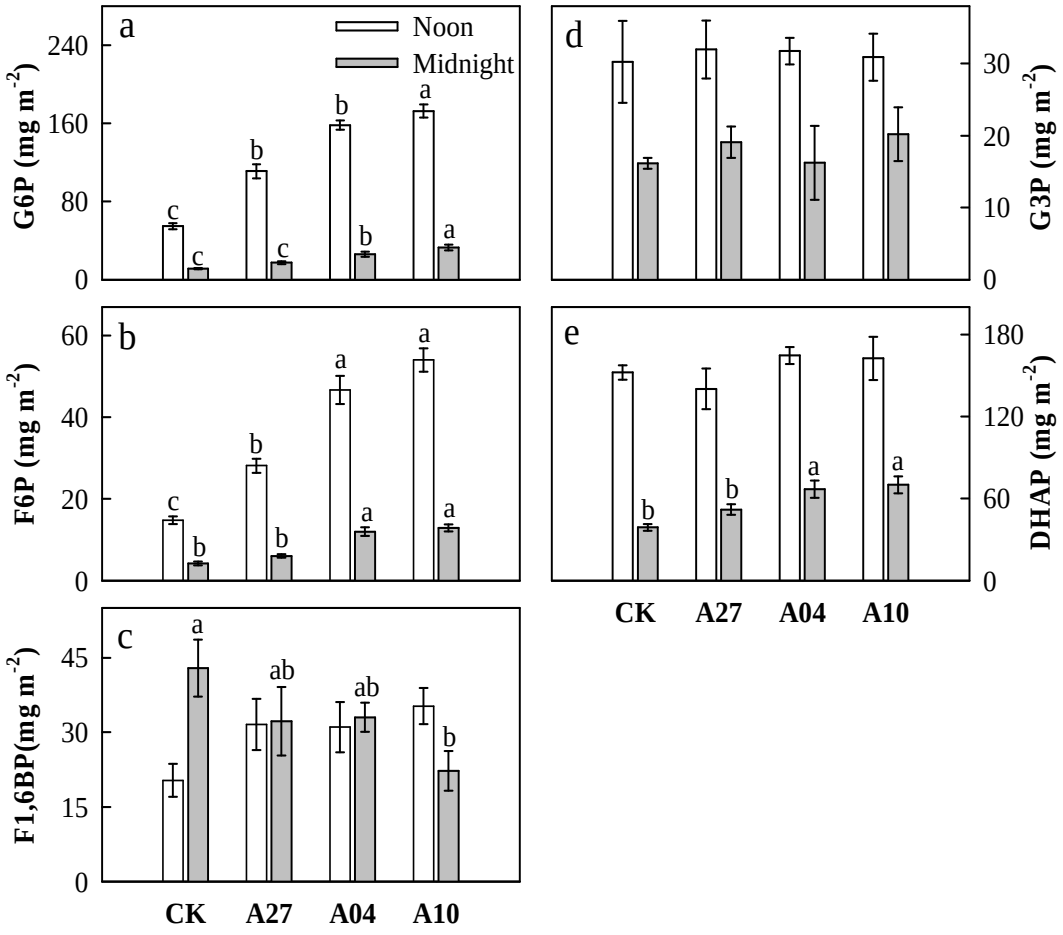

Figure 2-2. Contents of hexose phosphates (a-c) and triose phosphates (d-e) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Respiration rate

The dark respiration rates of all three antisense lines were significantly higher than the control (Fig. 2-3).

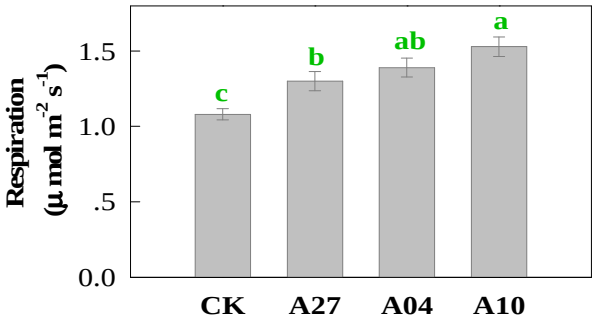

Figure 2-3. Respiration of mature 'Greensleeves' leaves. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$.

Organic acids

Compared with the untransformed control, contents of 3-PGA were lower in A04 and A10 at noon, but no difference was found at midnight (Fig. 2-4 a); antisense line A10 had significantly higher contents of PEP at both noon and midnight (Fig. 2-4b); the contents of pyruvate were higher in A04 and A10 at noon and in all three antisense lines at midnight (Fig. 2-4c); the contents of shikimate were higher in three antisense lines at noon and in A04 and A10 at midnight (Fig. 2-4d).

Compared with the control, the content of citrate was significantly increased in all three antisense lines at both noon and midnight (Fig. 2-4e); the content of isocitrate did not change (Fig. 2-4f); the content of 2-OG was slightly increased in antisense lines only at noon (Fig. 2-4g); the content of succinate was significantly increased in both A04 and A10 at noon and only in A04 at midnight (Fig. 2-4h); the content of OAA was significantly increased in all three antisense lines at noon and in both A04 and A10 at midnight (Fig. 2-4k); the content of fumarate was significantly decreased in all three antisense lines at noon and in A10 at midnight (Fig. 2-4i); the content of malate in A04 and A10 was significantly decreased at both noon and midnight (Fig. 2-4j). Additionally, the content of glycerate was significantly decreased in all three antisense lines at midnight (Fig. 2-4l).

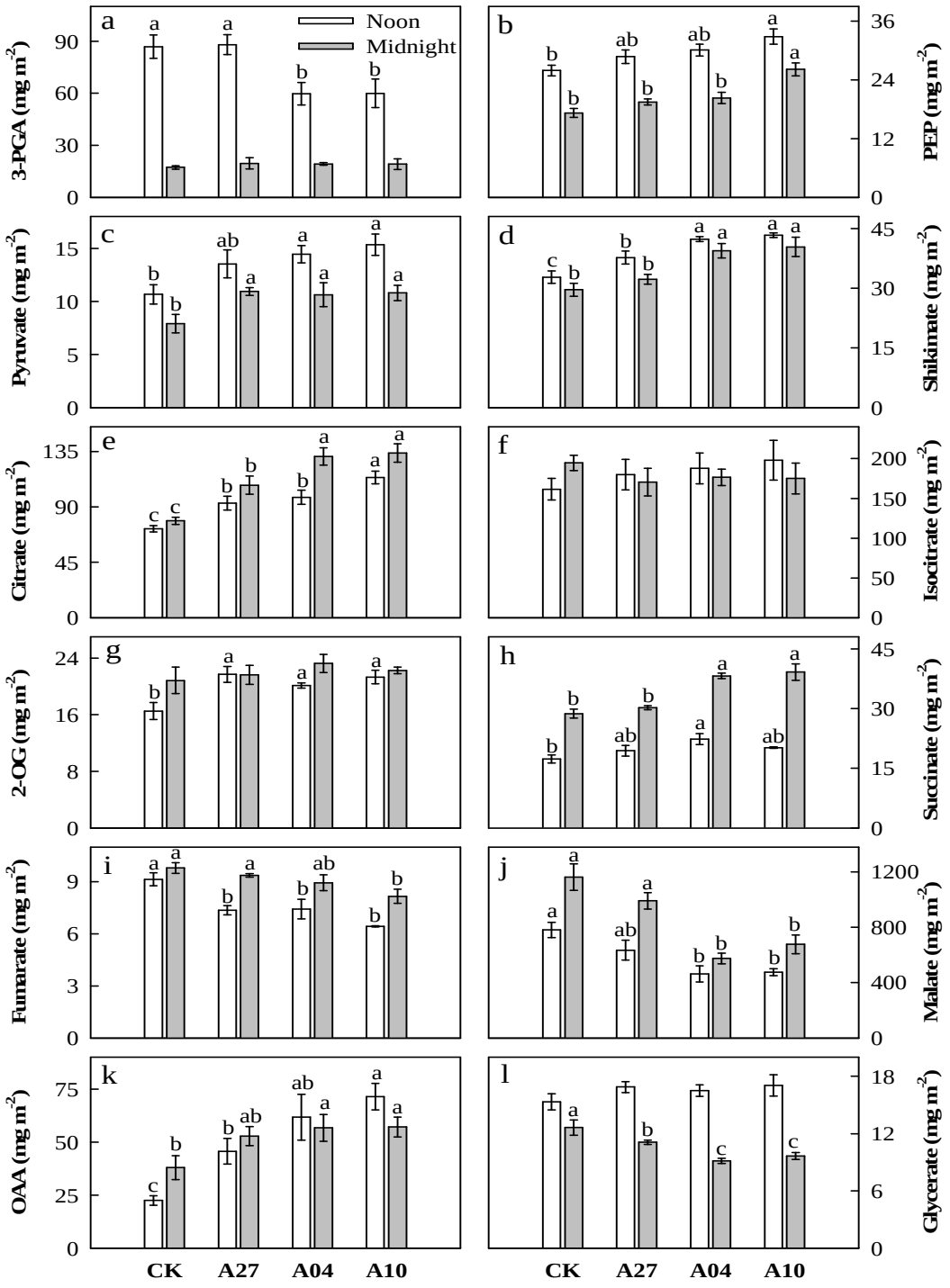

Figure 2-4. Contents of 3-phosphoglycerate (3-PGA, a), phosphoenolpyruvate (PEP, b), pyruvate (c), shikimate (d), citrate (e), isocitrate (f), 2-oxoglutarate (2-OG, g), succinate (h), fumarate (i), malate (j), Oxaloacetate (OAA, k), and glycerate (l) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Amino acids

Compared with the untransformed control, antisense lines A04 and A10 with 15% of the control A6PR activity had significantly higher contents of total amino acids at both noon and midnight (Fig. 2-5a).

For the seven amino acids in the Glu family, the content of Glu was significantly increased in all three antisense lines at noon and in both A04 and A10 at midnight (Fig. 2-5b). The contents of Gln and Arg were significantly increased only in A10 at noon (Fig. 2-5c, e). The content of Pro was significantly increased in all three antisense lines at noon, but was not altered at midnight (Fig. 2-5d). The content of GABA was significantly increased in all three antisense lines at both noon and midnight (Fig. 2-5f). The content of Orn was increased in A04 and A10 at midnight (Fig. 2-5g). The content of His was decreased in all three antisense lines at noon and in A10 at midnight (Fig. 2-5h).

For the six amino acids in Asp family, the content of Asp was significantly increased in both A04 and A10 at noon and in A10 at midnight (Fig. 2-6a). Asn content was significantly increased in both A04 and A10 at noon and in all three antisense lines at midnight (Fig. 2-6b). Thr content was significantly higher in A04 and A10 at both noon and midnight (Fig. 2-6c). The content of Lys was only slightly increased in A10 (Fig. 2-6d). No significant difference was detected in Met or Ile contents between antisense lines and the control at noon, but A10 had a slightly higher content of Met at midnight (Fig. 2-6e, f).

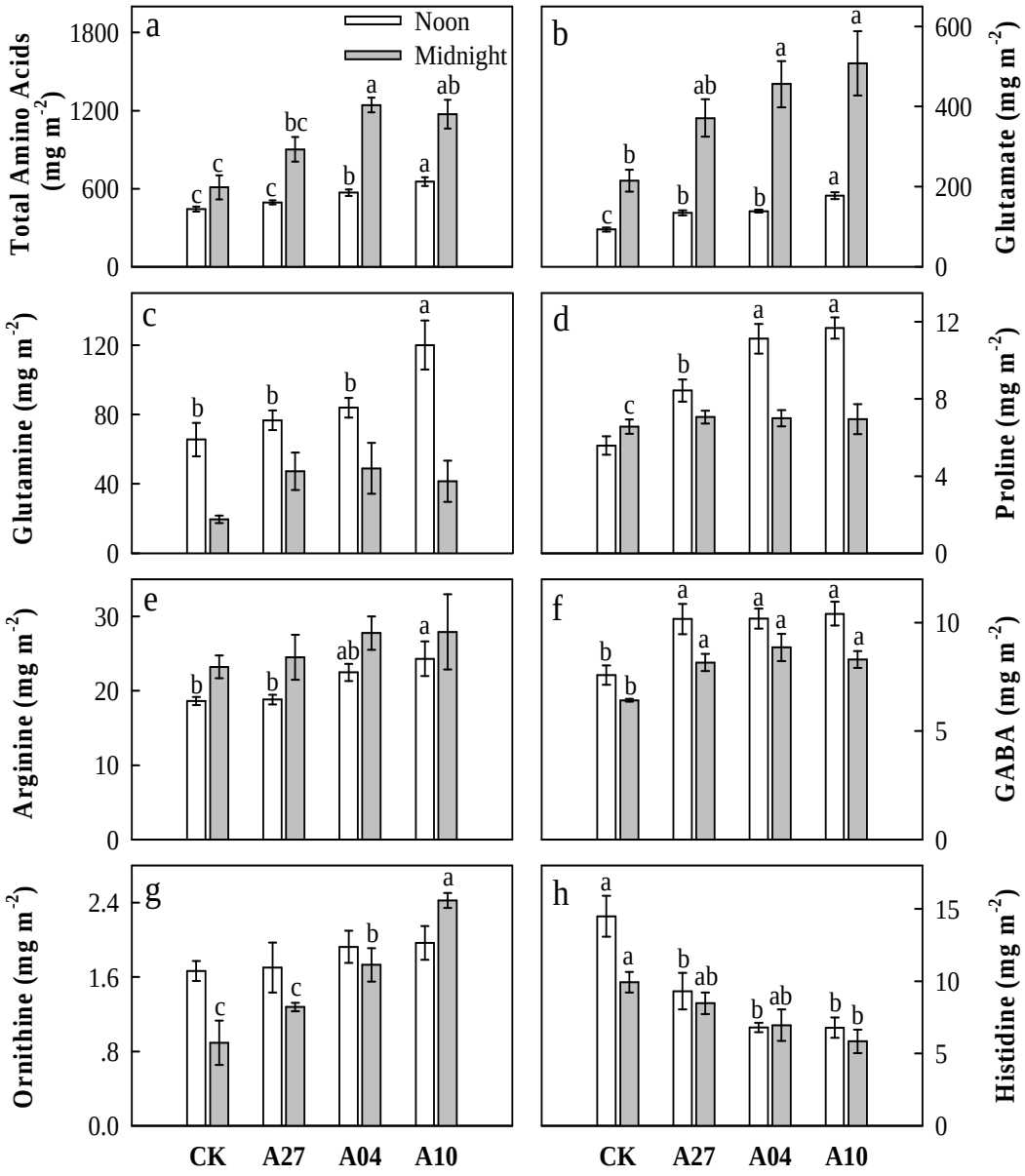

Figure 2-5. Contents of total amino acids (a) and glutamate family amino acids (b-h) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

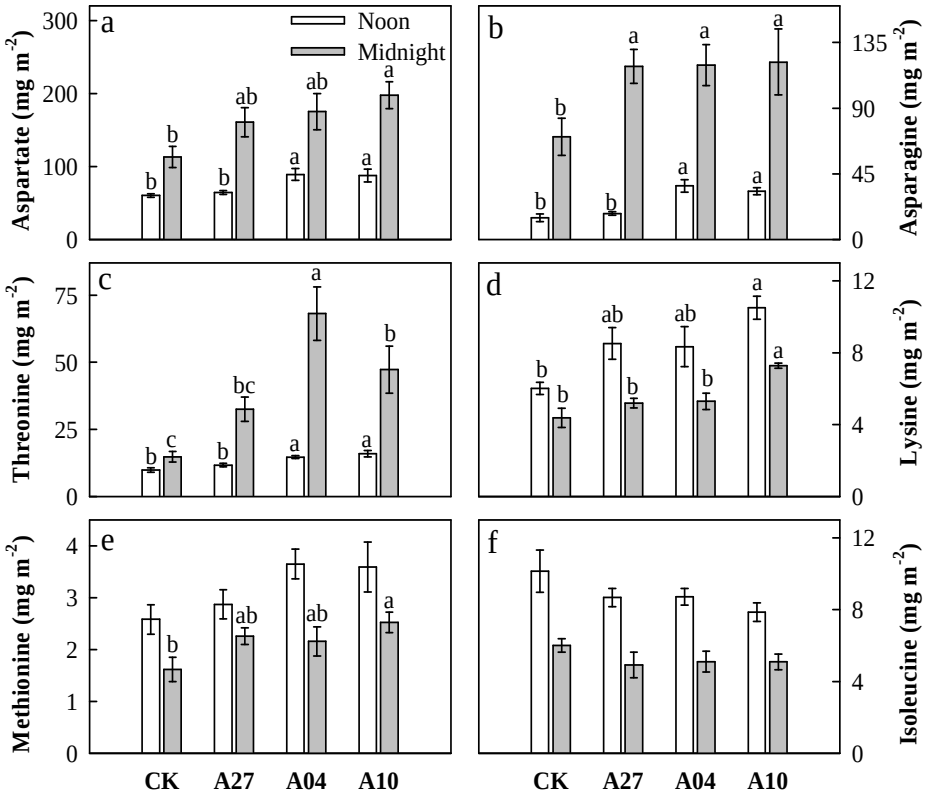

Figure 2-6. Contents of aspartate family amino acids (a-f) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

For three amino acids sharing pyruvate as the presursor, the content of Ala was significantly increased in all three antisense lines only at midnight (Fig. 2-7a); the content of Val was significantly decreased in all three antisense lines at noon and in both A04 and A10 at midnight (Fig. 2-7b) whereas the content of Leu was slightly increased only in A10 at midnight (Fig. 2-7c). The content of Ser was significantly increased in both A04 and A10 at noon and in A10 at midnight (Fig. 7d). Gly content was decreased in all three antisense lines at midnight (Fig. 2-7e). And for the two aromatic amino acids, Tyr content was significantly increased in both A04 and A10 at noon and in A10 at midnight (Fig. 2-7f); the content of Phe was significantly increased only in A10 at both noon and midnight (Fig. 2-7g).

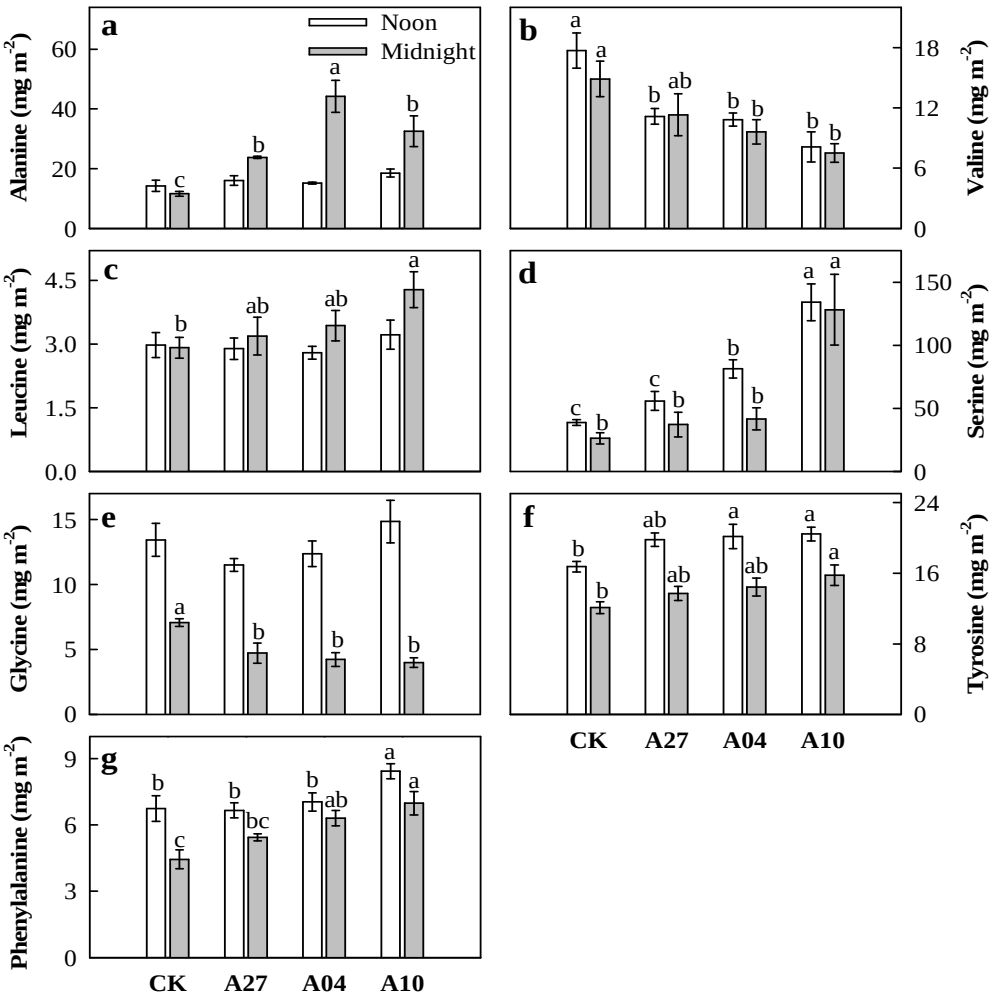

Figure 2-7. Contents of other free amino acids (a-g) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Enzymes in glycolysis

Compared with the untransformed control, the activities of ATP-PFK, PPi-PFK, NAD-G3PDH and NADP-G3PDH in A04 and A10 were all significantly higher at noon; whereas at midnight, the activities of ATP-PFK, NAD-G3PDH and NADP-G3PDH were not altered and the activity of PPi-PFK was significantly higher only in A10 (Fig. 2-8a, b, c, d). The activity of aldolase tended to be higher in antisense lines at noon, but only A10 had a statistically higher

aldolase activity (Fig. 2-8e). The activity of enolase was significantly higher only in A04 and A10 at midnight (Fig. 2-8f). The activity of PK was significantly higher in all three antisense lines at noon, but was not altered at midnight (Fig. 2-8g). The activity of PEPP was not altered at either noon or midnight (Fig. 2-8h). The activity of PGI was significantly higher in A10 at noon, but significantly lower in A04 and A10 at midnight (Fig. 2-8i). No significant difference was found in HK activity between antisense lines and the control at either noon or midnight (Fig. 2-8j).

Enzymes in anaplerotic pathway and pentose phosphate pathway

Compared with the untransformed control, the activity of PEPC was significantly higher in all three antisense lines at both noon and midnight (Fig. 2-9a). The activity of NAD-MDH was not altered (Fig. 2-9b). The activity of NAD-ME was significantly higher in all three antisense lines at noon and only in A10 at midnight (Fig. 2-9c). The activity of NADP-ME was statistically higher only in A10 at noon (Fig. 2-9d). For the first two enzymes in oxidative pentose phosphate pathway, the activity of G6PDH was significantly higher in both A04 and A10 only at midnight (Fig. 2-9e) whereas the activity of 6PGDH was significantly higher in both A04 and A10 at both noon and midnight (Fig. 2-9f).

