Local and systemic regulation of photosynthesis by carbohydrate accumulation in leaves of *Phaseolus vulgaris* L.

（邦題　インゲン葉における炭水化物による光合成の調節）

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Contents

Chapter 3

Manipulation of light and CO_2 environments of the primary leaves of bean (*Phaseolus vulgaris* L.) affects photosynthesis in both the primary and the first trifoliate leaves: Involvement of systemic regulation.

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General Introduction

Carbohydrates are major products of photosynthesis and are used for respiration of all living organisms. Carbohydrates also act as signals. In plants, carbohydrates are mainly produced as photosynthates, and accumulation of photosynthates in the leaf is known to repress gene expression of photosynthetic components. This regulation of photosynthesis by carbohydrates is called the feedback regulation of photosynthesis.

On the other hand, the carbohydrates are translocated from leaves (source) to other organs, such as roots, stems, meristems, flowers and fruits (sink). When carbohydrate production of a given leaf exceeds the demand for the carbohydrate consumption by the other organs, carbohydrates accumulate in the leaf. Thus, the carbohydrate content reflects balance between the photosynthetic rate of the leaf itself and the demand for carbohydrates by the sink organs, namely, the carbon status of the plant. The feedback regulation of photosynthesis by carbohydrates would thus contribute to the maintenance of the carbon status of a plant.

Nitrogen limitation enhances the feedback regulation of photosynthesis and carbohydrates tend to accumulate in the leaves, when grown under nitrogen-limited conditions. This suggests that the carbohydrate regulation of photosynthesis would relate to the repression of photosynthesis due to nitrogen limitation.

In a plant canopy, upper leaves received stronger light than lower leaves (Monsi and Saeki 1953). For maximization of photosynthetic production in the whole plant in the dense stand, upper leaves should have more nitrogen and higher photosynthetic activities than the lower leaves (Hirose and Werger 1987). Since the carbohydrate content of the leaf reflects the carbon and nitrogen balance of the whole plant, the carbohydrate would also play roles in formation of the gradient in the leaf nitrogen content among the leaves. The roles of carbohydrate on regulation of photosynthesis of the leaf and leaf canopy have not been elucidated yet. In this study, I studied (1) effects of carbohydrate accumulation on photosynthesis of the leaf, (2)

effects of nitrogen nutrition on regulation of photosynthesis by carbohydrates, and (3) effects of carbohydrate accumulation in one leaf on photosynthesis of the other leaves. These results suggest that the carbohydrates in one leaf are involved not only in regulation of photosynthesis of the leaf itself, but also in regulation of carbon status of the whole plant.

Carbon metabolism in plants

Carbon is one of the most important elements in living organisms. Carbon is assimilated by algae and plants through photosynthesis and the assimilated carbon are consumed by themselves and heterotrophic organisms. 220 Pg carbon is assimilated by autotrophic organisms per year, and the photosynthetic assimilation of CO_2 is a major sink of atmospheric CO_2 (Grace 2004, Woodward 2007). Terrestrial higher plants contribute to about half of this flux. Assimilated carbon is stored mainly as carbohydrates (sucrose and starch).

Carbohydrates are synthesized in chloroplasts (Fig. 1). At the initial step of the Calvin-Benson cycle, two molecules of phosphoglyceric acid (PGA) are synthesized from ribulose-1, 5-bisphosphate (RuBP) and atmospheric CO_2 in the stroma (RuBP carboxylation reaction). Riburose-1, 5-bisphosphate carboxylase/ oxygenase (Rubisco) catalyzes this reaction. The RuBP is regenerated from PGA using the reductant (NADPH) and ATP, which are synthesized in thylakoids (RuBP regeneration reaction). PGA is converted to glyceraldehyde-3-phosphate (GA3P), and GA3P in the chloroplast is exported to the cytosol in exchange with phosphate (Pi) in the cytosol by the Pi-triose phosphate (TP) translocator. GA3P is used for synthesis of substrates such as glucose and sucrose in the cytosol. Sucrose is exported from the leaf and is translocated to the sink organs. A part of sucrose is stored in the vacuole. Starch is synthesized from the GA3P in the chloroplast (for a review, see Lytovchenko et al. 2007). Starch is mainly synthesized in daytime, and is degraded in the night. The degradation products of starch are also used for the sucrose

synthesis in the cytosol, and the sucrose is mainly exported during the night period (Kaiser and Bassham 1979*ab*, Mitchell et al. 1992, Lloyd et al. 2005, Zeeman 2007).