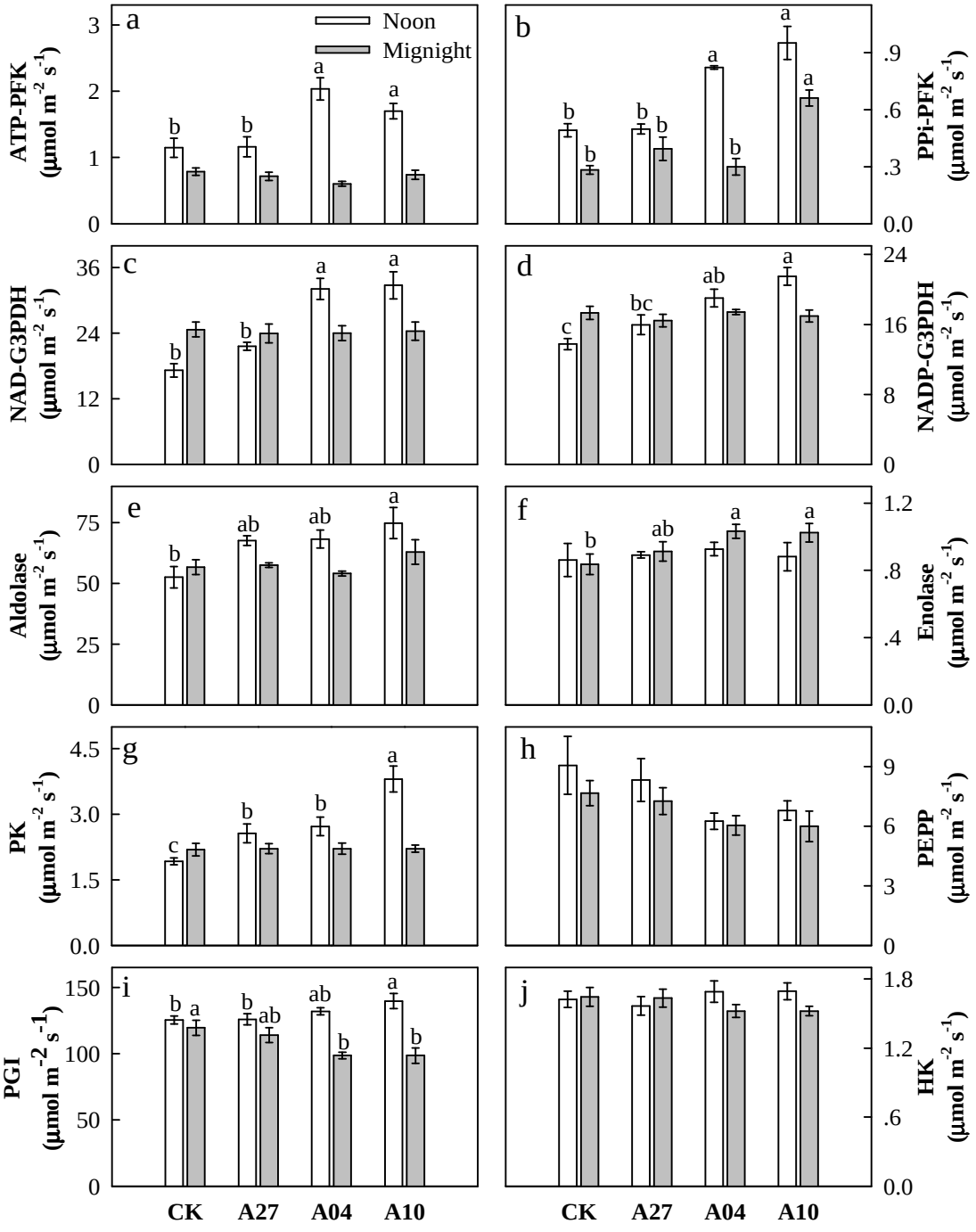

Figure 2-8. Activities of ATP-phosphofructokinase (ATP-PFK, a), PPi- phosphofructokinase (PPi-PFK, b), NAD-glyreraldehyde-3-phosphate dehydrogenase (NAD-G3PDH, c), NADP-G3PDH (d), aldolase (e), enolase (f), pyruvate kinase (PK, g), PEP phosphatase (PEPP, h), phosphoglucose isomerase (PGI, i), and hexokinase (HK, j) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

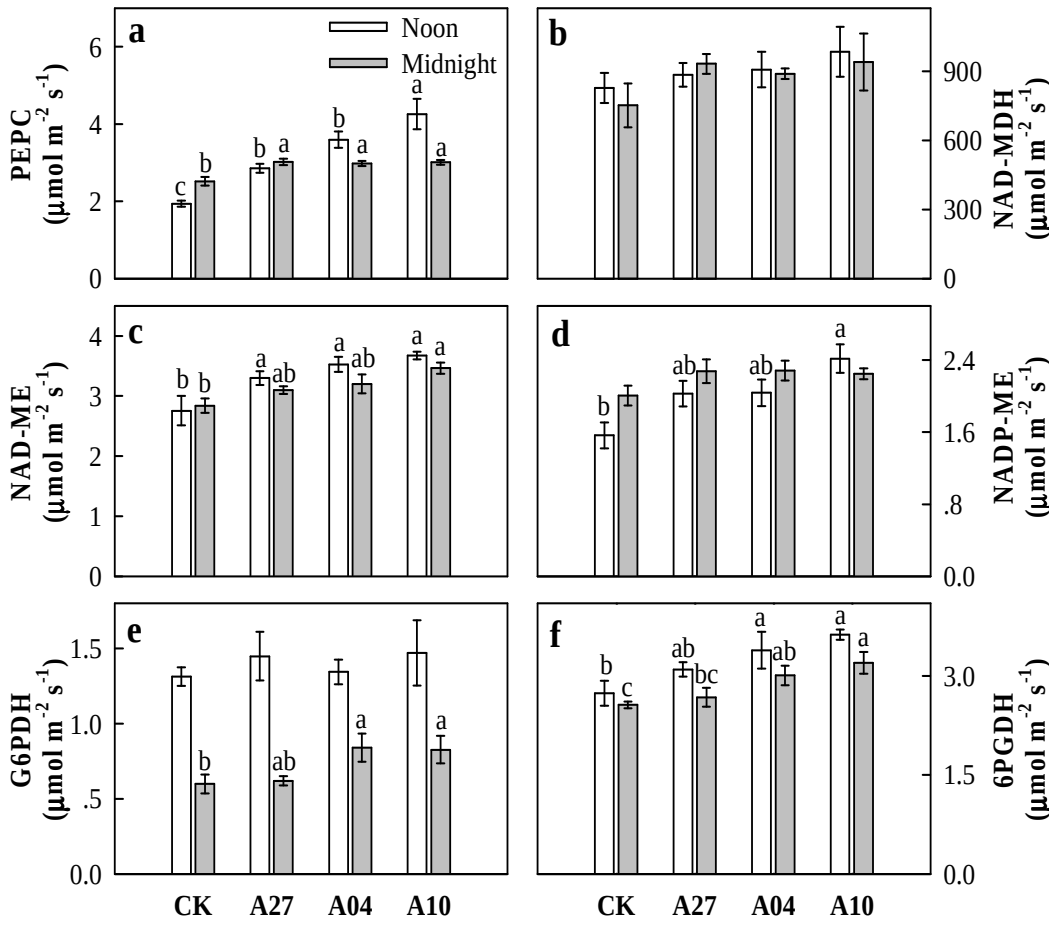

Figure 2-9. Activities of PEP carboxylase (PEPC, a), NAD-malate dehydrogenase (NAD-MDH, b), NAD-malic enzyme (NAD-ME, c), NADP-ME (d), glucose-6-phosphate dehydrogenase (G6PDH, e), and 6-phosphogluconate dehydrogenase (6PGDH, f) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Enzymes in TCA cycle

Compared with the untransformed control, the activities of both citrate synthase and aconitase were significantly higher in A04 and A10 at noon and only in A10 at midnight (Fig. 2-10a, b). No difference was found in the activity of NADP-ICDH or fumarase between antisense lines and the control at noon or midnight (Fig. 2-10c, d).

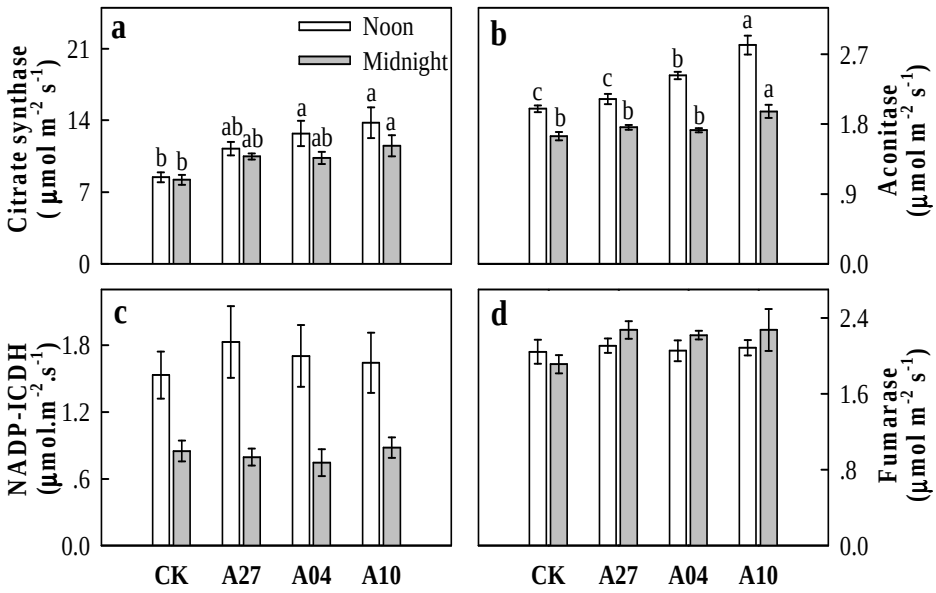

Figure 2-10. Activities of citrate synthase (a), aconitase (b), NADP-isocitrate dehydrogenase (NADP-ICDH, c), and fumarase (d) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Enzymes in N metabolism and amino acid synthesis

The activity of NR was not altered in antisense lines at noon but significantly lower than the control at midnight (Fig. 2-11a). The activity of Asp-AT was higher in antisense lines at midnight (Fig. 2-11b). No difference was detected in the activity of AS and GS between antisense lines and the control (Fig. 2-11c, f). Asparaginase activity was lower in antisense lines at noon (Fig. 2-11d). The activity of Fd-GOGAT and glutaminase was significantly higher in all three antisense lines at both noon and midnight (Fig. 2-11e, g). GDH activity was significantly lower in all three antisense lines at noon (Fig. 2-11h). GPT activity was significantly lower in all three antisense lines at noon and in both A04 and A10 at midnight (Fig. 2-11i). The activity of PGDH, which is important for serine synthesis in the dark was significantly lower in both A04 and A10 at noon, but significantly higher in A10 at midnight (Fig. 2-11j).

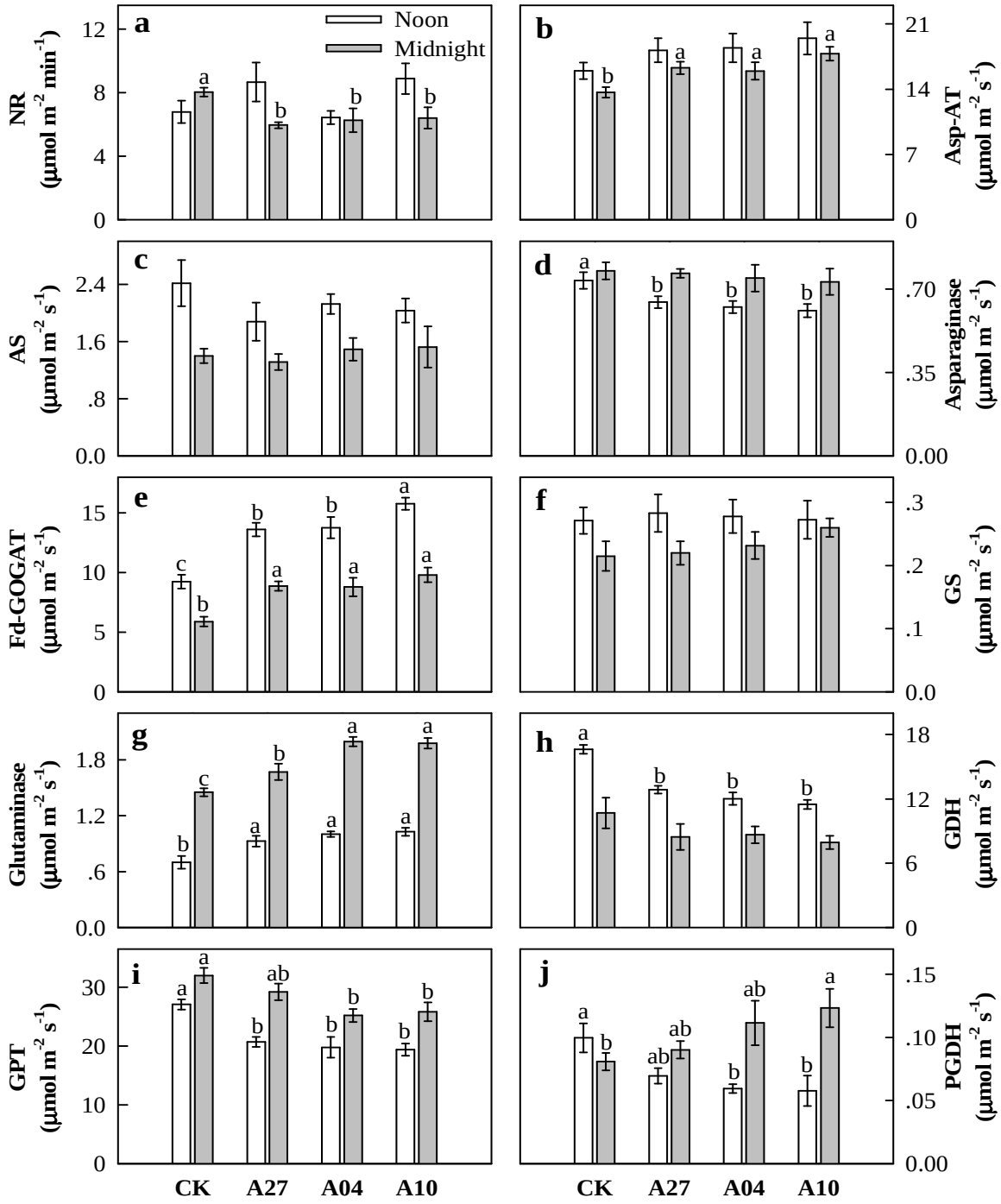

Figure 2-11. Activities of nitrate reductase (NR, a), aspartate aminotransferase (Asp-AT, b), asparagine synthetase (AS, c), asparaginase (d), Fd-glutamate synthase (Fd-GOGAT, e), glutamine synthetase (GS, f), glutaminase (g), glutamate dehydrogenase (GDH, h), glutamate pyruvate transaminase (GPT, i), and 3-PGA dehydrogenase (PGDH, j) in mature 'Greensleeves' leaves at noon and midnight. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$, for samples taken at noon and midnight, respectively.

Non-structural carbohydrates in H_2O, sucrose or mannose-fed leaves

Compared with H_2O-fed leaves, the contents of sucrose, glucose, fructose and galactose in sucrose-fed were significantly higher; the content of maltose was significantly lower; and the content of myo-inositol was not altered. In mannose-fed leaves, the contents of glucose, galactose, and maltose were significantly higher; the content of sorbitol was slightly lower; and the contents of sucrose, fructose and myo-inositol were not altered (Fig. 2-12a).

For hexose phosphates and triose phosphates, compared with H_2O-fed leaves, the contents of G6P and DHAP were significantly higher in sucrose-fed leaves whereas the contents of F6P and F1, 6BP were not altered and the content of G3P was even lower. In mannose-fed leaves, contents of G6P and F6P were significantly higher but contents of F1, 6BP, G3P and DHAP were not altered (Fig. 2-12b).

Organic acids in H_2O, sucrose or mannose-fed leaves

Compared with H_2O-fed leaves, only the content of succinate was significantly higher in sucrose-fed leaves whereas the content of all the other organic acids was not altered. However, mannose-fed leaves had significantly higher contents of pyruvate, succinate and OAA, and significantly lower contents of 3-PGA, glycerate and 2-OG. The content of PEP, shikimate, citrate, isocitrate, fumarate or malate was not altered in mannose-fed leaves (Fig. 2-13).

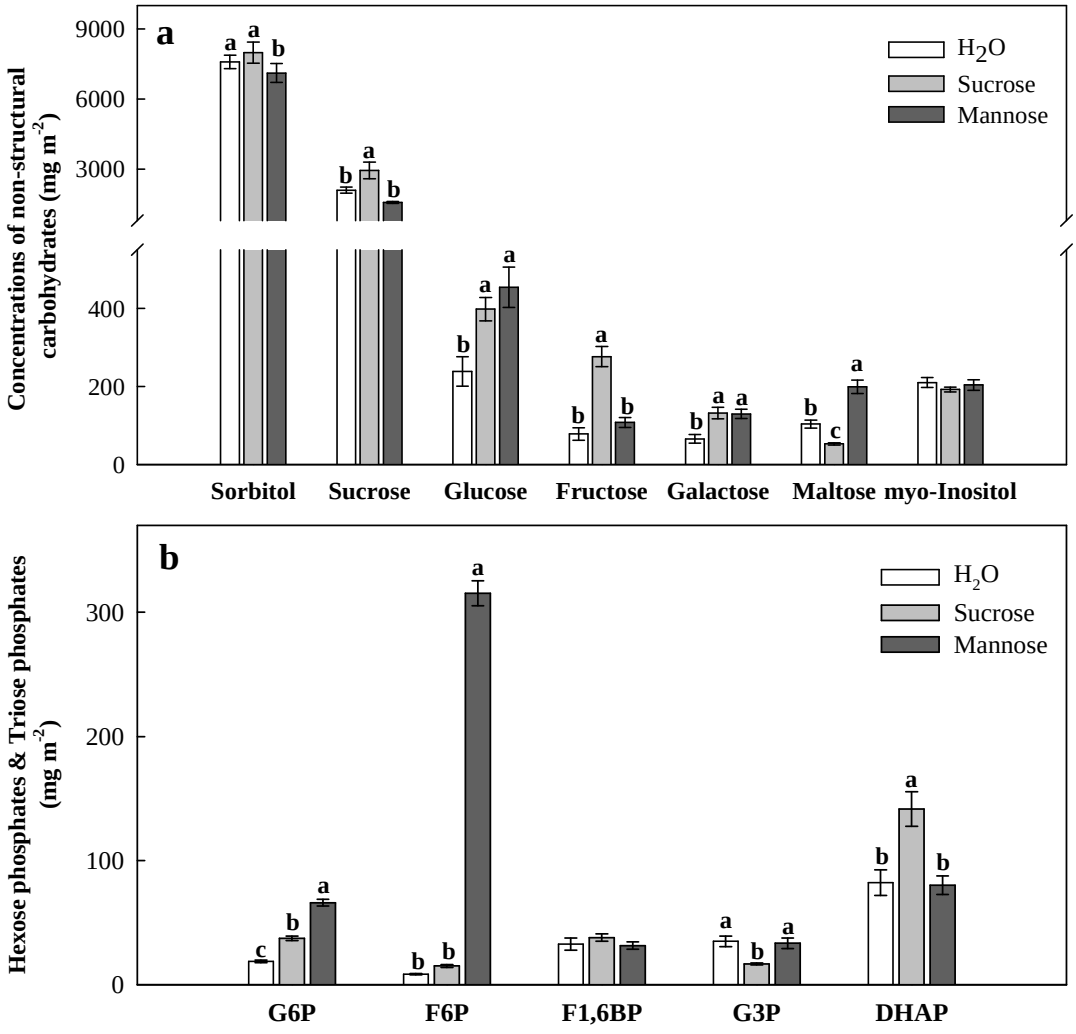

Figure 2-12. Contents of non-structural carbohydrates (a) and hexose phosphates & triose phosphates (b) in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

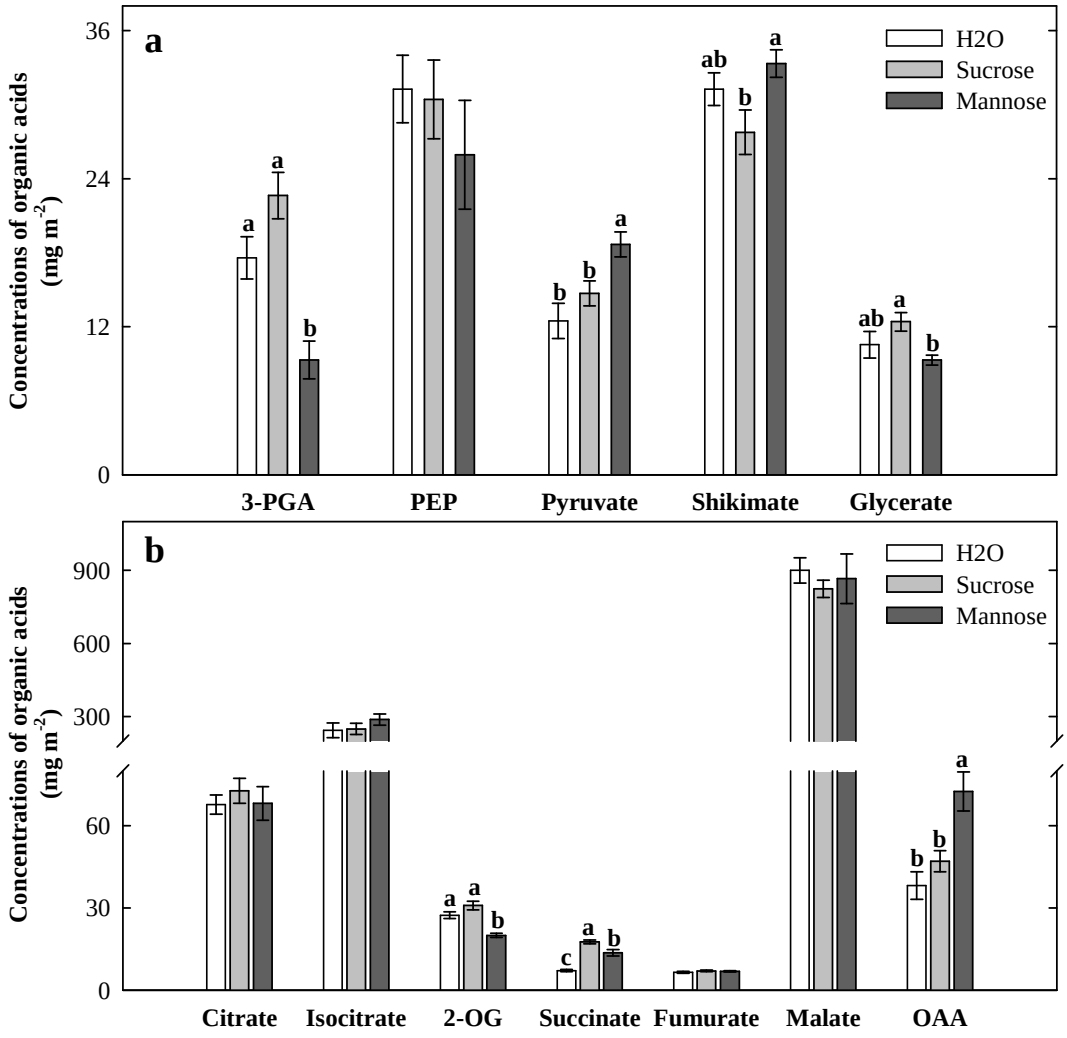

Figure 2-13. Contents of organic acids (a and b) in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

Amino acids in H_2O, sucrose or mannose-fed leaves

First of all, the contents of a large proportion of free amino acids in H_2O, sucrose and mannose-fed leaves were lower than those measured in control and antisense Leaf tissues taken at noon or midnight. For the seven amino acids in glutamate family (Fig. 2-14a), the contents of most amino acids were not altered in sucrose-fed leaves whereas only the content of GABA was slightly lower compared with H_2O-fed leaves. In mannose-fed leaves, the content of Pro was significantly higher; the contents of Glu and GABA were significantly lower; and the contents of Gln, Arg, Orn and His were not altered.

For the six amino acids in aspartate family (Fig. 2-14b), sucrose-fed leaves had a higher content of Thr but a lower content of Asp. The content of Ile was slightly higher in mannose-fed leaves whereas the contents of Asp, Thr and Lys were all significantly lower than those in H_2O-fed leaves.

For other amino acids (Fig. 2-14c), sucrose-fed leaves had significantly higher contents of Ala, Tyr and Phe but the same contents of Val, Leu, Ser and Gly. In mannose-fed leaves, the content of Ser was significantly higher whereas the contents of Ala and Leu were significantly lower. No significant difference was found between mannose-fed leaves and H_2O-fed leaves in terms of the contents of Val, Gly, Tyr and Phe.

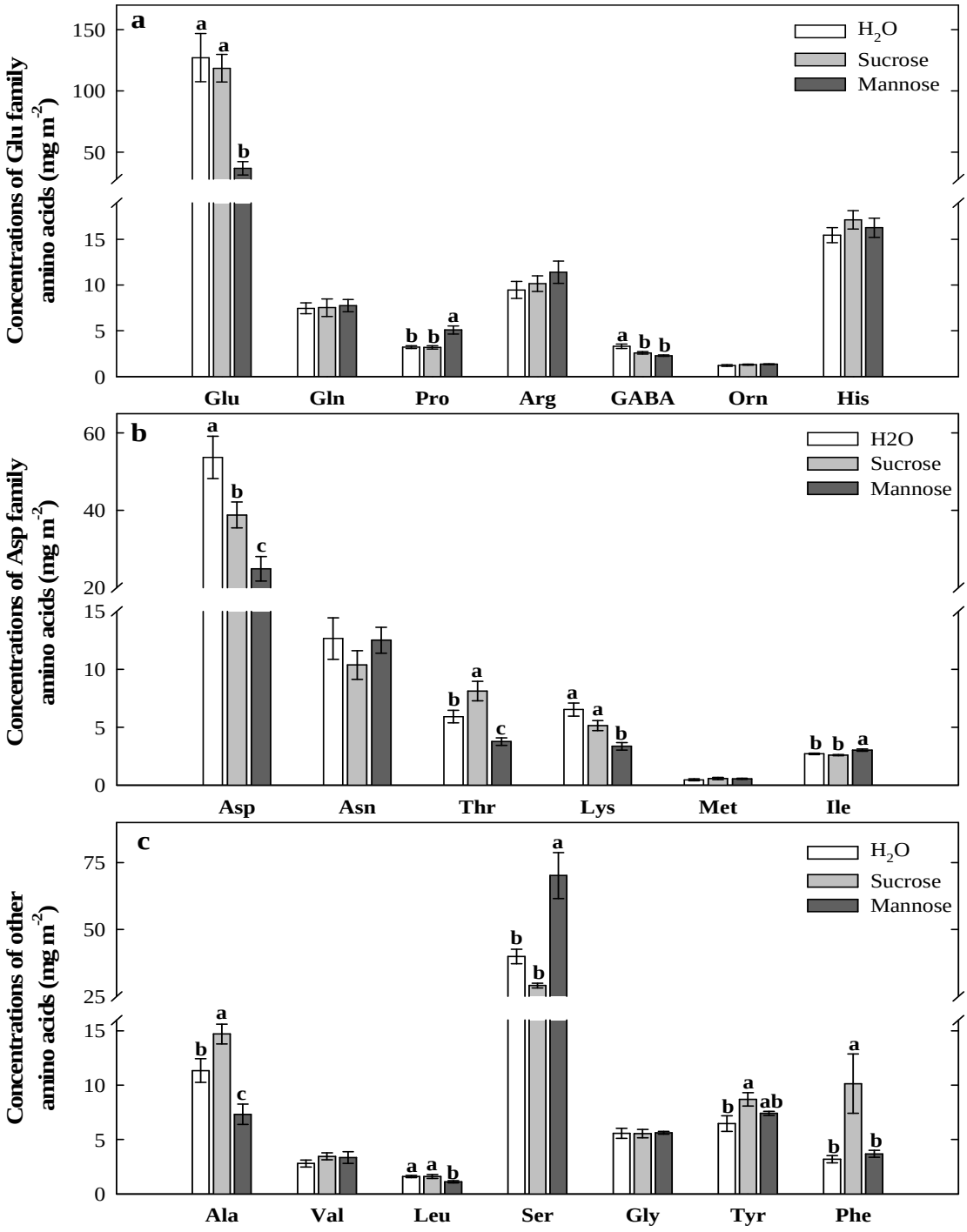

Figure 2-14. Contents of amino aicds (a-c) in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

Enzymes involved in primary metabolism in H_2O, sucrose and mannose-fed leaves

For key enzymes in glycolysis, no significant difference was found in the activity of ATP-PFK, PPi-PFP, NAD-G3PDH, NADP-G3PDH, aldolase, enolase, PK or PEPP between H_2O-fed leaves and sucrose-fed leaves. However, the activity of ATP-PFK in mannose-fed leaves was significantly higher than that of H_2O-fed leaves. The activity of PPi-PFP, NAD-G3PDH, NADP-G3PDH, aldolase, enolase, PK and PEPP was not altered in mannose-fed leaves (Fig. 2-15a).

For key enzymes involved anaplerotic and OPPP, the activity of PEPC was higher, but the activity of G6PDH was lower in sucrose-fed leaves whereas the activities of NAD-MDH, NAD-ME, NADP-ME and 6PGDH were not altered. However, mannose-fed leaves had significantly higher activities of PEPC, NAD-ME, G6PDH and 6PGDH. The activities of NAD-MDH and NADP-ME were not altered in mannose-fed leaves (Fig. 2-15b).

For the activity of citrate synthase, aconitase, NADP-ICDH and fumarase in TCA cycle, neither sucrose-fed leaves nor mannose-fed leaves showed any significant difference from those in H_2O-fed leaves (Fig. 2-16).

For the key enzymes involved in N and amino acid metabolism (Table 2-1.), sucrose-fed leaves had a significantly lower activity of Fd-GOGAT whereas mannose-fed leaves had a significantly higher activity of NR than H_2O-fed leaves. The activities of ASP-AT, asparaginase, GC, glutaminase, GDH and GPT were not altered in either sucrose-fed leaves or mannose-fed leaves.

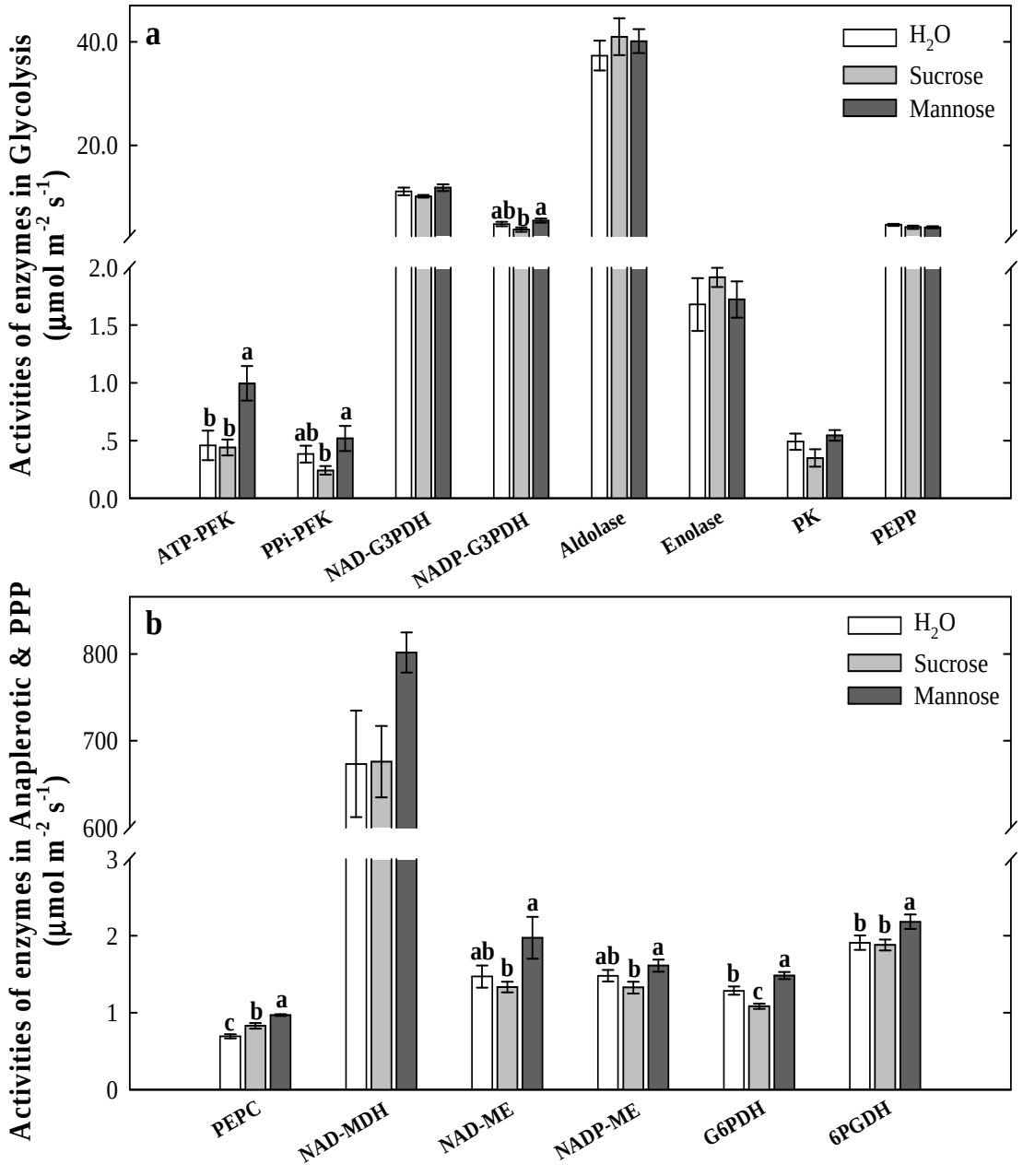

Figure 2-15. Activities of enzymes in glycolysis (a) andanaplerotic pathway & OPPP (b) in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

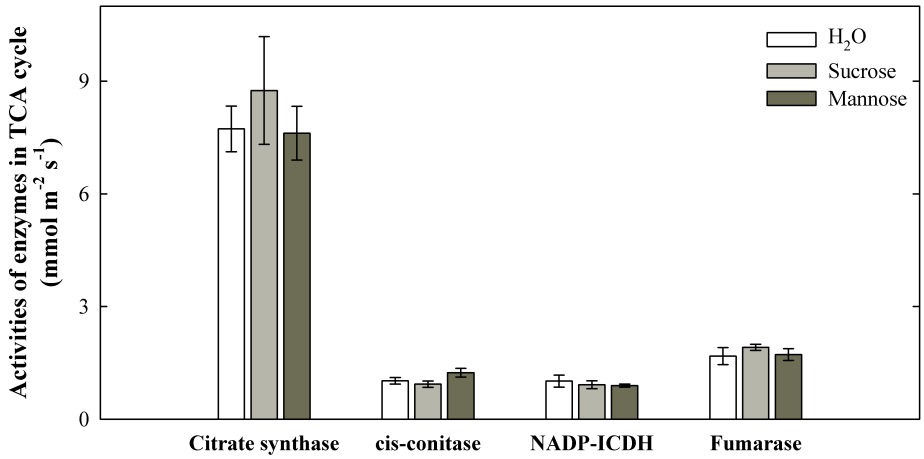

Figure 2-16. Activities of TCA cycle enzymes in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose. Each bar is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

Table 2-1. Activities of key enzymes in nitrogen metabolism in mature 'Greensleeves' leaves fed with H_2O, sucrose or mannose.

Enzymes	**H_2O**	50mM **Sucrose**	10mM **Mannose**
NR (μmol m^{-2} min^{-1})	2.61±0.47 **b**	1.7±0.26 **b**	4.14±0.56 **a**
ASP-AT (μmol m^{-2} s^{-1})	12.99±1.22	12.56±0.60	11.93±0.63
Asparaginase (μmol m^{-2} s^{-1})	0.77±0.03	0.76±0.04	0.75±0.02
Fd-GOGAT (μmol m^{-2} s^{-1})	0.89±0.08 **a**	0.57±0.08 **b**	0.73±0.03 **ab**
GS (μmol m^{-2} s^{-1})	0.21±0.03	0.21±0.02	0.22±0.02
Glutaminase (μmol m^{-2} s^{-1})	1.08±0.04	1.22±0.08	1.18±0.09
GDH (μmol m^{-2} s^{-1})	11.03±1.59	9.33±0.61	9.7±0.80
GPT (μmol m^{-2} s^{-1})	30.50±1.97	28.85±0.81	28.44±0.72

Each number is mean of five replicates with standard error. Different letters above the bars indicate significant difference between feeding treatments using Duncan's multiple range test at $P < 0.05$.

Discussion

I. Up-regulation of glycolysis in the leaves of transgenic plants is initiated by accumulation of hexose phosphates

Accumulation of G6P and F6P in the transgenic lines resulting from antisense inhibition of A6PR activity at both daytime and nighttime not only alters photosynthetic carbon partitioning (Cheng et al. 2005), but also leads to changes in down-stream processes that utilize carbon in source leaves. The conversion of F6P to F1, 6BP is considered as a pivotal step in regulating the carbon flux through glycolysis, which involves the action of three enzymes (ATP-PFK, PPi-PFK and FBPase). Although both cytosolic and plastidial ATP-PFKs are strongly inhibited by PEP and up-regulated by Pi in higher plants via a negative feedback mechanism (Plaxton, 1996), the slightly higher content of PEP and the expected lower Pi in transgenic plants at daytime (Fig. 2-4b) did not result in lower activity of ATP-PFK. Instead, higher activities both ATP-PFK and PPi-PFK in A04 and A10 were found at daytime (Fig. 2-8a, b), which suggests an enhanced conversion from F6P to F1,6BP. Higher activities of many other key enzymes involved in glycolysis, including NAD-G3PDH, NADP-G3PDH, aldolase, enolase, and PK (Fig. 2-8c-g) indicate that glycolysis is up-regulated in the transgenic plants. Apparently, the general 'bottom up' regulation of glycolysis that operates under most conditions is overridden by accumulation of hexose phosphates in the transgenic plants. The concept of 'bottom-up' regulation of plant respiration has been well established that, downstream intermediates can feedback to the upstream steps to simulate or inhibit enzymes there (Beaudry et al, 1989; Hatzfeld and Stitt, 1991; Plaxton, 1996). Therefore, generally speaking, plant respiration is demand-driven and the futile accumulation of intermediates as well as the waste of energy can be avoided. However, in the transgenic plants, this 'top-down' feedforward regulation, initiated by accumulation of hexose phosphates drives the central metabolism. Since both PEP and pyruvate are not only the

lower end intermediates of glycolysis but also the substrates for TCA cycle and the biosynthesis of amino acids, a large proportion of PEP and pyruvate generated are used in TCA cycle and amino acid synthesis while their contents are kept relatively stable (Fig. 2-4b, c).

The expected low Pi in the cytosol resulting from accumulation of hexose phosphates in the transgenic plants may have also contributed to the up-regulation of glycolysis. First, the activity of non-phosphorylating NADP-G3PDH was significantly higher in both A04 and A10 than in the control at daytime, suggesting the Pi-independent conversion of G3P to 3-PGA was more active, a typical response to Pi limitation (Fig. 2-8d). In addition, mannose-fed leaves had higher activity of ATP-PFK than the H_2O-fed leaves (Fig. 2-15a). However, this up-regulation of ATP-PFK activity by mannose-feeding is apparently not consistent with the bottom-up regulation in which higher Pi enhances ATP-PFK activity. The fact that sucrose-feeding did not alter the activity of ATP-PFK activity or the activity of any other key enzymes in glycolysis suggests that the higher level of sucrose might not be involved in the up-regulation of glycolysis in the transgenic plants (Fig, 2-15; 2-16).

While higher activities were observed for many key enzymes in glycolysis in the transgenic plants, contents of most glycolytic intermediates either remained unchanged (G3P, DHAP, and F1,6BP) or even decreased (3-PGA), with only PEP and pyruvate showing slight increases (Fig. 2-2; 2-4). These relatively small changes are not inconsistent with up-regulation of glycolysis because fluxes can be increased while maintaining the intermediates fairly stable. The lower contents of 3-PGA in both A04 and A10 at daytime (Fig. 2-4a) are most likely related to the up-regulation of shikimate pathway and the subsequent synthesis of aromatic amino acids as indicated by higher contents of shikimate and Tyr and Phe (Fig. 2-4d; Fig. 2-7f, g). The higher content of DHAP in transgenic plants at nighttime (Fig. 2-2e) might come from the increased starch degradation (Cheng et al., 2005). This was also consistent with the functions of glycolysis

in the dark including participating in starch degradation and generating carbon skeletons and energy compounds for anabolic pathways.

II. Both the anaplerotic pathway and the TCA cycle are up-regulated in the leaves of transgenic plants

In addition to direct entry into the mitochondria via pyruvate, PEP generated in glycolysis can be converted to OAA by PEPC, and then to malate by NAD-MDH in the cytosol, both of which can enter the mitochondria to replenish TCA cycle intermediates for several biosynthetic pathways including biosynthesis of amino acids and phenolic compounds (Andrews 1986; Melzer and O'Leary 1987; Noguchi and Yoshida 2008). Malate can also be decarboxylated in the cytosol by NADP-ME or in the mitochondrial matrix by NAD-ME to produce pyruvate, which is then oxidized in the TCA cycle. The activities of both PEPC and NADP-ME were higher in the transgenic lines than in the control, which clearly indicate up-regulation of the anaplerotic pathway in the transgenic plants (Fig. 2-9a, d). This is consistent with enhanced amino acid biosynthesis in the transgenic plants. Since up-regulation of the PEPC-MDH-ME bypass of PK is an important feature of Pi-deficiency response (Plaxton and Podestá, 2006), higher activities of PEPC, NAD-ME and NADP-ME in mannose-fed leaves suggest that the expected low level of Pi in the cytosol is involved in the up-regulation of these enzymes (Fig. 2-15b). And following the significant accumulation of G6P and F6P, the PEP metabolism is the secondary most activated step of carbon metabolism in transgenic plants.

TCA cycle was up-regulated in the transgenic plants as indicated by higher activities of citrate synthase and aconitase at both daytime and nighttime (Fig. 2-10a, b). The contents of some TCA intermediates, citrate, 2-OG, succinate and OAA were higher in the transgenic plants at both daytime and nighttime or at least at daytime (Fig. 2-4e, g, h, k) whereas contents of both

malate and fumarate were lower (Fig. 2-4h, j). The elevated OAA content in the transgenic plants are likely the result of 1) increased activity of PEPC (Fig. 2-9a) and 2) enhanced TCA cycle whereas the lower contents of fumarate and malate (Fig. 2-4i, j) are related to higher activities of NAD-ME and NADP-ME (Fig. 2-9c, d). In addition to the conventional TCA cycle, it is possible that some specific non-cyclic TCA fluxes also operated in the transgenic plants, which could adjust the carboxylic acid metabolism to be more suitable for the biosynthesis of amino acids (Sweetlove et al., 2010; Tcherkez et al., 2009; Hanning and Heldt, 1993). The up-regulation of both the anaplerotic pathway and the TCA cycle provided more carbon skeletons and energy for amino acid synthesis, which is consistent with higher levels of amino acids in the transgenic plants (Fig. 2-5a). Since mannose feeding did not change the activity of any TCA cycle enzymes (Fig. 2-16), the lower Pi level in the cytosol of the transgenic plants might not be directly involved in the up-regulation of TCA cycle. Meanwhile, no changes on the activities of these TCA enzymes were found in sucrose-fed leaves (Fig. 2-16), which suggests that sucrose was not involved either.

III. Oxidative pentose phosphate pathway is up-regulated in the transgenic plants at nighttime

OPPP is generally running at a low rate in the light (Taiz and Zeiger, 2006). It appears that the up-regulation of OPPP in the transgenic plants primarily occurred at night as the activity of the key regulatory enzyme, G6PDH, was significantly higher in A04 and A10 only at nighttime (Fig. 2-9e). Since one of the most important functions of OPPP is to provide NADPH for nitrogen assimilation in the dark (Oji et al., 1985; Wright et al., 1997; Jin et al., 1998), this is also consistent with the fact that the three antisense lines had higher contents of total amino acids, particularly at night (Fig. 2-5a). Mannose-fed leaves also had significantly higher activities of

both G6PDH and 6PGDH, which suggests that the expected low Pi content in the cytosol of the transgenic plants might have also contributed to the up-regulation of OPPP (Fig. 2-15b). Similar results were reported by Liu et al. (2007) on red kidney bean roots that, an inhibitor of G6PDH, Na_3PO_4 could blocked the increase of G6PDH induced by salt stress. Mannose-induced higher activity of G6PDH was also reported by Hauschild and Schaewen (2003), but they pointed out that it was the altered sugar availability not the Pi sequestration led to the enhanced cytosolic G6PDH expression. They also came up with a model for G6PDH regulation, which highlighted the function of substrate availability on G6PDH activation, and suggested that the major trigger of higher activities of G6PDH and 6PGDH in antisense plants at nighttime was still the accumulation of G6P and F6P.

Compared with the enzyme data obtained at daytime, fewer enzymes showed higher activities at nighttime in the transgenic plants, especially for those in glycolysis. However, the accumulation of hexose phosphates at night still resulted in higher contents of downstream intermediates, such as pyruvate, shikimate, citrate, succinate and OAA (Fig. 2-4c, d, e, h, k). Still, the accumulation of pyruvate, the end-product of glycolysis, in three antisense lines at nighttime, is the most obvious sign for the up-regulated glycolysis.

IV. The enhanced amino acid synthesis in transgenic plants was mainly caused by the increased substrate availability

Transgenic plants accumulated higher levels of free amino acids at both daytime and nighttime (Fig. 2-5a) which suggests that amino acid metabolism was up-regulated in the transgenic plants. Percentage wise, more amino acids were accumulated in transgenic plants at nighttime than at daytime (80.9% vs. 29.4% increase relative to the control, respectively).