Carbohydrates accumulation and photosynthesis

In general, carbohydrate accumulation in the leaf represses its rate of photosynthesis. This regulation is called "feedback inhibition of photosynthesis" or "sugar repression of photosynthesis." Thorne and Koller (1974) reported that shading of the soybean plants keeping one leaf exposed decreased its starch content by 20%, and enhanced the photosynthetic rate of the leaf. In tobacco plants overexpressing cell wall invertase, carbohydrates accumulated in the leaves and the photosynthesis was repressed (Schaewen et al. 1990, Stitt et al. 1991). Krapp et al. (1991) fed glucose solution to spinach leaves via their petioles, and found that the photosynthetic rate of the glucose-fed mature leaves decreased. Cold-girdling of petioles of the leaves in *Chenopodium album* also increased the carbohydrate contents and decreased the photosynthetic rate of the leaves (Krapp and Stitt 1995). These results suggest that the carbohydrate accumulation represses photosynthesis. Such the feedback inhibition of photosynthesis would have some ecological meanings for plants to survive in fluctuating environments. However, the mechanisms and the ecological meaning of the feedback repression of photosynthesis have not been clarified yet. Elucidation of the meaning and mechanisms of feedback inhibition is crucial, because the elevated atmospheric CO_2 typically enhances the carbohydrate accumulation in the leaves.

Effects of long-term cultivation at elevated CO_2 on photosynthesis

Atmospheric CO_2 concentration is increasing because of the industrial use of fossil fuels. The CO_2 concentration of the atmosphere had been stable and about 280 $\mu L\ L^{-1}$ for 10 thousand years before the industrial revolution. It has been increasing gradually and attains about 380 $\mu L\ L^{-1}$ [National Oceanic and Atmospheric Administration (NOAA) in America, http://www.esrl.noaa.gov/, Fig. 2] in 2007. The global increase in the atmospheric

CO_2 concentration affects not only the air temperature through the greenhouse effect, but carbon assimilation of the plants.

Elevated CO_2 instantaneously increases the photosynthetic rate on leaf area basis, because Rubisco is not saturated at the ambient CO_2 concentration of 380 μL L^{-1} and the oxygenation reaction of Rubisco is repressed by the elevated CO_2 (Furquhar et al. 1980). However, in many cases, long-term cultivation in elevated CO_2 decreases the photosynthetic rate. Long-term cultivation in elevated CO_2 causes the carbohydrate accumulation in the leaves (Brown and Escombe 1902, Madsen 1968, Apel 1976, Sasek et al. 1985, Chang et al. 1998, Moore et al. 1998, Sawada et al. 2001). This carbohydrate accumulation is thought to repress the photosynthetic rates of the leaves (Makino 1994, van Oosten and Besford 1996, Griffin and Seemann 1996, Ainsworth and Long 2005).

Limitation of growth of sink organs is also suggested to cause the repression of photosynthesis, in particular, under the elevated CO_2 conditions. Arp (1991) showed that the decrease in the rate of photosynthesis by the long-term cultivation in elevated CO_2 was large in plants cultivated in small pots (pot size effect). Thomas and Strain (1991) also showed similar results. However, even when the plants are treated with elevated CO_2 condition in the field (free air CO_2 enrichment: FACE), the photosynthetic rate of the leaves also decreases (Nie et al. 1995, Long et al. 2004, Ainsworth and Long 2005). Thus, the pot size effect is not a sole reason for the photosynthetic repression in elevated CO_2.