Among the glutamate family amino acids, the level of Glu was increased mostly (Fig. 2-5b),

which is highly consistent with the significantly elevated activity of Fd-GOGAT in all three antisense lines at both daytime and nighttime (Fig. 2-11e). Although GDH might catalyze the synthesis of Glu with the presence of sufficient ammonium, its main function is to deaminate glutamate (Miyashita and Good, 2008; Labboun et al., 2009). So, the decreased activity of GDH in transgenic plants at daytime could also contribute to the accumulation of Glu (Fig. 2-11h). No difference in GS activity was detected between transgenic lines and the control and (Fig. 2-11f) and only A10 had slightly higher level of Gln at daytime (Fig. 2-5c). However, the activity of glutaminase, catalyzing the catabolism of Gln, was found to be significantly higher in all three antisense lines at both daytime and nighttime (Fig. 11g), which may have prevented the transgenic plant from accumulating Gln. GABA was found to be accumulated in all three antisense lines at both daytime and nighttime (Fig. 5f). The higher content of GABA in all three antisense lines might come from higher content of Glu (Fig. 2-5f). GABA could be transported into the mitochondria to produce more succinate (Fig. 2-4h). In most cases, GABA accumulates in response to biotic and abiotic stresses (Shelp et al., 1999; Kinnersley, 2000) although the regulation of GABA shunt is still not clear (Bouché and Fromm, 2004). In addition, the levels of Pro (Fig. 2-5d) and Arg (Fig. 2-5e) were higher in transgenic plants only at daytime. This elevated level of Glu family amino acids combined with limited changes in the enzymes in their synthesis suggests that the higher contents of amino acids in transgenic plants were mainly caused by elevated levels of their substrates, such as citrate and 2-OG as discussed above.

The contents of three major members in Asp family amino acids, Asp, Asn and Thr were all increased in transgenic plants at both daytime and nighttime (Fig. 2-6a, b, c). However, for the two key enzymes functioning in Asp and Asn biosynthesis, Asp-AT and AS, only the activity of Asp-AT was slightly higher in transgenic plants at nighttime (Fig. 2-11b). The decreased catabolism of Asn due to the slightly decreased activity of asparaginase at daytime (Fig. 2-11d)

might be one reason for the accumulation of Asn in transgenic plants. Still, these results suggest that the accumulation of both Asp and Asn was largely related to the more available OAA in the transgenic plants (Fig. 2-4k). For aromatic amino acids, higher levels of both Tyr and Phe were also closely related to higher shikimate content (Figs 2-4d; 2-7f, g). Therefore, in the transgenic plants, glycolysis, anaplerotic pathway and TCA cycle were up-regulated in a coordinated manner to provide more carbon skeletons to amino acid synthesis. At the same time, most enzymes involved in nitrate assimilation and amino acid enzymes remained unchanged or were changed to a much lesser extent compared to the changes in metabolite levels.

However, based on this point anout more substrat availability, it is difficult to explain why transgenic plants also accumulated significantly higher content of Ser at daytime since the content of 3-PGA, the substrate for Ser synthesis, was found to be significantly decreased in A04 and A10 at daytime. Different from other amino acids, Ser is also involved in photorespiration and its metabolism is less affected by the glycolytic pathway. The activity of PGDH, the enzyme catalyzing the first step in Ser synthesis mainly in dark, was found to be slightly higher in transgenic plants at nighttime, which might explain the accumulation of Ser in transgenic plants at nighttime (Fig. 2-11j) .

Not all of the amino acids were increased in the transgenic plants. For example, although Ala, Val and Leu share the same substrate, pyruvate, only Ala and Leu contents were slightly increased at nighttime (Fig. 2-7a, b, c). Additionally, the activity of GPT, which catalyzes the transamination between Glu and Ala, was lower at both daytime and nighttime in transgenic plants (Fig. 2-11i). These data suggest that, instead of being used for the synthesis of more Ala, Leu and Val, more pyruvate was directed towards the downstream TCA cycle to generate more organic acids for the synthesis of both Glu and Asp families of amino acids.

Conclusions

In response to accumulation of hexose phosphates, glycolysis, the anaplerotic pathway, the TCA cycle, and the OPPP are up-regulated in the transgenic plants with decreased sorbitol synthesis which amy provide more organic acids and energy for amino acid synthesis, leading to enhanced amino acid synthesis and significant accumulation of amino acids. The lower Pi level in the cytosol may also contribute to the metabolic changes observed in the transgenic plants.

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CHAPTER 3

CARBON AND NITROGEN METABOLISM IN THE LEAVES OF TRANSGENIC APPLE TREES WITH DECREASED SORBITOL SYNTHESIS IN RESPONSE TO NITROGEN DEFICIENCY

Abstract

Sorbitol is the main photosynthetic end-product and the primary translocated form of carbohydrates in apple. Previous study indicated that, when the expression of aldose-6-phosphate (A6PR), the key enzyme in sorbitol synthesis, was decreased via antisense inhibition in 'Greensleeves' apple, both A6PR activity and sorbitol content were significantly decreased. As a result, hexose phosphates accumulated in the cytosol at the expense of inorganic phosphate, leading to up-regulation of starch synthesis, organic acid and amino acid metabolism in the antisense plants. In this study, a transgenic line (A10) and the untransformed control (CK) were supplied with 0.5, 2.5, 7.5 or 15mM nitrogen to determine the responses of the transgenic plants to N deficiency. The net CO_2 assimilation rate of A10 was significantly higher than that of CK under N deficiency and A10 had significantly higher contents of chlorophylls and higher activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), stromal fructose- 1,6-bisphosphate phosphatase (FBPase), and ribulose-5-phosphate kinase (PRK) than CK under N deficiency. Under N deficiency, organic acid metabolism including glycolysis, the tricarboxylic acid (TCA) cycle and other alternative pathways was maintained at a higher level in A10 compared to CK. Moreover, A10 had significantly higher contents of soluble proteins under N deficiency and total free amino acids across the entire range of N treatments. Activities of nitrate reductase (NR) and several

enzymes in amino acid synthesis also displayed an overall increase in A10 under N deficiency. These results indicate that under N deficiency, the transgenic plants shifted metabolic flux toward organic acids which were utilized to supply amino acid and protein synthesis with more carbon skeletons to maintain the higher photosynthesis. In addition, A10 accumulated a dramatically higher level of maltose than CK under N deficiency.

Introduction

Nitrogen (N) is critical for plant growth and development because it plays important roles in fundamental cellular functions as the primary constituent of macromolecules such as proteins and nucleic acids. The majority of the total N in leaves is in proteins (Chapin and Kedrowski, 1983) and 70-80% of these proteins perform their functions in chloroplasts in C_3 plants (Morita and Kono, 1975). For example, rubisco, the key enzyme in photosynthesis, typically accounts for 20-30% of leaf total N in C_3 plants (Evans, 1989). Under nitrogen deficiency, plants develop smaller leaf area and have lower photosynthesis, leading to less biomass accumulation, lower yield, and higher root to shoot ratio (Rufty et al., 1984; Stitt and Krapp, 1999; Hermans et al., 2009). In terms of carbon and nitrogen metabolism, low N availability restricts amino acid and protein synthesis, leading to accumulation of non-structural carbohydrates in leaves. The basic underlying mechanism of plants responses to N deficiency is that N starvation can modify N remobilization and recycling as well as carbon partitioning between source leaves and sink tissues by affecting nitrate uptake and reduction, photosynthesis (Chen and Cheng, 2003; Huang et al., 2004), sugar metabolism, organic acids production, and amino acid biosynthesis.

Although N fertilizers are routinely applied for producing higher yields worldwide, the enormous loss of N fertilizers due to various environmental factors results in low nitrogen use efficiency (NUE) in crops and significant waste of natural resources and energy. Based on its

original definition (Moll et al., 1982), the term NUE combines two processes: the N uptake efficiency (NupE) and the N utilization efficiency (NutE). Both are influenced by crop, environmental and management factors, which can be boiled down to how N is applied and how N is lost (Balasubramanian et al, 2004). In addition to optimizing agricultural practices, one strategy to increase NUE is to breed plants or new hybrids with improved tolerance to N deficiency to meet the requirements of sustainable crop production. A key to this strategy is to understand the regulation of metabolic processes that determine NUE. Since assimilation of nitrogen into amino acids depends on the supply of carbon skeletons from organic acid metabolism (Stitt et al., 2002), plants with enhanced organic acid metabolism are expected to have higher nitrogen use efficiency and better tolerance to nitrogen deficiency. Indeed, potato plants with overexpression of phosphoenolpyruvate carboxylase (PEPC) (Rademacher et al., 2002) and Arabidopsis plants with overexpression of Dof transcriptional factor (Yanagisawa et al., 2004) had enhanced organic acid metabolism, which led to higher levels of amino acids; moreover, the broad metabolic alterations induced by Dof1 made the transgenic plants grow better under low N conditions and have larger fresh weight than the control. These findings were consistent with the hypothesis that, improving the flexibility of metabolic pathways could generate a more efficient driving force for N metabolism, which in turn results in an enhanced NUE (Foyer et al., 2011). Some recently released maize hybrids were reported to be more tolerant of N deficiency than older hybrids due to their more slowly decreased photosynthetic capacity caused by being able to maintain the contents of chlorophylls and soluble proteins as well as the activity of PEPC at low levels of applied N fertilizer (Ding et al., 2005). However, this enhancement of organic acid metabolism on nitrogen use has not been tested in woody perennial plants, whose nitrogen assimilation is thought to occur primarily in the roots (Andrews, 1986; Black et al., 2002).

When sorbitol synthesis was decreased in apple leaves by expressing A6PR in the antisense orientation, transgenic plants accumulated high levels of hexose phosphates in leaves. Both organic acid metabolism and amino acid metabolism are up-regulated under sufficient N supply. These transgenic plants provided us with a unique opportunity to gain insights into the relationship between organic acid metabolism and nitrogen use in woody plants. It is predicted that these transgenic plants with enhanced organic acid metabolism and amino acid biosynthesis will be better able to adapt to low N conditions and show more tolerance to N deficiency than the untransformed control.

Methods and materials

Transgenic lines and N treatments

Generation of transgenic 'Greensleeves' apple with decreased expression of A6PR was described in detail in Cheng et al. (2005). RNA gel blotting showed that all the transformed lines had significantly lower A6PR transcript levels compared with the untransformed control. Line A27 had about 30% of the control A6PR activity; all the other lines (A04, A05, A10, A14, A18, A41, and A75) had about 15% of the control A6PR activity (Cheng et al., 2005). The untransformed control (CK) and antisense line A10 were used in this study.

One-year-old tissue-cultured CK and A10 were transplanted into two-gallon pots containing sand before budbreak, pruned to about 30 cm, and grew outdoors at Cornell Orchards. After budbreak, two shoots were allowed to grow on each plant. CK and A10 were supplied twice a week with 0.5, 2.5, 7.5 or 15 mM N in Hoagland's solution via a fertigation system from mid May 2009, which lasted for seven weeks. Fungicides and pesticides were sprayed at regular intervals during the growing season. After 7-week of N treatment, fully expanded, mature leaves of CK and A10 were taken at noon (12:30PM-1:30PM), frozen in liquid N_2, ground into fine

powder in liquid N_2, and stored at -80°C for the following biochemical and physiological measurements.

Sucrose and G6P feeding experiment

In a separate experiment, two-year-old untransformed control on M.26 rootstock were supplied twice a week with 0.5 mM N in Hoagland's solution via a fertigation system from mid May 2010, which lasted for eight weeks. At 8:00AM, mature leaves were detached from the plants at the bottom of the petiole using a razor blade and were re-cut under water immediately, and then brought to the lab. The detached leaves were fed with water, 50 mM sucrose, 3 mM glucose-6-phosphate (G6P), or 50 mM sucrose plus 3 mM G6P in a fume hood under fluorescent lights at a photon flux density of 100 µmol m^{-2} s^{-1} for a total of 48 hours. Each treatment was replicated five times in a randomized design. Leaf discs were taken at both 24 hrs and 48 hrs for analysis.

Gas exchange measurements

Leaf CO_2 assimilation and respiration were measured via a CIRAS-1 portable photosynthesis system (PP systems, Herts, UK). Leaf CO_2 assimilation was measured under ambient CO_2 (360 µmol mol^{-1}), photon flux density (PFD) of 1500 ± 50 µmol m^{-2} s^{-1} and air temperature 23±1 °C from 10:00 AM to 11:30AM after 7-week of N treatment. Leaf dark respiration was measured at 3:00 PM after adapting detached leaves in dark for 30 min at 26°C.

Analysis of leaf chlorophylls, N and soluble proteins

Leaf chlorophylls were extracted with 80% (v/v) acetone and measured according to Arnon (1949). Total leaf N content per unit leaf area was measured with a C/N analyzer using

combustion analysis. Leaf soluble protein content per unit leaf area was assayed according to Bradford (1976).

Analysis of primary metabolites

Non-structural carbohydrates, such as sugars, sugar alcohols, hexose phosphates, and most organic acids (citrate, 2-oxoglutarate (2-OG), succinate, fumarate, malate, glycerate and shikimate) were extracted, derivatized and analyzed according to the protocol of Lisec et al. (2006) with some modifications. Leaf tissues (about 100 mg FW) were extracted in 1.4 ml 75% (v/v) methanol with 60µl ribitol (0.6mg/ml) added as the internal standard. After fractionating the non-polar metabolites into chloroform, the non-diluted, aqueous extracts were used to determine low abundance metabolites and the extracts at 1:20 dilution were used to measure high abundance metabolites, such as sorbitol, sucrose and malate. After sequential derivatization with methoxyamine hydrochloride and N-methyl-N- trimethylsilyl- trifluoroacetamide (MSTFA), metabolite profiling was operated at an Agilent 7890A GC /5975C MS (Agilent Technology, Palo Alto, CA, USA). Injection, chromatography and MS parameters were described in detail in Wang et al. (2010). Metabolites were identified by comparing fragmentation patterns against a mass spectral library generated on our GC-MS system and an annotated quadrupole GC-MS spectral library and quantified based on standard curves generated for each metabolite and internal standard.

3-phosphoglycerate (3-PGA), pyruvate, phosphoenolpyruvate (PEP), oxaloacetate (OAA), isocitrate, fructose-1,6-bisphosphate (F1, 6BP), glyceraldhyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) were extracted according to Chen et al. (2002) with modifications. Leaf tissues (about 100mg FW) were pulverized with a liquid N_2 pre-cooled mortar and pestle in 2 ml of 5% (v/v) $HClO_4$ and 100 mg insoluble PVPP ($(C_6H_9NO)_X$, MW

111.1). After centrifugation at 16100 g for 10 min, the supernatant was withdrawn and neutralized with 5M KOH/1M triethanolamine, keeping on ice for assay. 3-PGA was measured according to Chen and Cheng (2003). Pyruvate, PEP, OAA, isocitrate, and F1, 6BP ere measured according to Chen et al. (2002) with minor modifications. Pyruvate was assayed in 1ml reaction medium containing 100 mM Hepes-KOH (pH 7.5), 1 mM $MgCl_2$, 1 mM ADP, 0.1 mM NADH, and 1 unit of LDH (EC 1.1.1.27). PEP was assayed in the same reaction medium of pyruvate assay by adding 1 unit of PK (EC 2.7.1.40). OAA was assayed in a 1ml reaction medium containing 100 mM triethanolamine-HCl (pH 7.6), 0.1 mM NADH, and 1 unit of MDH (EC 1.1.1.37). Isocitrate was assayed in 1ml reaction medium containing 100 mM Tris-HCl (pH 7.6), 3.3 mM $MnSO_4$, 0.15 mM NADP, and 1 unit of ICDH (EC 1.1.1.42). F1, 6BP was assayed in 1ml reaction medium containing 100 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADH, 2 units of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 4 units of TPI (EC 5.3.1.1), and 0.5 unit of aldolase (EC 4.1.2.13). G3P and DHAP were assayed according to Copper et al. (1958).

Starch was digested and determined enzymatically as glucose equivalents (Chen et al., 2002). The residue after 75% methanol extraction for GC/MS analysis was re-extracted with 1.5 ml 80 % (v/v) ethanol at 80 ^{o}C for three times, and the pellet was digested with 30 units of amyloglucosidase (EC 3.2.1.3) and retained for glucose determination using enzyme-link assay.

NH_4^+ and twenty free amino acids glutamate (Glu), glutamine (Gln), proline (Pro), Arginine (Arg), γ-aminobutyric acid (GABA), ornithine (Orn), histidine (His), asparate (Asp), asparagine (Asn), threonine (Thr), lysine (Lys), methionine (Met), isoleucine (Ile), alanine (Ala), valine (Val), leucine (Leu), serine (Ser), glycine (Gly), tyrosine (Tyr), and phenylalanine (Phe) were extracted from 50 mg (FW) leaf tissues in 1.5 ml 20 mM HCl with norleucine added as an internal standard. Measurements were performed at a HP1100 Liquid Chromatograph equipped with an Agilent 1200 fluorescence detector (Agilent Technology, Palo Alto, CA, USA).

Derivatization of free amino acids and chromatography parameters were described in detail in Wang et al. (2010). Amino acids were indentified and quantified according to Cohen and Michaud (1993) with modifications.

Assay of photosynthetic enzymes

Leaf tissues (about 100mg FW) were homogenized in 1.5 mL extraction mixture containing 50 mM Hepes-KOH (pH7.5), 10 mM $MgCl_2$, 2 mM EDTA, 10 mM DDT, 1%(v/v) Triton X-100, 5% (w/v) insoluble PVPP, 1% (w/v) BSA, and 10% (v/v) glycerol. The extract was centrifuged at 13000g for 5 min and the supernatant was used immediately for enzyme activity assay according to Chen and Cheng (2003) with slight modifications. Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) activity was determined in a reaction mixture containing 100 mM Bicine-KOH (pH 8.0), 25 mM $KHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 5 units glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 5 units 3-phosphoglyceric phosphokinase (EC 2.7.2.3), 17.5 units creatine phosphokinase (EC 2.7.3.2), 0.25 mM NADH and 0.5 mM RuBP. NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was determined at 340nm in a reaction mixture containing 100 mM Tricine-KOH (pH 8.0), 4 mM 3-phosphoglycerate, 5 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADPH, 20 units 3-phosphoglyceric phosphokinase (EC 2.7.2.3). Stromal fructose- 1,6-bisphosphate phosphatase (stromal FBPase) was assayed in a reaction mixture of 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM fructose 1,6-bisphosphate (FBP), 0.5 mM NAD(P), 4 units of phosphoglucose isomerase (EC 5.3.1.9), and 2 units of G6PDH (EC1.1.1.49). Cytosolic fructose- 1,6-bisphosphate phosphatase (cytosolic FBPase) was assayed in a reaction mixture of 50 mM Hepes-NaOH (pH 7.0), 2 mM $MgCl_2$, 0.1 mM FBP, 0.5 mM NAD(P), 4 units of PGI (EC 5.3.1.9), and 2 units of G6PDH (EC1.1.1.49). Ribulose-5-phsophate kinase (PRK) activity

was assayed in a reaction mixture containing 100 mM Tricine-KOH (pH 8.0), 0.5 mM ribose 5-phosphate, 1 mM ATP, 10 mM $MgCl_2$, 50 mM KCl, 5 mM phosphenolpyruvate, 0.4 mM NADH, 7 units PK (EC 2.7.1.40), 10 units lactate dehydrogenase (EC 1.1.1.27), 1 unit R5P isomerase (EC 5.1.3.6).

Assay of starch, sorbtiol and sucrose metabolism enzymes

ADP-Glucose pyrophosphorylase (AGPase) and sucrose-6-phosphate synthase (SPS) were extracted and assayed according to Chen and Cheng (2003) with slight modifications. Leaf tissues (about 100mg FW) were ground with a liquid N_2 pre-cooled mortar and pestle in 1.5 ml extract buffer containing 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2%(w/v) polyethylene glycol-20000 (PEG-20), 1% (w/v) BSA, 0.4 % (v/v) Triton X-100, and 5% (w/v) insoluble PVPP. The extract was then centrifuged at 13000 g for 5 min and the supernatant was used immediately for AGPase activity measurement with the reaction mixture containing 50 mM Hepes-KOH (pH 8.0), 1 mM ADPG, 5 mM $MgCl_2$, 0.6 mM NAD, 1.5 mM PP_i, 3 mM PGA, 2 units G6PDH (EC 1.1.1.49, NAD-linked), and 2 units phosphoglucomutase (EC 5.4.2.2). For SPS activity assay, leaf tissues (about 100mg FW) were homogenized in 1.5 mL extraction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, 10 mM DDT, 1%(v/v) Triton X-100, 5% (w/v) insoluble PVPP, 1% (w/v) BSA, and 10% (v/v) glycerol. The extract was centrifuged at 13000g for 5 min and 20ul supernatant was mixed with 50ul assay mixture containing 70mM Hepes-NaOH (pH 7.5), 15 mM UDPG, 10 mM F6P, 40 mM G6P and kept at 25 ºC for 30 min. The reaction was stopped by heating in the boiling water for 3min. The control was carried out with heat-denatured enzyme. After brief centrifugation at full speed for 5min, the supernatant was used to determine UDP production with the reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 5 mM $MgCl_2$, 0.3 mM NADH, 0.8 mM PEP, 14 units

LDH (EC 1.1.1.27), and 4 units PK (EC 2.7.1.40). Aldose-6-phosphate reductase (A6PR) was extracted according to Negm and Loescher (1981) with some modifications. Leaf tissues (about 100mg FW) were ground with a liquid N_2 pre-cooled mortar and pestle in 1.5 ml extract 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 13000 g for 5 min and the supernatant was used immediately for enzyme activity assay with a reaction mixture containing 100 mM Tris-HCl (pH 9.0), 0.11 mM NADPH, and 50 mM G6P.

β-amylase activity was measured following the protocol of Laby et al. (2001) with modifications. Leaf tissues (about 100mg FW) were ground in 2 ml extraction buffer containing 50 mM potassium acetate (pH 6.0) 1 mM EDTA, 5 mM DTT and 3 mM $CaCl_2$ plus PVPP. β -amylase reaction mixtures were prepared by mixing 85 µl of the enzyme extract with 75 µL of 20 mg mL^{-1} amylopectin and 150 µL of 0.1 M KAc-HAc (pH 4.5). Immediately after mixing, 95 µL aliquots of each β-amylase reaction mixture were transferred to new microtubes, and the reactions were stopped by placing the microtubes in boiling water for 2 to 3 min (as the zero time point). The remainder of each reaction mixture was incubated at 37°C for 60 min before removing and boiling additional 95 µL aliquots. To quantify the amount of sugar present in each sample, 20 µL aliquots of each sample were mixed with 230 µL of ddH_2O and 750 µL of *p*-hydroxybenzoic acid hydrazide (PAHBAH)/NaOH solution. The PAHBAH/NaOH solution was prepared immediately prior to use by mixing one part 5% (w/v) PAHBAH in 0.5 M HCl with 4 parts 0.5 M NaOH. The sugar assay reaction mixtures were incubated at 100°C for 5 min and allowed to cool, and then absorbance at 410 nm were determined and compared with the values obtained using a maltose standard curve. Glycosyl transferase (DPE2) was extracted and determined according to Chia et al (2004) with slight modifications. Leaf tissues (about 100mg FW) were extracted in a total volume of 1.5 mL of 100 mM Mops-KOH (pH 7.0), 10% (v/v)

glycerol, and 5 mM DTT, 0.3% Triton X-100, with 50 mg PVPP and a pinch of sand. After centrifuged at 16100 g for 20 min, the supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK) and used immediately for assay. The activity of DPE2 was measured as the difference between the amount of glucose generated in the incubations that contained both maltose and the second substrate (glycogen), and the sum of the amounts of glucose generated in separate incubations that contained either maltose or glycogen alone. Incubation buffer contained 100 mM of Mops-KOH (pH 7.0), 10% glycerol, 30 mM maltose, 1.5% (w/v) oyster glycogen and 0.1 mL of the extract. Control incubations were operated without either maltose or glycogen. After 2 hr at 25 °C, incubations were stopped by heating at 90 °C for 2 min, and the glucose was assayed spectrophotometrically at 340nm with hexokinase and glucose-6-phosphate dehydrogenase in the presence of NAD.