On the other hand, low nitrogen nutrition reinforces the photosynthetic repression in elevated CO_2 (Stitt and Krapp 1999). For example, Rogers et al. (1996) showed that Rubisco content of wheat leaves cultivated in elevated CO_2 markedly decreased in the nitrogen deficient plants. Photosynthetic rate of leaves of the nodulated soybean plants is less affected by the long-term cultivation in the elevated CO_2 (Rogers et al. 2006). Plants that are fertilized with sufficient nitrogen are less affected by the elevated CO_2 than the nitrogen deficient plants (Ludewig et al. 1998, Geiger et al. 1999). Oren et al. (2001) treated a pine forest stand in North Carolina by the FACE. In the well fertilized area, the FACE treatment increased the carbon gain of the stand. However, in the nutrient deficient area, the carbon gain of the forest was not affected, or even decreased by the FACE

treatment. In general, nitrogen nutrition improves plant growth, and the demand for carbon by the sink organs increases in the well fertilized plant (Lehmeier et al. 2005). Much carbohydrate assimilated in the source leaves is transported and consumed by the sink organs in the well fertilized plants even in the elevated CO_2. Thus, the carbohydrates are less accumulated, and the effects of cultivation in elevated CO_2 on photosynthesis are less pronounced in the well fertilized plants than in the nitrogen limited plants (Stitt and Krapp 1999). From these studies, the carbohydrate accumulation seems to one of the main reasons for the photosynthetic repression in elevated CO_2.

However, some experimental results suggest that the carbohydrate accumulation of the leaves is not the main reason of photosynthetic repression by the long-term cultivation in elevated CO_2. Elevated CO_2 does not affect the photosynthetic rate and the *rbcs* transcript level of the leaves in some species and cultivars (Moore et al. 1998). Sims et al. (1998) treated two leaflets of a trifoliate leaf of soybean at 1000 μL L^{-1} CO_2 while keeping the other part of plant including one leaflet of the trifoliate leaf in air containing CO_2 at 250 μL L^{-1}. Although the treated trifoliate leaves accumulated carbohydrates, the photosynthetic rate of the trifoliate leaves was not affected by the treatment. They also treated the whole soybean plants at 1000 μL L^{-1} CO_2 but the two leaflets of the trifoliate leaf at 250 μL L^{-1} CO_2. The carbohydrates contents of the leaflets were unchanged, however, the photosynthetic rates of the leaflets significantly decreased. From these, they claimed that the CO_2 environment itself, but not the carbohydrate contents, affected the photosynthetic rate of the leaves. These results suggest that the elevated CO_2 accelerates leaf ontogeny and that carbohydrate accumulation is not the main cause for the decrease in the photosynthetic rate of the leaves. However, the leaves studied in their study maintained photosynthetic capacity, and the effect of the carbohydrate accumulation of photosynthesis would be regulated by the leaf age. Thus, age-dependent changes of the effect of elevated CO_2 should be needed for the elucidation of CO_2 effects on photosynthesis.

There are three proposed hypotheses to explain the feedback repression of photosynthesis: 1) sugar repression of photosynthetic gene expression, 2) phosphate limitation by accumulation of sugar-phosphate, and 3) decrease in CO_2 conductance from

the intercellular space to the Rubisco catalytic site in the chloroplast stroma because of the starch accumulation.

Sugars act as signaling molecules

Carbohydrates act as signaling molecules (Rolland et al. 2001). In higher plants, many physiological characteristics are regulated by carbohydrates (Tsukaya et al. 1991, Herbers et al. 1995, Herbers et al. 1996, Pien et al. 2001, Riou-Khamlichi et al. 2000, Ohto et al. 2001, Takahashi et al. 2003, Heyer et al. 2004, Chen et al. 1994, Mita et al. 1995, Chiou and Bush 1998, Aoki et al. 1999, Barker et al. 2000, Koch 2004).