Assay of enzymes involved in organic acid metabolism and nitrogen metabolism

Enzymes in carbon and nitrogen metabolism were extracted according to the protocols of Häusler et al. (2000), Häusler et al. (2001) and Chen and Cheng (2003) with modifications. Enzymes used in assays were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the unit of enzymes was in standard form (IU). Contents and combinations of reagents used in assays were optimized according to standard enzyme assay procedures described in Bergmeyer et al. (1983) and preliminary assays performed in our lab.

Assay of enzymes in organic acid metabolism

ATP-phosphofructokinase (ATP-PFK), PPi-phosphofructokinase (PPi-PFP), NAD-glyreraldehyde-3-phosphate dehydrogenase (NAD-G3PDH) and non-phosphorylating NADP-G3PDH were extracted from leaf tissues (about 100mg FW) with 1.5 mL extraction mixture

containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% (w/v) insoluble PVPP, and a pinch of sand. The homogenate was centrifuged at 16,000 g for 10 min and the resultant supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK). ATP-PFK and PPi-PFP were measured according to Moorhead and Plaxton (1988) and Botha et al. (1992) with modifications. ATP-PFK was assayed in a reaction mixture containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM NADH, 10 mM F6P, 25 mM NaH_2PO_4, 1mM ATP, 5 units of aldolase (EC 4.1.2.13), 5 units of TPI (EC 5.3.1.1), and 5 units of G3PDH (EC 1.1.1.8). PPi-PFP was assayed in a reaction mixture containing 100 mM Hepes-NaOH (pH 8.0), 5 mM $MgCl_2$, 0.1 mM NADH, 10 mM fructose-6-phosphate, 1 mM PPi, 0.01 mM F2,6BP, 5 units of aldolase (EC 4.1.2.13), 5 units of TPI (EC 5.3.1.1), and 5 units of G3PDH (EC 1.1.1.8). NAD-G3PDH was assayed in a reaction mixture containing 50 mM triethanolamine-HCl (pH 8.5), 4 mM NAD, 10 mM sodium arsenate, 1 mM F1, 6BP, and 6 units of alsolase (EC 4.1.2.13). Non-phosphorylating NADP-G3PDH was assayed in a reaction mixture containing 50 mM tricine-NaOH (pH 8.5), 0.4 mM NADP, 1 mM F1, 6BP, and 6 units of alsolase (EC 4.1.2.13).

Aldolase, pyruvate kinase (PK), PEP phosphatase (PEPP), phosphoglucose isomerase (PGI), hexokinase (HK), triose phosphate isomerase (TPI), PEP carboxylase (PEPC), NAD-malate dehydrogenase (NAD-MDH), NAD-malic enzyme (NAD-ME), NADP-ME, glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), citrate synthase, aconitase, NADP-isocitrate dehydrogenase (NADP-ICDH), NAD-ICDH, succinate dehydrogenase, and fumarase were extracted from leaf tissues (about 100 mg FW) with 1.5ml of extraction mixture containing 100 mM Hepes-KOH (pH 7.5), 30% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and 0.3% (v/v) Triton X-100, 4% (w/v) insoluble PPVP, and a pinch of sand. After centrifugation at 16000g for 10 min at 4 °C, the supernatant was desalted with

Sephadex® G-25 M PD10 columns (GE Healthcare, UK). Aldolase was assayed in a 1ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM FBP, 0.2 mM NADH, 10 units of TPI (EC 5.3.1.1) and 1 unit of α-glycerophosphate dehydrogenase (EC 1.1.99.5). PK was assayed in a reaction mixture containing 100mM Hepes-KOH (pH 7.0), 2 mM PEP, 2 mM ADP, 10 mM $MgCl_2$, 0.2 mM NADH, 0.2 mM ammonium molybdate, 0.2 mg/ml BSA, 2 mM DTT, and 2 units of LDH (EC 1.1.1.27). PEPP was assayed in the same mixture as PK in the absence of ADP and ammonium molybdate. PGI was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM NAD, 2 mM F6P and 2 units NAD-G6PDH (EC 1.1.1.49). HK was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 1 mM NAD, 1 mM ATP, 30 mM glucose and 2 units NAD-G6PDH (EC 1.1.1.49). Triose phosphate isomerase (TPI) was assayed in a reaction mixture containing 100mM triethanolamine -KOH (pH 7.6), 10 mM EDTA, 1 mM G3P, 0.2 mM NADH, and 1 unit of α-glycerophosphate dehydrogenase (EC 1.1.99.5). PEPC was assayed in a reaction mixture containing 50 mM Tris-HCl (pH 9.2), 10 mM $MgSO_4$, 4 mM PEP, 10 mM $NaHCO_3$, 0.2 mM NADH and 5 units MDH (EC 1.1.1.37). NAD-MDH was assayed in a reaction mixture containing 50 mM Bicine-KOH (pH 8.0), 1 mM DDT, 0.2 mM NADH, 1 Mm EDTA, 5 mM $MgCl_2$, 1 mM OAA and 0.01% BSA. NAD-ME was assayed in a reaction mixture containing 100 mM imidazole-HCl (pH 7.0), 10 mM MgSO4, 30 μM coenzyme A (CoA), 2 mM NAD, and 20 mM malate. NADP-ME was assayed in the same reaction mixture with NAD-malic enzyme except that 0.25mM NADP instead of NAD. G6PDH was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM $MgSO_4$, 5 mM KCl, 0.5 mM NADP and 3 mM glucose-6-P. 6PGDH was assayed in the same reaction mixture with G6PDH only except that 3mM 6-phosphogluconate was used instead of G6P. Citrate synthase was assayed in a 1ml reaction mixture containing 50 mM Tris-HCl (pH 7.8), 0.1 mM DTNB, 0.2

mM acetyl-CoA, and 0.5 mM OAA (Chen et al., 2009). Aconitase was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgSO_4$, 5 mM $MnCl_2$, 2 mM DTT, 5 mM NADP, 10 mM cis-aconitate, and 2 units of NADP-ICDH (EC 1.1.1.42). NADP-ICDH activity was measured in a reaction mixture containing 100 mM KH_2PO_4-KOH (pH 7.5), 5 mM $MgCl_2$, 0.25 mM NADP, and 250 mM isocitrate. NAD-isocitrate dehydrogenase (NAD-ICDH) activity was measured in a reaction mixture containing 100 mM KH_2PO_4-KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM NAD, and 5 mM isocitrate. Succinate dehydrogenase was assayed in a reaction mixture containing 50 mM KH_2PO_4-KOH (pH 7.5), 0.1mM 2,6-dichlorophenolindoplenol, 10 mM potassium cyanide, 40 mM sodium succinate, and 0.4 mg/ml (w/v) phenazine methosulfate. Fumarase was assayed in a reaction mixture containing 100 mM KH_2PO_4 (pH 7.3), 50 mM L-malate followed by measuring the formation of fumarate at 240nm.

Assay of enzymes involved in N and amino acid metabolism

Nitrate reductase (NR), glutamine synthetase (GS), aspartate aminotransferase (Asp-AT), glutamate pyruvate transaminase (GPT), asparaginase, glutaminase, glutamate dehydrogenase (GDH), and 3-PGA dehydrogenase (PGDH) were extracted from leaf tissues (about 100 mg FW) with 2 ml of extraction medium containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.3% (v/v) Triton X-100, and 5% (w/v) PVPP. The homogenate was centrifuged at 16,000g for 10 min and the supernatant was desalted with Sephadex® G-25 M PD10 columns (GE Healthcare, UK). NR was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM KNO_3, and 0.25 mM NADH, and incubated at 30°C for 30 min for the determination of nitrite production according to Hageman et al. (1980). The reaction was stopped by adding 0.5 ml 1% sulfanidamide (in 3M HCl), followed by addition of 0.5 ml 0.2% 1-naphthylamine and held for 15min. The mixture was then centrifuged at 13000 g for 5min. A standard curve was made using

authentic KNO_2 and the absorbance was measured at 540 nm. GS was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 20 mM $MgSO_4$, 10 mM NH_2OH, 5 mM ATP, 30 mM Glu, incubating at 30 °C for 20 min, followed by addition of 0.7 ml $FeCl_3$ reagent (2.5% $FeCl_3$, 5% (w/v) TCA in 1.5 M HCl). The resulting precipitate was spun down at 16100 g for 5 min and the absorbance was measured at 540nm (González et al., 1995). A standard curve was made using authentic r-glutamyl hydroxamate. Asp-AT was assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 25 mM Asp, 0.2 mM NADH, 25 mM 2-OG and 5 units MDH (EC 1.1.1.37) according to Murray and Kennedy (1980). GPT was assayed in a 1ml reaction mixture containing 100 mM Tris-HCl (pH 7.5), 25 mM Ala, 0.2 mM NADH, 10 mM 2-OG and 5 units LDH (EC 1.1.1.27). Asparaginase was assayed in a reaction system containing 62.5 mM KH_2PO_4-KOH (pH 7.5), 6.25 mM 2-OG, 12 mM Asn, 0.2 mM NADH and 1 unit of GDH (EC 1.1.1.47) according to Murray and Kennedy (1980). Glutaminase was assayed in the same reaction system with asparaginase except that 12mM Gln was used instead of asparagine. GDH was assayed in a reaction system contained 100 mM Tris-HCl (pH 7.0), 10 mM 2-OG, 50 mM ammonium chloride, and 0.1mM NADH according to Lutts et al. (1999). PGDH was assayed in a reaction mixture containing 200 mM Tris-HCl (pH 9.0), 25 mM EDTA, 5 mM 3-PGA, 2.5 mM DTT, and 0.5mM NAD according to Ho et al. (1999).

Asparagine synthetase (AS) was extracted and assayed according to Bellucci et al. (2004). Leaf tissues (about 100 mg FW) were extracted with 2 ml extraction buffer containing 200 mM Hepes-KOH buffer (pH 7.5), 10 mM DTT, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol (v/v), and 10% PVPP. After centrifugation at 15,000 g for 20 min at 4 ºC, the enzymatic activity of the Gln-dependent AS was determined by measuring the generation of Glu via HPLC using the same method for free amino acid quantification described above. The 1 ml substrate mixture contained 200 mM KH_2PO_4-KOH (pH 7.5), 2 mM Gln, 10 mM ATP, 17 mM magnesium acetate, 2 mM L-

Asp, and 400 μl of enzyme extract. The mixture was incubated at 37 ºC for 15 min and the assay was stopped by adding 1 ml of 80% ethanol followed by centrifugation at 5000 g for 5 min. Controls were assayed in the same conditions without Asp and ATP.

Fd-glutamate synthase (Fd-GOGAT) activity was assayed according to (Lea et al., 1990). The reaction mixture consisted of 200 mM KH_2PO_4-KOH (pH 7.5), 10 mM Gln, 10 mM 2-OG, 15 mM methyl viologen (the electron donor), and 1 mM amino-oxyacetic acid (transaminase inhibitor). Pre-incubatiing at 30 ºC for 5 min, the reaction was started by the addition of reductant solution (47 mg $Na_2S_2O_4$, 50 mg $NaHCO_3$ in 1 ml of water). After 30 min of incubation at 30 ºC, the reaction was stopped by adding 1 ml of 100% ethanol followed by vigorous vortex. Fd-GOGAT activity was determined by measuring the Glu generation via HPLC using the same method for free amino acid quantification described above.

Results

Leaf N status

Total N content, soluble protein content, total amino acids, and NH_4^+ content of both A10 and CK leaves all decreased as N supply decreased. Total leaf N content of A10 was consistently higher than that of CK across the entire range of N treatments (Fig. 3-1a) whereas leaf NH_4^+ content of A10 was consistently lower than that of CK (Fig. 3-1d). The content of NO_3^- in A10 decreased as N supply decreased whereas it increased in CK as N supply decreased; under the three highest N treatments, the content of NO_3^- in A10 was significantly higher than CK (Fig. 3-1e). Leaf soluble proteins in A10 decreased to a lesser extent than that of CK, and under the two lowest N treatments A10 leaf soluble protein content was significantly higher than that of CK (Fig. 3-1b). Total amino acids in A10 leaves were also consistently higher than that of CK over the entire range of N supply (Fig. 3-1c).

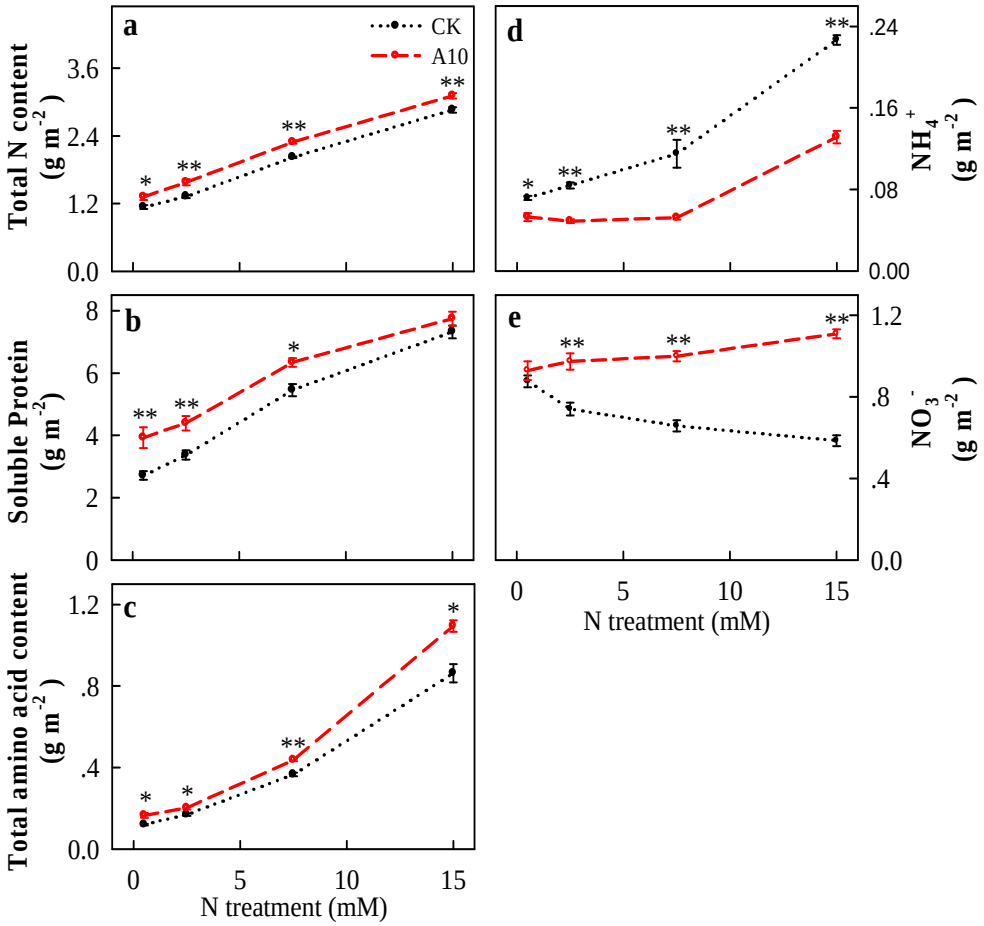

Figure 3-1. Total N leaf content (a), soluble proteins (b), total amino acid content (c), NH_4^+ (d), and NO_3^- (e) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. Specific leaf weight (SLW, g m^{-2}) in CK and A10 in response to N supply (lower to higher N level) are as follows: 99.3±2.9, 100.9±0.9, 103.4±1.7, 105.2±2.1 for CK; 113.1±2.3, 109.7±2.0, 114.1±1.6, 116.7±1.7 for A10. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*-test at $P < 0.05$, and 0.01, respectively.

CO_2 assimilation and dark respiration

In response to decreasing N supply, net CO_2 assimilation of both A10 and CK decreased (Fig. 3-2a). At the two highest N supply levels, no difference was found in net CO_2 assimilation between A10 and CK; at the two lowest N supply levels, however, the net CO_2 assimilation of A10 was significantly higher than that of CK (51% higher at the lowest N supply).

Dark respiration rates of A10 leaves were significantly higher than those of CK leaves

under the two highest N treatments whereas no difference was found at the two lowest N treatments (Fig. 3-2b).

Chlorophylls

Chla and Chlb decreased in both A10 and CK as N supply decreased. No difference was detected in either Chla or Chlb between A10 and CK at the two highest N supply levels. However, at the two lowest N supply levels, both Chla and Chl b of A10 leaves were significantly higher than those of CK leaves (Fig. 3-2c, d).

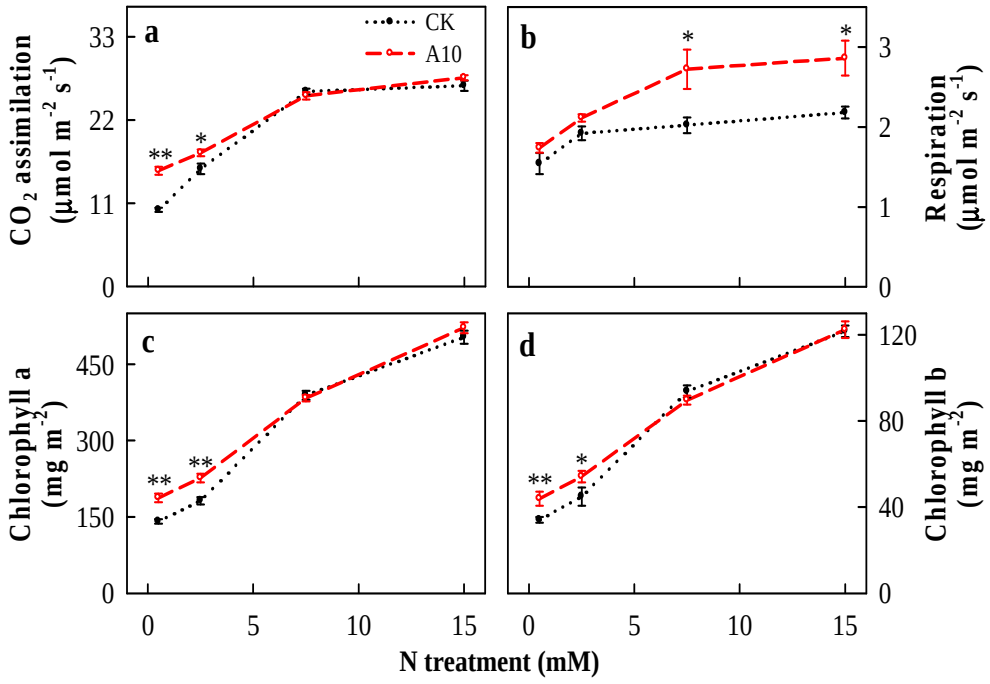

Figure 3-2. CO_2 assimilation (a), dark respiration (b) and contents of chlorophylls (c and d) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Key photosynthetic enzymes

Activities of rubisco, GAPDH, stromal FBPase and PRK in both A10 and CK all decreased as N supply decreased. No significant difference was detected in rubisco activity between A10 and CK at the two highest N treatments. However, under the two lowest N treatments, the activity of rubisco in A10 was significantly higher than that of CK (Fig. 3-3a). The activities of GAPDH, stromal FBPase and PRK in A10 were all significantly higher than those in CK leaves except that no significant difference in PRK activity was found between A10 and CK under the highest N treatment (Fig. 3-3b, c, d).

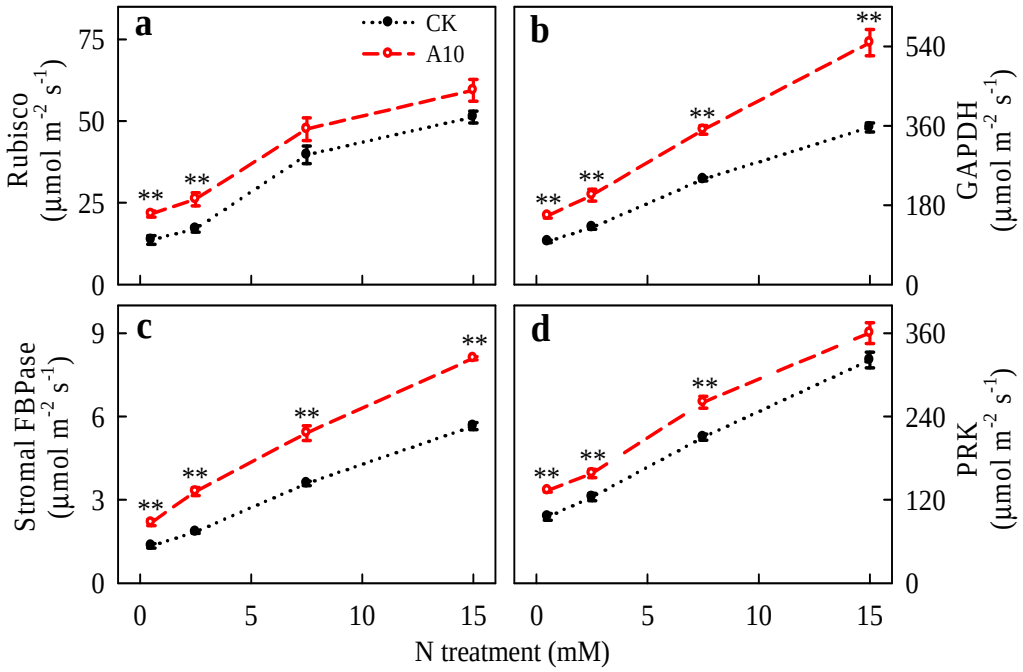

Figure 3-3. Activities of ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco, a), NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, b), stromal fructose- 1,6-bisphosphate phosphatase (FBPase, c), and ribulose-5-phosphate kinase (PRK, d) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Metabolite levels

Starch, soluble sugars and sugar alcohols

In response to decreasing N supply, contents of starch, fructose and maltose increased in both A10 and CK leaves (Fig. 3-4a, d, h); contents of sorbitol, sucrose, and myo-inositol decreased (Fig. 3-4b, c, g); contents of glucose and galactose did not change much (Fig. 3-4e, f). At each given N supply, contents of starch, sucrose, and myo-inositol were significantly higher in A10 than in CK whereas contents of sorbitol, fructose, glucose, and galactose were significantly lower in A10 than in CK. Relative to its content in CK, maltose in A10 accumulated to levels about 10-fold higher under the two lowest N treatments (Fig. 3-4h).

Hexose phosphates and triose phosphates

Contents of G6P and F6P increased slightly in both A10 and CK as N supply decreased, with higher contents of G6P and F6P detected in A10 at any given N supply (Fig. 3-5a, b). F1, 6BP content decreased in both A10 and CK with decreasing N supply, but A10 had significantly higher F1, 6BP content than CK under the three lowest N treatments (Fig. 3-5c). G3P content did not change much in either A10 or CK in response to decreasing N supply, but A10 had significantly higher G3P content under the two lowest N treatments (Fig. 3-5d). The content of DHAP in A10 and CK decreased as N supply decreased, with no significant difference found between A10 and CK at any given N supply (Fig. 3-5e).

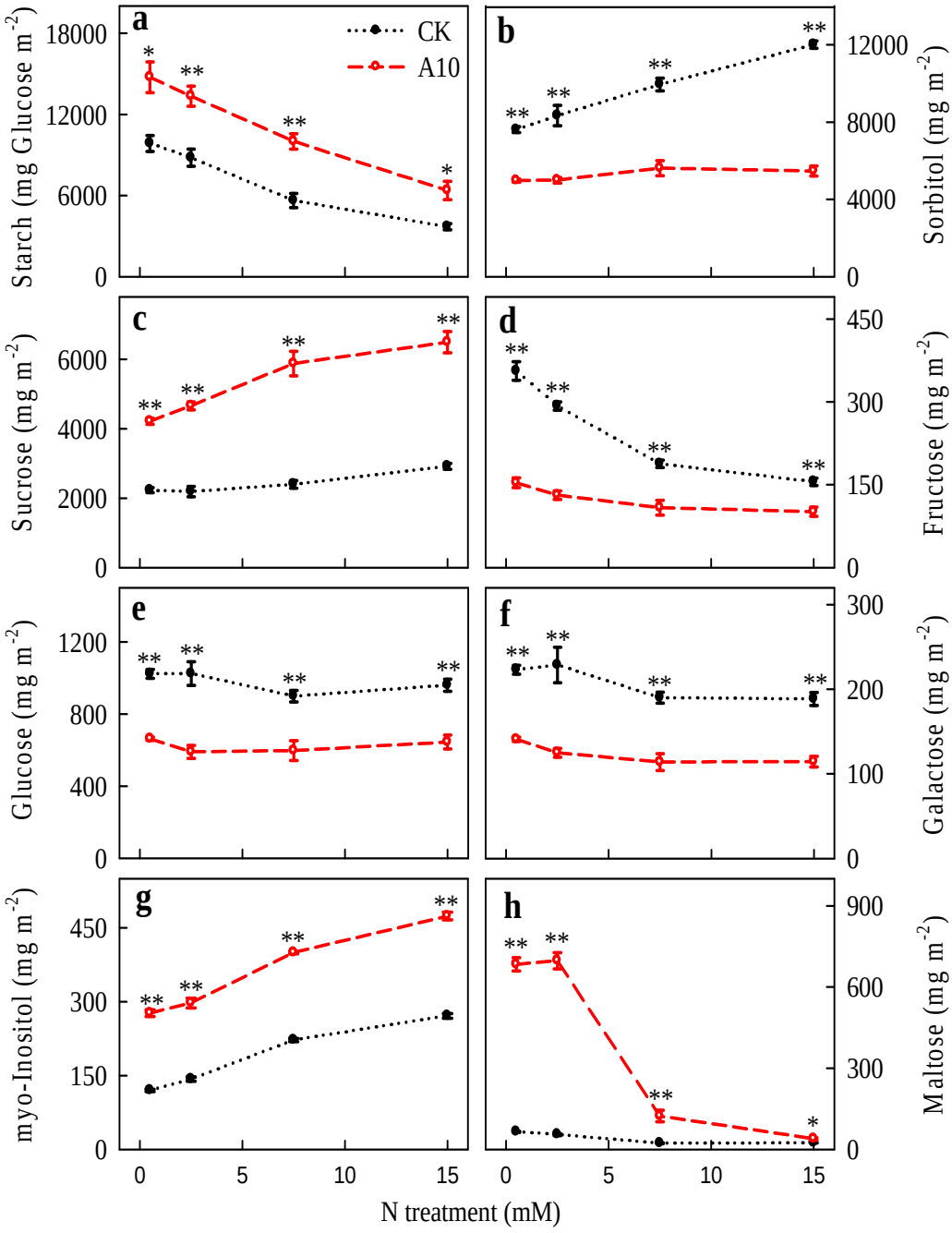

Figure 3-4. Contents of starch (a), sorbitol (b), sucrose (c), fructose (d), glucose (e), galactose (f), myo-inositol (g), and maltose (h) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

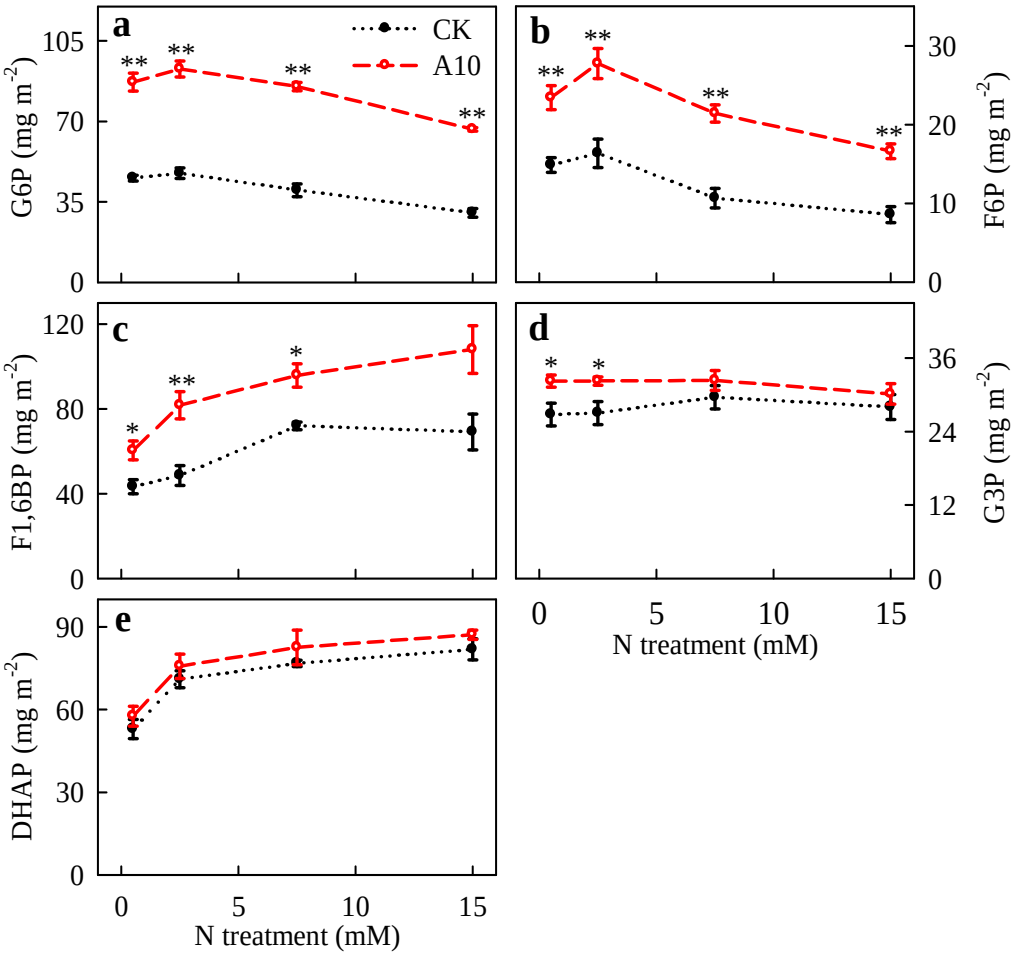

Figure 3-5. Contents of glucose-6-phosphate (G6P, a), fructose-6-phosphate (F6P, b), fructose-1,6-bisphosphate (F1,6BP, c), glyceraldhyde-3-phosphate (G3P, d), and dihydroxyacetone phosphate (DHAP, e) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Organic acids

At the highest N treatment, A10 had significantly lower 3-PGA content than CK; 3-PGA content decreased slightly in both A10 and CK with decreasing N supply; at the lowest N treatment, A10 had significantly higher content of 3-PGA than CK (Fig. 3-6a). PEP content in both A10 and CK did not change much as N supply decreased, but A10 had significantly higher PEP content than CK at the lowest N treatment (Fig. 3-6b). Pyruvate content in both A10 and CK decreased with decreasing N supply; however, the content in A10 was consistently higher than that of CK at any given N supply (Fig. 3-6c). OAA content increased slightly in both A10 and CK with decreasing N supply, but A10 had significantly higher OAA contents under the two highest N treatments (Fig. 3-6d). Malate content decreased in both A10 and CK with decreasing N supply, but A10 had consistently lower malate contents than CK over the entire range of N supply (Fig. 3-6e). Under the three highest N treatments, citrate content in A10 was consistently higher than that of CK whereas it was lower in A10 at the lowest N treatment, (Fig. 3-6f). As N supply decreased, isocitrate content in both A10 and CK decreased, with no difference detected between A10 and CK at any given N supply (Fig. 3-6g). In response to decreasing N supply, 2-OG content did not change in A10, but increased in CK. A10 had significantly lower 2-OG than CK over the entire range of N supply, especially under the two lowest N supply levels (Fig. 3-6h). Succinate content in both A10 and CK did not change much in response to decreasing N supply, with higher content detected in A10 only under the highest N treatment (Fig. 3-6i). As N supply decreased, fumarate content decreased in both A10 and CK, with A10 having slightly higher content only at the lowest N supply (Fig. 3-6j). Shikimate content in both A10 and CK decreased in response to decreasing N supply, with A10 having higher contents at the two highest N treatments (Fig. 3-6k). Glycerate content decreased in both A10 and CK as N supply decreased, with higher contents detected in CK under the two highest N treatments (Fig. 3-6l).

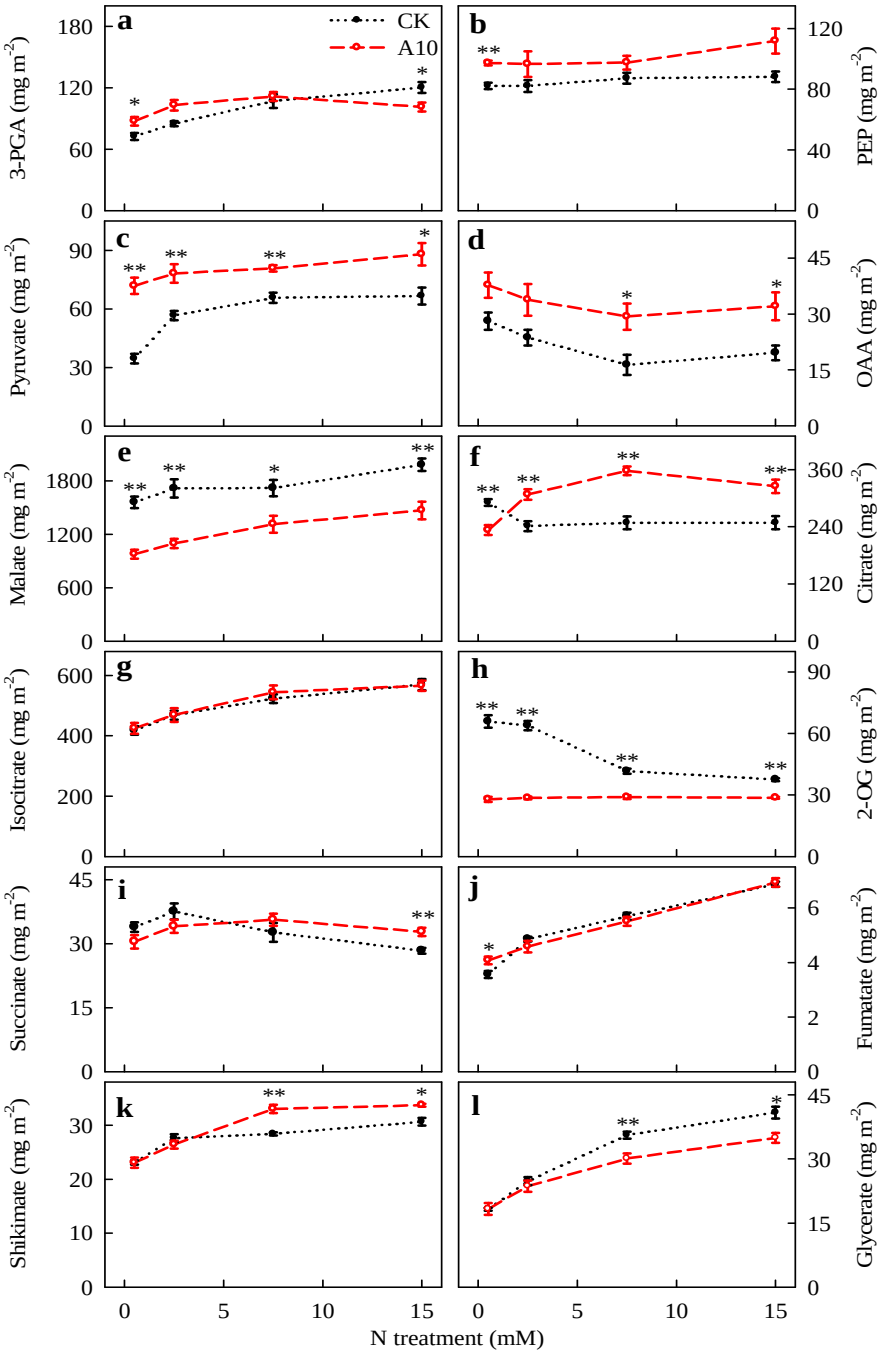

Figure 3-6. Contents of 3-phosphoglycerate (3-PGA, a), phosphoenolpyruvate (PEP, b), pyruvate (c), oxaloacetate (OAA, d), malate (e), citrate (f), isocitrate (g), 2-oxoglutarate (2-OG, h), succinate (i), fumarate (j), shikimate (k), and glycerate (l) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Amino acids

Contents of six out of seven amino acids in the Glu family decreased in response to decreasing N supply (Fig. 3-7). Contents of Glu, Pro, Arg, and GABA were consistently higher in A10 than in CK over the entire range of N supply (Fig. 3-7a, c, d, e). The content of Gln in A10 was significantly higher than that of CK only under the highest N treatment (Fig. 3-7b). No difference was found in Orn between A10 and CK at any given N supply (Fig. 3-7f). His content in A10 was consistently lower than that of CK over the entire range of N treatments (Fig. 3-7g).

Contents of all six amino acids in Asp family decreased in response to decreasing N supply (Fig. 3-8). Asp content was significantly higher in A10 than in CK over the entire range of N treatments (Fig. 3-8a). No difference was detected in Asn content between A10 and CK at any given N supply (Fig. 3-8b). Content of Thr was significantly higher in A10 under the two lowest N treatments (Fig. 3-8c). Contents of Lys and Ile were higher in A10 only under the lowest N treatment (Fig. 3-8d, f). No difference was found in Met content between A10 and CK at any given N supply (Fig. 3-8e).

Contents Ala, Val, and Leu all decreased in response to decreasing N supply (Fig. 3-9a, b, c). Contents of Ala and Leu did not show significant differences between A10 and CK at any given N supply; Val content in A10 was lower than that of CK at the highest N treatment, but was higher than that of CK at the lowest N treatment. Contents of Ser and Gly both decreased as N supply decreased; A10 had significantly higher contents of Ser than CK across the entire range of N treatments whereas Gly content showed no difference between A10 and CK (Fig. 3-9d, e). Tyr contents in both A10 and CK decreased as N supply decreased, with A10 having significantly higher content only at the second lowest N treatment (Fig. 3-9f). Phe content in both A10 and CK decreased with decreasing N supply, but A10 had significantly higher content of Phe than CK under the two highest N treatments (Fig. 3-9g).

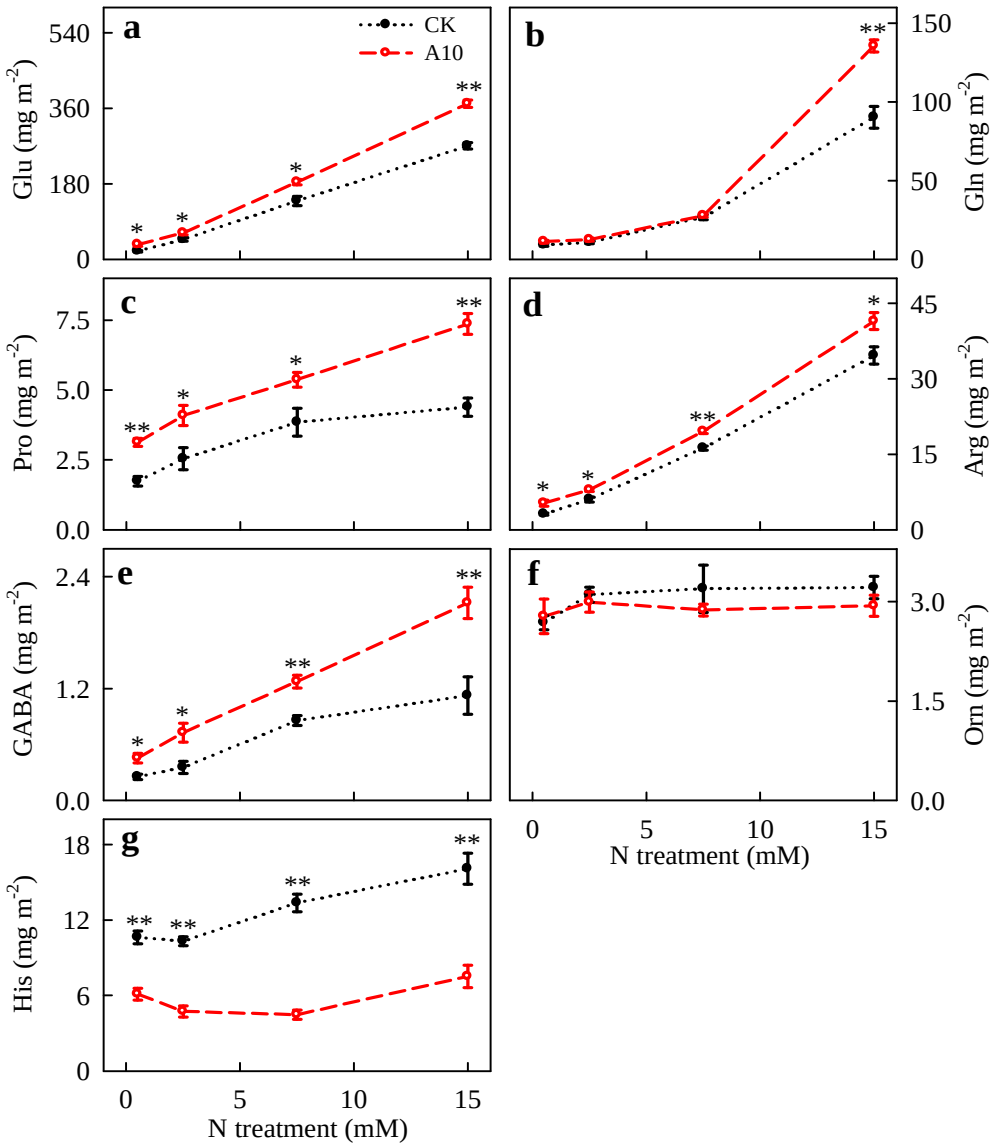

Figure 3-7. Contents of glutamate (Glu, a), glutamine (Gln, b), proline (Pro, c), Arginine (Arg, d), γ-aminobutyric acid (GABA, e), ornithine (Orn, f), and histidine (His, g) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

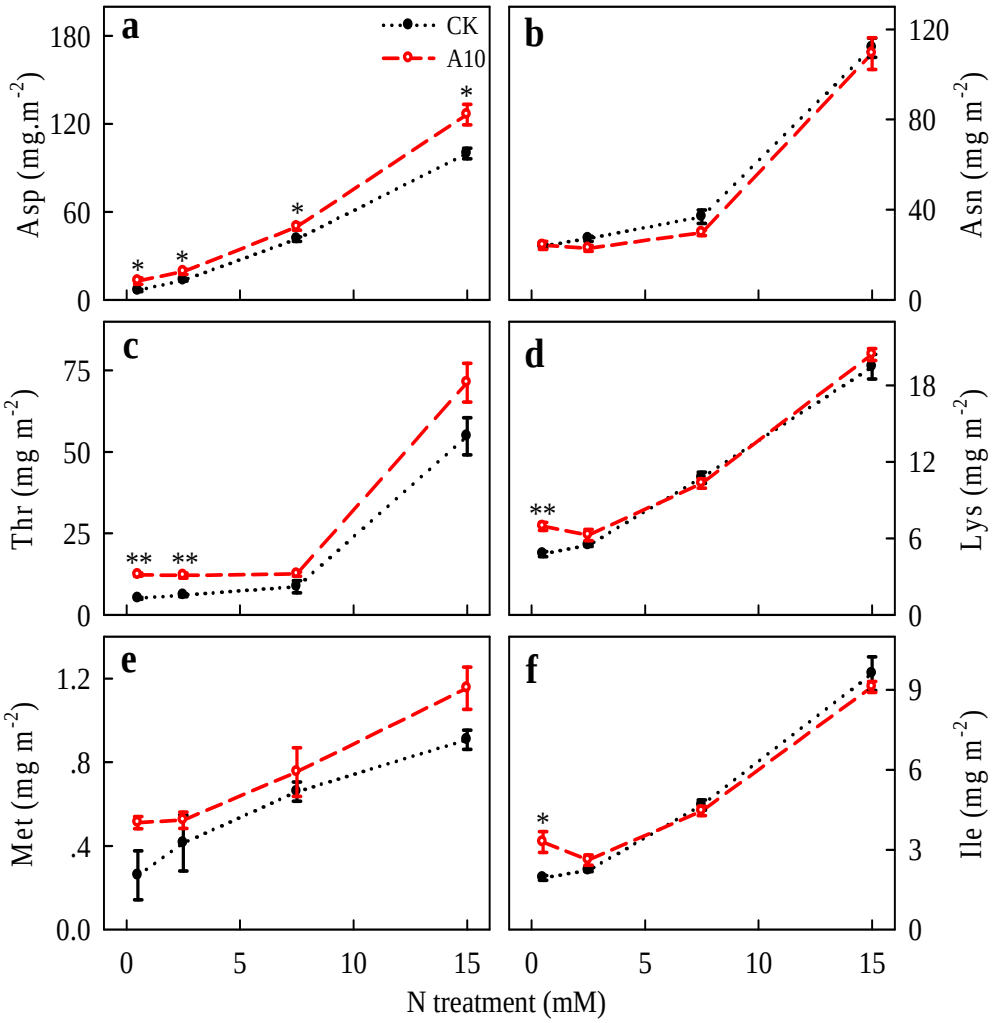

Figure 3-8. Contents of asparate (Asp, a), asparagine (Asn, b), threonine (Thr, c), lysine (Lys, d), methionine (Met, e), and isoleucine (Ile, f) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

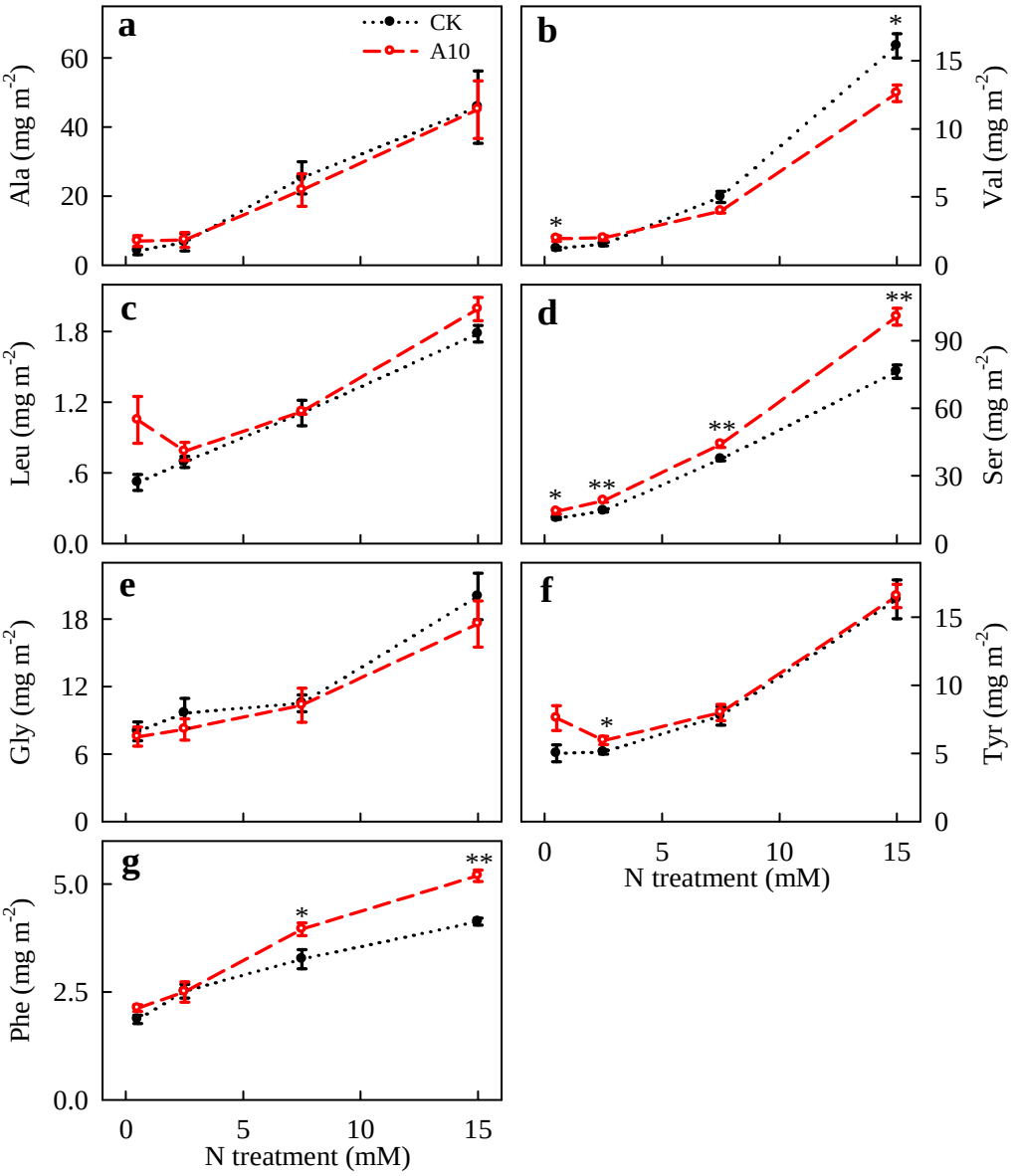

Figure 3-9. Contents of alanine (Ala, a), valine (Val, b), leucine (Leu, c), serine (Ser, d), glycine (Gly, e), tyrosine (Tyr, f), and phenylalanine (Phe, g) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Key enzymes in carbon metabolism

Enzymes in starch, sorbitol and sucrose metabolism

In response to decreasing N supply, the activities of four key enzymes for sucrose, sorbitol, and starch synthesis, cytosolic FBPase, A6PR, SPS, and AGPase all decreased (Fig. 3-10a, b, c, d). A10 had consistently higher activity of cytosolic FBPase than CK over the entire range of N supply (Fig. 3-10a). Compared with CK, the activity of A6PR in A10 decreased to a much less extent and was consistently lower across the entire range of N supply (Fig. 3-10b). Under the three lowest N treatments, SPS activity in A10 was significantly higher than that of CK (61% higher at the lowest N supply) (Fig. 3-10c). Under the two highest N treatments, no difference in AGPase activity was detected between A10 and CK, but under the two lowest N treatments, AGPase activity in A10 was significantly higher than that of CK (On average 71% higher; Fig. 3-10d). The activities of two key enzymes involved in starch degradation, β-amylase and DPE2, decreased in both A10 and CK as N supply decreased. However, both enzyme activities were significantly higher in A10 than in CK at any given N supply (Fig. 3-10e, f).

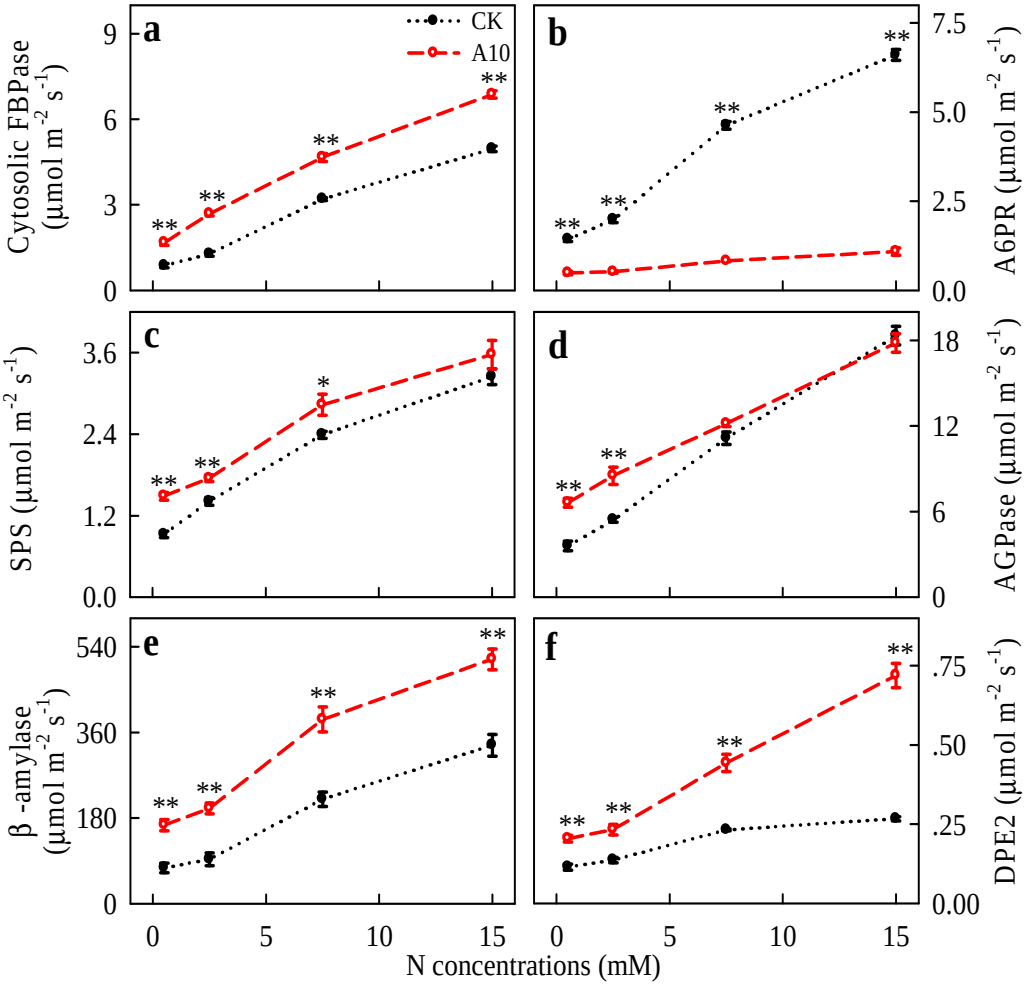

Figure 3-10. Activities of cytosolic fructose-1,6-bisphosphatase (cytosolic FBPase, a), aldolase-6-phosphate reductase (A6PR, b), sucrose-6-phosphate synthase (SPS, c), ADP-glucose pyrophosphorylase (AGPase, d), β-amylase (e), and glycosyl transferase (DPE2, f) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Enzymes in glycolysis

The activities of ten key enzymes involved in glycolysis all decreased in both A10 and CK as N supply decreased (Fig. 3-11; Fig. 3-12a, b). The activities of aldolase, TPI, NAD-G3PDH, NADP-G3PDH, PGI, and PK in A10 were consistently higher than those of CK across the entire range of N treatments (Fig. 3-11c, d, e, f, h; Fig. 3-12a). A10 had significantly higher activity of ATP-PFK only under the highest N treatment (Fig. 3-11a) and significantly higher activity of HK only under the two lowest N treatments (Fig. 3-11g). No difference in the activity of PPi-PFK or PEPP was detected between A10 and CK at any given N supply (Fig. 3-11b; Fig. 3-12b).

Enzymes in anaplerotic pathway

The activities of PEPC, NAD-MDH, NAD-ME and NADP-ME all decreased in both A10 and CK as N supply decreased (Fig. 3-12c, d, e, f). PEPC activity in A10 was significantly higher than that of CK across the entire range of N supply (Fig. 3-12c). A10 also had significantly higher activities of NAD-MDH under the three lowest N treatments (Fig. 3-12d). NAD-ME activity in A10 was significantly higher than that of CK under both the highest and the lowest N treatments (Fig. 3-12e) whereas NADP-ME showed no difference between A10 and CK at any given N supply (Fig. 3-12f).

Key enzymes in oxidative pentose phosphate pathway

The activity of G6PDH decreased in both A10 and CK as N supply decreased, but G6PDH activity in A10 decreased to a lesser extent and was significantly higher than that of CK under the two lowest N treatments (On average 38% higher; Fig. 3-12g). The activity of 6PGDH also decreased in both A10 and CK as N supply decreased, but A10 had significantly higher activity than CK across the entire range of N supply (Fig. 3-12h).

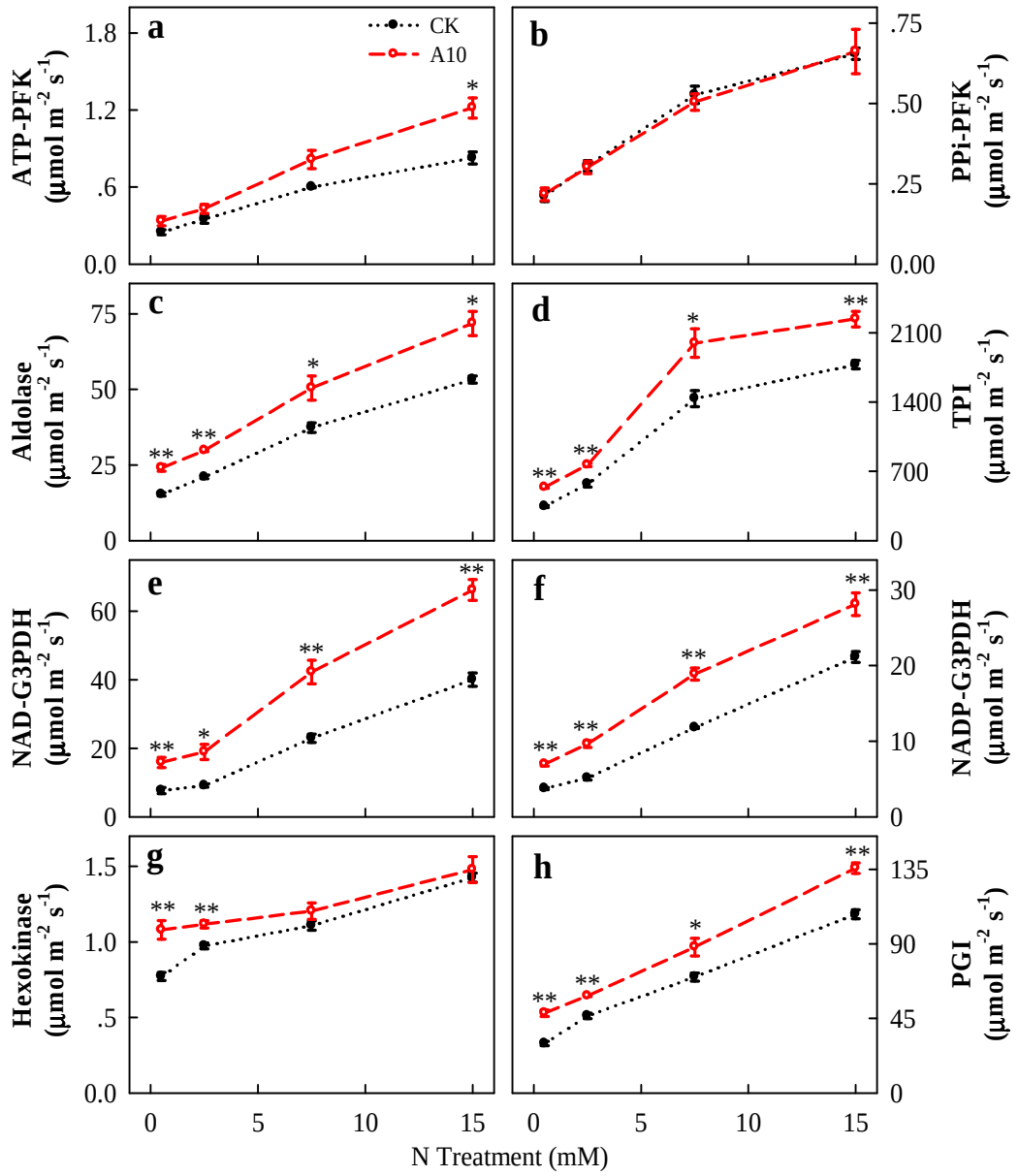

Figure 3-11. Activities of ATP-phosphofructokinase (ATP-PFK, a), PPi- phosphofructokinase (PPi-PFK, b), aldolase (c), triose phosphate isomerase (TPI, d), NAD-glyreraldehyde-3-phosphate dehydrogenase (NAD-G3PDH, e), NADP- glyreraldehyde-3-phosphate dehydrogenase (NADP-G3PDH, f), hexokinase (HK, g), and phosphoglucose isomerase (PGI, h) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at P < 0.05, and 0.01, respectively.

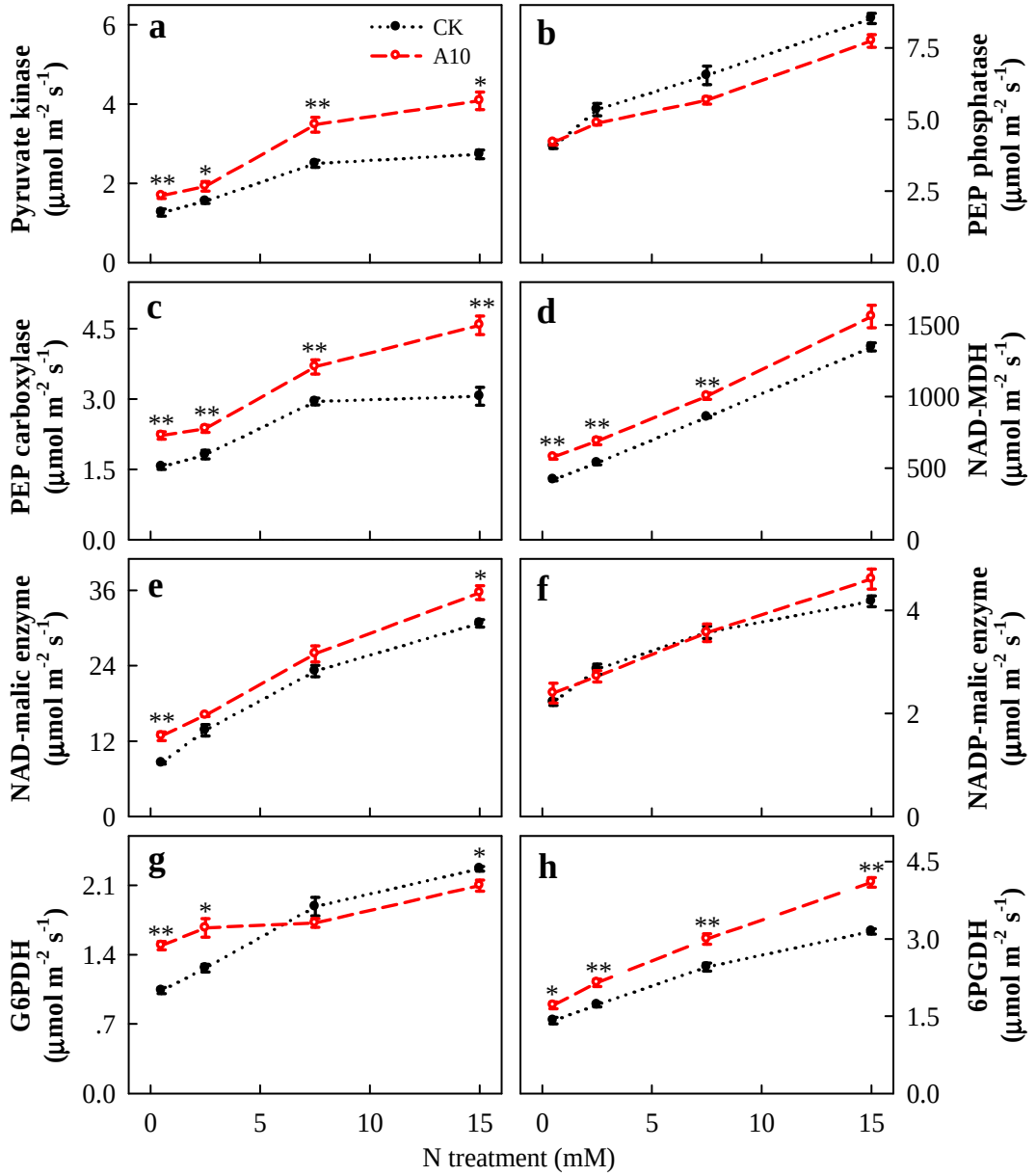

Figure 3-12. Activities of pyruvate kinase (PK, a), PEP phosphatase (PEPP, b), PEP carboxylase (PEPC, c), NAD-malate dehydrogenase (NAD-MDH, d), NAD-malic enyzme (NAD-ME, e), NADP-malic enzyme (NADP-ME, f), glucose-6-phosphate dehydrogenase (G6PDH, g), and 6-phosphogluconate dehydrogenase (6PGDH, h) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Enzymes in TCA cycle

The activities of six enzymes in TCA cycle all decreased in both A10 and CK as N supply decreased (Fig. 3-13). The activity of citrate synthase of A10 decreased to a lesser extent than that of CK, with significantly higher activity detected at the three lowest N treatments (Fig. 3-13a). The activity of aconitase in A10 was significantly higher than that of CK under the two highest N treatments (Fig. 3-13b). NADP-ICDH activity in A10 was higher than that of CK only under the lowest N treatment and NAD-ICDH activity in A10 was significantly higher than that of CK under the two lowest N treatments (Fig. 3-13c, d). The activity of succinate dehydrogenase in A10 was lower than that of CK under the two highest N treatments, but was significantly higher under the lowest N treatment (Fig. 3-13e). No significant difference was found in fumarase activity between A10 and CK (Fig. 3-13f).

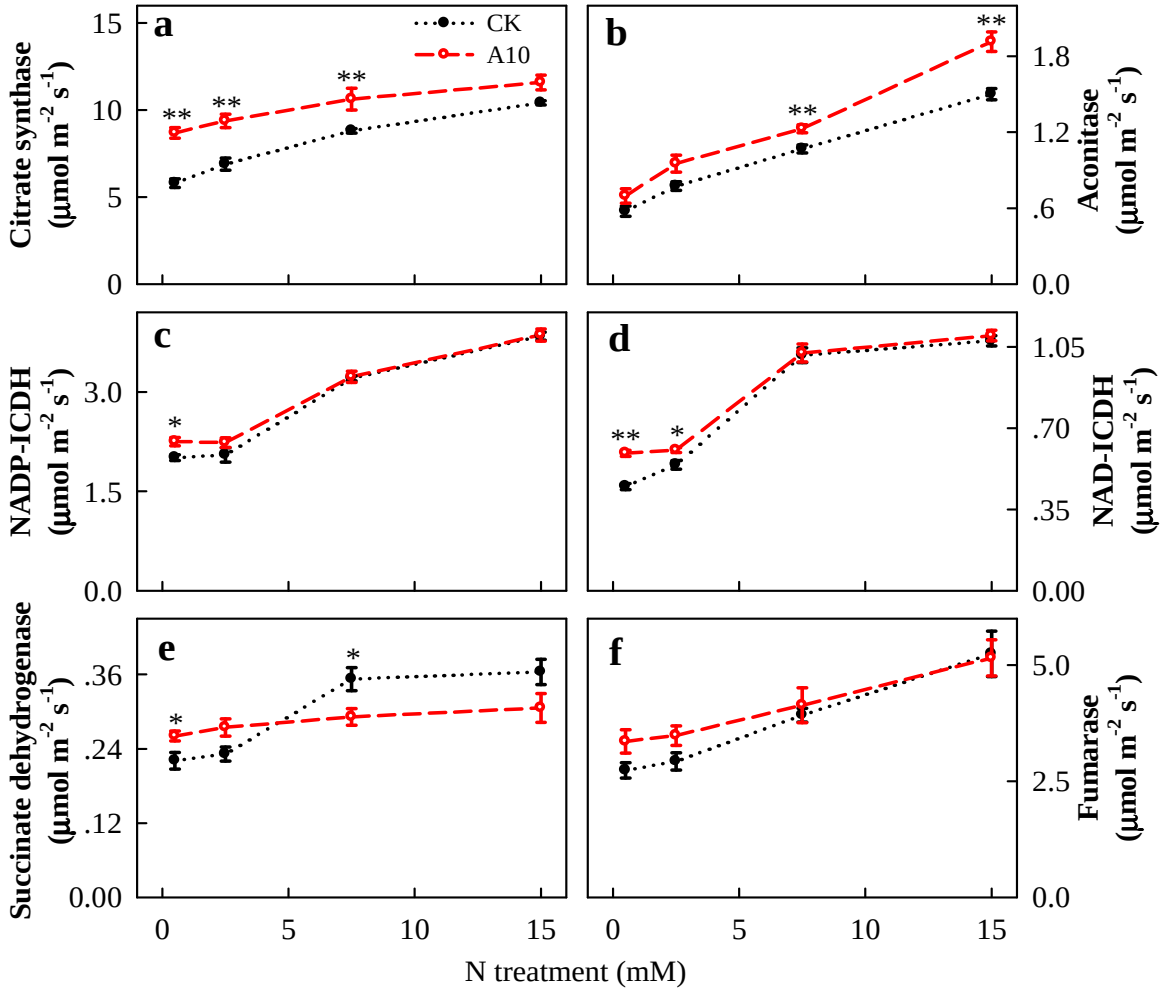

Figure 3-13. Activities of citrate synthase (a), aconitase (b), NADP-isocitrate dehydrogenase (NADP-ICDH, c), NAD-isocitrate dehydrogenase (NAD-ICDH, d), succinate dehydrogenase (e), and fumarase (f) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Enzymes in N metabolism

Key enzyme in N assimilation

No difference in NR activity was found between A10 and CK at the highest N supply; NR activity decreased dramatically in CK as N supply decreased to below 7.5 mM, but in A10, NR activity showed a much lesser decrease, with significantly higher activity detected in A10 than in CK at the two lowest N treatments (on average 73% higher activity; Fig. 3-14).

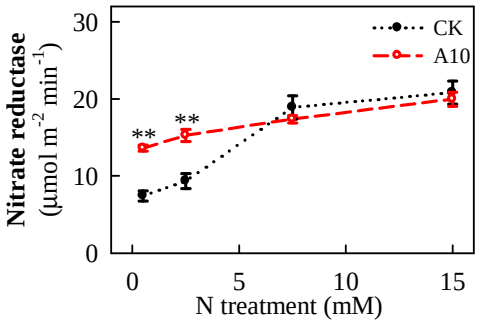

Figure 3-14. Activity of nitrate reductase (NR) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at $P < 0.05$, and 0.01, respectively.

Enzymes in amino acid biosynthesis

The activities of eight enzymes involved in amino acid biosynthesis except GDH in A10 all decreased as N supply decreased (Fig. 3-15). The activity of GS in A10 was significantly higher than that of CK across the entire range of N treatments (Fig. 3-15a). The activity of Fd-GOGAT in A10 was significantly higher than that of CK under the highest and the two lowest N treatments (Fig. 3-15b). AS activity was significantly lower in A10 than in CK under the three highest N treatments, but it was significantly higher under the lowest N treatment (Fig. 3-15c). Asp-AT activity was significantly higher in A10 only under the two lowest N treatments (Fig. 3-15d). The activities of GPT and asparaginase in A10 were significantly higher only under the lowest N treatment (Fig. 3-15e, h). GDH activity in A10 was significantly lower than that of CK

across the entire range of N treatments (Fig. 3-15f). Glutaminase activity in A10 was significantly higher than that of CK under both the highest and the lowest N treatments (Fig. 3-15g).

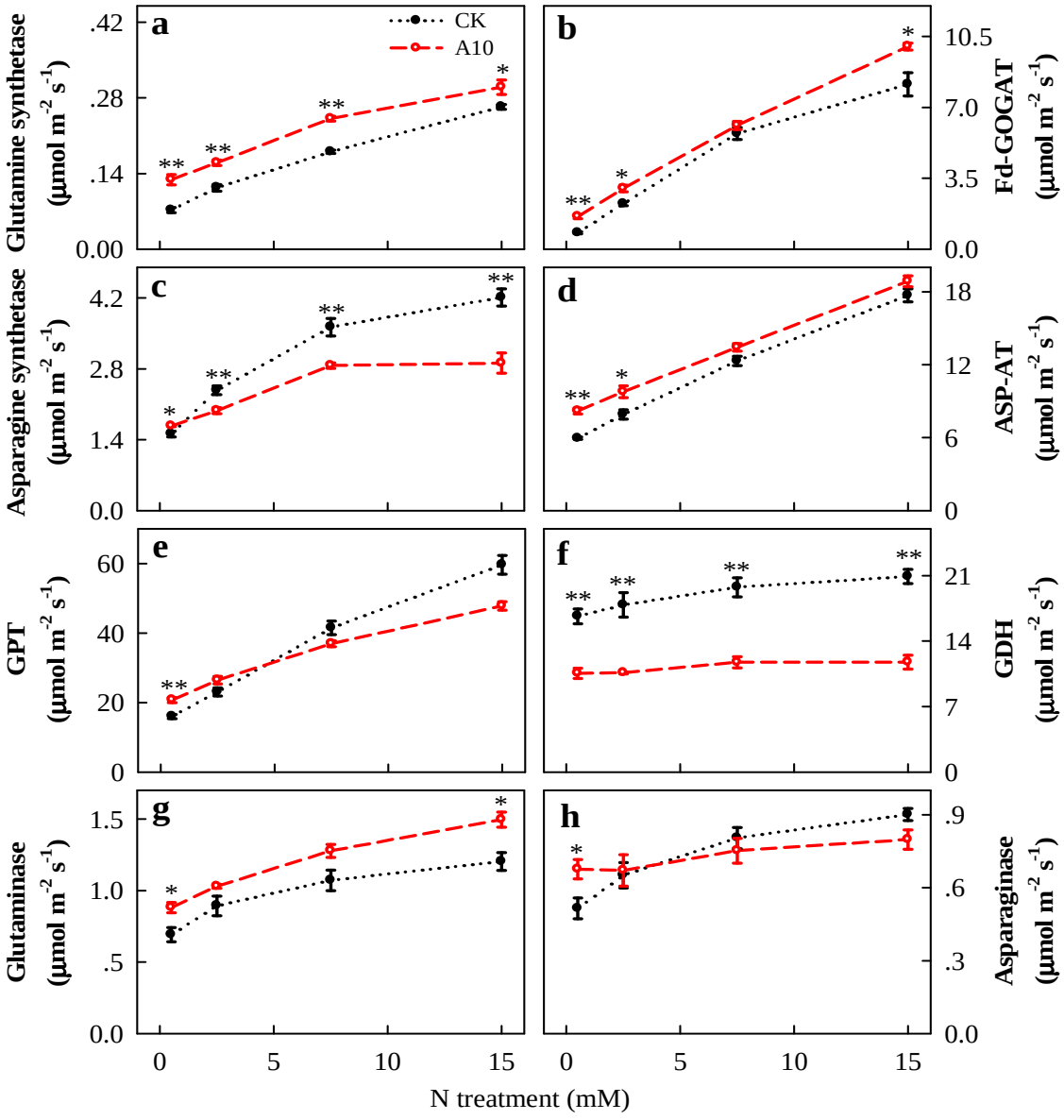

Figure 3-15. Activities of glutamine synthetase (GS, a), Fd-glutamate synthase (Fd-GOGAT, b), asparagine synthetase (AS, c), aspartate aminotransferase (Asp-AT, d), glutamate pyruvate transaminase (GPT, e), glutamate dehydrogenase (GDH, f), glutaminase (g), and asparaginase (h) in leaves of CK and A10 in response to N supply. Each point is mean of five replicates with standard error. CK: Untransformed control; A10: Antisense line. One asterisk and two asterisks above the bars indicate significant difference between A10 and CK using Student's *t*- test at P < 0.05, and 0.01, respectively.

Maltose level in response to sucrose and G6P feeding

At 24 hr, no difference in maltose content was found between water or 3 mM G6P-fed leaves and T_0 control leaves; however, leaves fed with 50 mM sucrose or 50 mM sucrose plus 3 mM G6P accumulated significantly higher content of maltose (127% and 75% higher, respectively). At 48 hr, leaves fed with sucrose, G6P or sucrose plus G6P all had significantly higher content of maltose than water -fed leaves. Water-fed leaves also had significantly higher maltose content at 48 hr than T_0 control leaves.

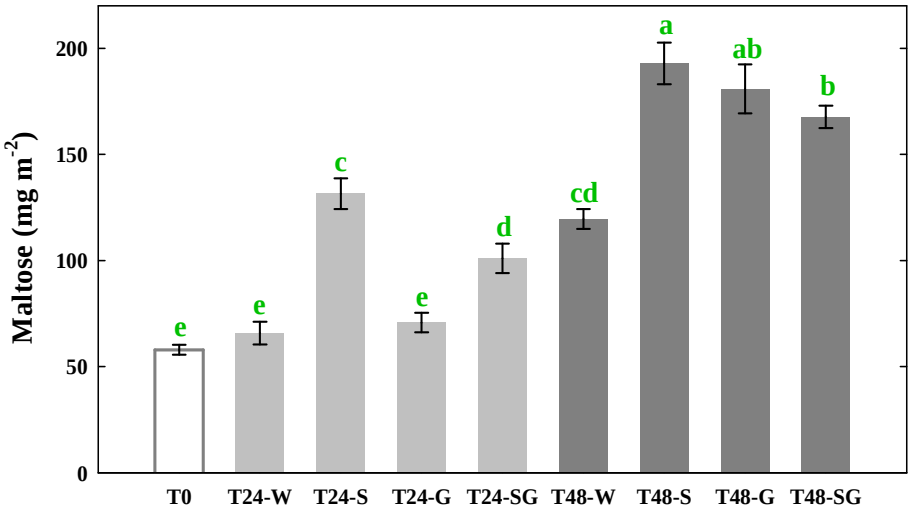

Figure 3-16. Content of maltose in detached 0.5mM N treated CK leaves fed with water, 50mM sucrose, 3mM G6P or 50mM sucrose plus 3 mM G6P for 24 hr and 48 hr. T0: CK leaves whitout any feeding treatment as the control; T24-W, T24-S, T24-G, and T24-SG: CK leaves fed with water, 50mM sucrose, 3mM G6P or 50mM sucrose plus 3mM G6P for 24 hr; T48-W, T48-S, T48-G, and T48-SG: CK leaves fed with water, 50 mM sucrose, 3 mM G6P or 50 mM sucrose plus 3mM G6P for 48 hr. Each bar represents mean of five replicates with standard error. Different letters above the bars indicate significant difference using Duncan's multiple range test at $P < 0.05$.

Discussion

I. Carbon metabolism is altered in transgenic plants to generate more substrates for ammonium assimilation under N deficiency

Data about soluble protein and total amino acid contents (Fig. 3-1b, c) clearly show that, more amino acids in A10 are utilized to synthesize proteins under N deficiency. First, the consistently higher level of hexose phosphates (Fig. 3-5a, b) in A10 than in CK across the entire range of N supply indicates that the "top-down" feedforward regulation of carbon metabolism operates under N deficiency as well as under N repletion. Under replete N, the accumulation of hexose phosphates in A10 appears to be entirely caused by inhibition of A6PR activity because no difference in HK activity was detected between A10 and CK. However, under N deficiency, HK may have also contributed to the accumulation of hexose phosphates in A10 because HK activity was significantly higher in A10 than in CK under low N supply (Fig. 3-11g). In fact, of all the enzymes in glycolysis, HK is the only enzyme that showed a preferential increase in A10 relative to CK under low N supply. In addition to its catalytic role, HK might also be involved in sensing sugar levels to regulate carbon metabolism as discussed extensively in recent reviews (Rolland et al., 2006; Granot, 2008; Bolouri-Moghaddam et al., 2010). In contrast to HK, the activity of ATP-PFK in A10 was higher than that of CK only at the highest N supply. Neither ATP-PFK nor PPi-PFK was responsible for the elevated carbon flow from hexose phosphates to G3P in A10 because no difference in activity were detected between A10 and CK for either enzyme under N deficiency (Fig. 3-11a, b).

Second, transgenic plants maintained a higher activity of glycolysis, TCA cycle and OPPP under N deficiency than CK. Both higher contents of 3-PGA and higher activities of NAD-G3PDH and NADP-G3PDH in A10 under N deficiency suggest that more G3P was converted to 3-PGA in A10 relative to CK under N deficiency (Fig. 3-6a; Fig. 3-11e, f). Higher G3P content

(Fig. 3-5d) along with a possibly higher requirement for NADPH is consistent with this up-regulation of G3PDH. It is known that NADP-G3PDH plays a role in generating NADPH for sugar alcohol synthesis and facilitating the transfer of reducing equivalents from chloroplasts to the cytosol (Loescher and Everard, 2000). NADP-G3PDH also allows the bypass of the ATP-consuming reaction catalyzed by 3-PGA kinase during energy-limitation stage (Van Dongen et al., 2011). As discussed in Chapter 2, the direct conversion of G3P to 3-PGA catalyzed by NADP-G3PDH was important for transgenic plants to mitigate the Pi limitation in the cytosol. Therefore, the up-regulation of NAD-G3PDH and NADP-G3PDH in A10 not only supplies the lower-end glycolysis with more 3-PGA but also generates more reductants to meet other biosynthetic needs, such as N assimilation or bypassing the Pi and ATP consuming step to help A10 maintain a more efficient glycolysis. Moreover, A10 consistently had significantly higher pyruvate content (Fig. 3-6c) and higher PK activity than CK across the entire range of N supply (Fig. 3-12a), which clearly indicates that A10 maintaines the activity of glycolysis under N limitation to a larger degree than CK.

In contrast to higher contents of most intermediates in glycolysis, contents of key organic acids in TCA cycle in A10 were either lower (2-OG and malate) or remained the same (isocitrate, succinate and fumarate) relative to CK under N deficiency (Fig. 3-6d-j). When combined with higher activities of citrate synthase, isocitrate dehydrogenase and succinate dehydrogenase in A10 under N deficiency (Fig. 3-13a, c, d, e), the data suggest that more TCA intermediates are withdrawn for amino acid synthesis in A10 under N deficiency. These changes in organic acids of A10 in response to decreasing N supply are similar for the most part to the results reported by Tschoep et al (2009) that N limitation induced decreased contents of malate, fumurate, cirtrate, succinate and 2-OG. They are also consistent with higher contents of amino acids and soluble proteins in A10 under N deficiency (Fig. 3-1b, c). Why most intermediates in glycolysis are

maintained at higher contents whereas those in TCA cycle are kept at lower contents or remained unchanged? We think that the significant accumulation of G6P and F6P in A10 under N deficiency is powerful enough to maintain a higher activity of glycolysis with the intermediates elevated whereas, TCA cycle, as a downstream process, driven by upstream intermediates, operates at a higher rate with lower or the same contents of intermediates when some of its intermediates are withdrawn for amino acid synthesis. To replenish the TCA cycle intermediates that were withdrawn for amino acid synthesis, the anaplerotic pathway must be up-regulated. This is indeed the case as higher activities of PEPC, NAD-MDH and NAD-ME in A10 under the highest N treatment were maintained under lower N supply (Fig. 3-12c, d, e).

It was found previously in Chapter 2 that, G6PDH, the key regulatory enzyme in OPPP, was up-regulated mainly at nighttime in transgenic plants to support N metabolism under N repletion. Under the two lowest N treatments, however, G6PDH activity in A10 during daytime was on average 38% higher than that of CK, indicating the up-regulation of the OPPP in A10 under N deficiency (Fig. 3-12g). This is not inconsistent with previous work showing that the enzymes of the OPPP pathway is up-regulated in response to nitrate addition in Arabidopsis (Scheible et al., 2004) and other plants (Crawford, 1995) because in both cases more amino acids and proteins are synthesized. Since OPPP provides intermediates and NADPH for nucleotides synthesis, shikimake and aromatic amino acids synthesis, lignins, flavonoids, phenylpronoids synthesis and other cellular activities (Kruger and Von Schaewen, 2003), a more active OPPP helps to support the higher rates of amino acid synthesis and other metabolic activities in A10 under N deficiency. Hauschild and Von Schaewen (2003) reported that higher contents of sugars (sucrose and fructose) increased the mRNA level of cytosolic G6PDH in potato leaves, leading to the elevated G6PDH activity. The up-regulation of G6PDH in A10 under N deficiency belonged to the same mechanism and the involvement of sugar signaling would be discussed in details below.

II. Nitrogen metabolism is up-regulated in transgenic plants under N deficiency

Compared with the untransformed control, higher contents of amino acids (Fig. 3-7, 8 and 9) and soluble proteins (Fig. 3-1b) and higher activities of NR (Fig. 3-14) and key enzymes in amino acid synthesis, GS, Fd-GOGAT, AS, Asp-AT and GPT (Fig. 3-15a, b, c, d, e) found in the transgenic plants under low N supply clearly indicate that, N metabolism is up-regulated in the transgenic plants under nitrogen deficiency. Both metabolite and enzyme activity data point to the following scenario that eventually leads to up-regulation of nitrogen metabolism and synthesis of amino acids and proteins. Sustained accumulation of hexose phosphates in A10 relative to CK under N deficiency drives the operation of glycolysis, anaplerotic pathway and TCA cycle at a higher rate as discussed above. This provides more substrates for amino acid synthesis and subsequent protein synthesis, which is reflected in higher contents of amino acids and soluble proteins. The increased amino acid and protein synthesis demands more NH_4^+ in transgenic plants, which directly or indirectly up-regulates nitrate reduction. This explains why the content of NH_4^+ was lower in A10 than in CK under N deficiency (Fig. 3-1d). The higher NO_3^- content found in the transgenic plants (Fig. 3-1e) is likely the result of differential up-regulation of nitrate uptake and reduction (uptake exceeds reduction). In addition, accumulation of hexose phosphates up-regulates OPPP to produce more reducing equivalents requied for nitrate reduction as discussed above. However, higher SLW found in A10 may have also contributed to its consistently higher total leaf N content (Fig. 3-1a) across the entire range of N treatments.

III. The reprogrammed carbon and nitrogen metabolism in A10 under N deficiency is related to low nitrate, high sucrose, high HK activity, and maltose accumulation

As discussed above, changes on NR activity in A10 under N deficiency was a consequence of several factors functioning coordinately, so was the reprogrammed carbon and nitrogen metabolism. Sugar involvement in plants' responses to different N supply has been well established: sugar status could directly or indirectly influence NR transcription and low sugar resulted in NR inactivation at the post-translational level (Kaiser and Förster, 1989; Kaiser and Huber, 1994; Klein et al., 2000). According to Klein et al (2000), only falling below a critical level, could low level of sucrose repress the expression of NR. Morcuende et al (1998) reported that, the detached tobacco leaves supplied with 25 mM sucrose had elevated nitrate assimilation rate, ammonium assimilation rate, amino acid biosynthesis, and 2-OG content. Sucrose supplement stimulated carbon flow from glycolysis to organic acids and nitrate was also involved in this process. However, these metabolic changes brought by high sucrose or high nitrate were not consistent with what had been found in the experiment described in Chapter 2 that, the higher level of hexose phosphates, not the higher level of sucrose, was responsible for the changes in primary metabolism of transgenic plants in response to the decreased sorbitol synthesis under N repletion. In this chapter, the decreased sucrose content in both CK and A10 due to the decreased N supply apparently resulted in distinct metabolic responses in A10 most probably because A10 had higher content of sucrose than CK under N deficiency (Fig. 3-4c). Since sorbitol content in A10 and sucrose content in CK did not change much as N supply decreased (Fig. 3-4b, c), it had been postulated that the significant decrease but consistently higher content of sucrose in A10 might be the signal in response to N deficiency to bring about the metabolic changes listed above. Moreover, SPS activity in A10 under the lowest N treatment showed the highest increase, which indicated that sucrose synthesis was more active in A10

under N deficiency than what happened under N repletion (Fig. 3-10c). The cytosolic FBPase activity in A10 was on average 42.07% and 101.45% higher than that of CK under the highest N and lowest N treatments, respectively (Fig. 3-10a). This higher activity of cytosolic FBPase in A10 under N deficiency also suggests that more newly fixed carbon was partitioned to the cytosol supporting sucrose synthesis. A10 consistently accumulated a higher content of starch across the entire range of N treatments and percentage wise, but the extent of increase in A10 under N deficiency was even slightly smaller (Fig. 3-4a). Considering that AGPase activity in A10 was significantly higher than that of CK only under N deficiency (Fig. 3-10d), it was clear that, more starch was utilized in A10 under N deficiency. Of the two major products of starch degradation, glucose content did not change much in A10 and whereas maltose content in A10 showed the biggest increase ever under N deficiency (Fig. 3-4e, h). The content of maltose in A10 was 58.19%, 451.74%, 1165.20%, and 950.95% higher than that of CK under four N treatments from higher to lower N, respectively. The higher activities of both β-amylase and DPE2 in A10 suggest that, in response to decreasing N supply, less maltose was converted to glucose in the cytosol than generated by starch degradation in the chloroplast in A10 under N deficiency (Fig. 3-10e, f). This explains why maltose accumulated to such a high level in A10. Maltose accumulation has been suggested to play a role in some stress tolerance mechanism. Kaplan and Guy (2005) reported that, a starch-dependent maltose accumulation plus a maltose-dependent increase in glucose, fructose and sucrose might function in protecting the photosynthetic electron transport chain under freezing stress. As discussed above, HK activity was also significantly higher in A10 than in CK under N deficiency (Fig. 3-11g). Therefore, the response of A10 to N deficiency is the result of coordinated consequence regulated by sucrose, HK and maltose signals plus low nitrate. Additionally, as discussed in Chapter 2, when some upstream substrates of the respiratory pathways, such as G6P and F6P, accumulate to a critically

high level, the regular ‘bottom-up’ feedback inhibition could be released by the ‘top-down’ feedforward up-regulation. Since the levels of nitrate, sucrose, hexsoe phosphates, and maltose changed simultaneously, it is difficult to pinpoint which signal plays a more important role. However, the data from sucrose feeding experiment on control leaves with low N mimicked the response of transgenic plants under N deficiency in terms of maltose accumulation (FIG. 3-16). So, it’s likely that the higher sucrose level present in the transgenic leaves has made them more responsive to nitrogen deficiency to generate much higher levels of maltose.

IV. Transgenic plants have higher photosynthesis under N deficiency as a result of up-regulation of nitrogen metabolism

Compared with CK, A10 had significantly higher contents of chlorophylls and higher activities of rubisco, GAPDH, stromal FBPase, and PRK under N deficiency, thereby maintaining higher photosynthesis (Fig. 3-2a, c, d; Fig. 3-3). The relatively higher photosynthesis per unit leaf area in A10 under N deficiency indicates that the transgenic plants are more tolerant of N deficiency. Because N deficiency lowers leaf photosynthetic capacity by reducing the synthesis of proteins (Sugiharto et al., 1990; Scheible et al., 2004), a slow decrease of photosynthetic rate in response N deficiency in the recently released maize hybrids has been taken as a sign of tolerance to N deficiency (Ding et al., 2005). So, the fact that transgenic plants had less reduction in the activity of most enzymes in photosynthesis relative to the control under N deficiency can only be explained by up-regulation of nitrogen metabolism in the transgenic plants. In other words, a more active nitrogen and carbon metabolism in the transgenic plants under N deficiency has enabled them to synthesize proteins or enzymes at a higher level than the control plants, thereby rendering them more tolerant of nitrogen deficiency. These findings are consistent with the conclusions made by Foyer et al. (2011) that, improving the flexibility of

respiratory pathways can generate a more powerful driving force for N metabolism and enhanced NUE.

Conclusions

Under N repletion, glycolysis, TCA cycle and N metabolism is up-regulated in the transgenic plants because of the accumulation of hexose phosphates and the resulting Pi limitation. The elevated levels of both organic acid metabolism and nitrogen metabolism are maintained or even enhanced in the transgenic plants under N deficiency. This enables the transgenic plants to synthesize more proteins (enzymes), thereby maintaining a higher photosynthesis per unit leaf area relative to the untransformed control plants under N deficiency. As a result of having a more active organic acid and nitrogen metabolism, the transgenic plants are more tolerant of N deficiency.

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