Sugar signaling affects the gene expression of the photosynthetic components of the leaves. Sheen (1990) showed that the β-glucuronidase (β-GUS) gene, which were fused with the promoter regions of genes of photosynthetic components, such as pyruvate phosphodikinase (PPDK), phosphoenolpyruvate carboxylase (PEPC), apoprotein of light harvesting chlorophyll protein complex II, and Rubisco small subunit, were electroporated into freshly isolated maize mesophyll protoplasts, and the β-GUS activity was analyzed. When glucose or sucrose was fed to the maize protoplasts, expressions of β-GUS from the promoter regions of photosynthetic genes were repressed. Krapp and Stitt (1993) also showed similar results using spinach protoplasts. The gene encoding Pi-TP translocator is induced by sucrose feeding in tobacco seedlings (Knight and Gray 1994). Ono and Watanabe (1997) showed that the *rbcs* gene of sunflower leaves was induced by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treatment, a well known inhibitor of the photosynthetic electron transport. Because the carbohydrate content of the leaves was very low in the presence of DCMU, the enhanced *rbcs* gene expression was attributed to the low carbohydrate content. Many genes of the photosynthetic components such as *atp-D* (subunit of F_o-F_1 ATPase), *ca* (carbonic anhydrase), *psbA* (PSII subunit), *pc* (plastocyanin), and *lhcb* (apoprotein of chlorophyll *a*/*b* protein complex), are known to be regulated by sugar signals in higher plants(Koch 1996, Pego et al. 2000, Paul et al. 2001).

On the contrary, some studies suggest that the sugar feeding to the root even increases the photosynthetic rate and the enzymes related to the photosynthesis of the leaves. Kovtun and Daie (1995) showed that 90-300 mM glucose or sucrose feeding to the root of sugar beet (*Beta vulgaris*) enhanced expressions of *rbcs* and cytoplasmic fructose-1,6-bisphosphatase (cytFBPase) gene of the leaves, the photosynthetic rate, and the growth rate. Furbank et al. (1997) also showed marked increase in photosynthetic rate in tobacco and *Flaveria bidentis* plants fed with 5% sucrose from their roots. Thus, in some cases, the sugar feeding to the roots improves the photosynthetic activity of the leaves.

Leaf senescence is accelerated by carbohydrate accumulation in the leaves (Yoshida 2003). Dai et al. (1999) showed that the leaf senescence was accelerated in tomato plants overexpressing the hexokinase gene of *A. thaliana*. Leaves of *rbcs*-antisense tobacco plants accumulates smaller amount of carbohydrates than that of wt plants, and the leaf senescence delays (Miller et al. 2000). Heat-girdling of the major vein accelerates accumulation of the carbohydrate and breakdown of chlorophylls and proteins in the barley leaves (Parrott et al. 2005). The acceleration of the breakdown is marked in high light. Glucose feeding to the roots accelerated the decrease in maximum quantum yield of PSII (F_v/F_m, Wingler et al. 2006), and induced the expression of *sag12* (senescence associated gene) in *A. thaliana* (Pourtau et al. 2006). These results suggest that the carbohydrate accumulation accelerates the decrease in the photosynthetic rate, and thus leaf senescence.

Sugar sensor and the signaling mechanisms

In higher plants, hexokinase acts as a glucose sensor. Jang and Sheen (1994) fed glucose analogs to maize protoplasts, which express reporter genes that were fused with promoter regions of the photosynthetic components. When 2-deoxyglucose, which is phosphorylated by hexokinase but is not metabolized further, was fed to the protoplasts, the expressions of the reporter genes were repressed. On the contrary, feeding of 3-O-metylglucose or 6-oxoglucose, which are not metabolized by hexokinase, to the maize protoplasts did not affect the expression of the reporter genes. Furthermore,

hexokinase-antisense seedlings of *A. thaliana* do not show the yellowing cotyledons, or stunt elongation of hypocotyls when they are cultured in glucose-containing medium, although these phenomenons are observed in wt plants (Jang and Sheen 1997). Transfection of an yeast hexokinase gene to the hexokinase-antisense plants recovers the hexokinase activity, but does not rescue the seedling responses to high glucose concentration in *A. thaliana*. HXK mutants lacking catalytic activity still support glucose signaling functions (Moore et al. 2003). Thus, the signaling function of hexokinase is independent of the enzyme activity. Hexokinase is located in the cytosol or associated with the chloroplast envelope, mitochondrial outer membrane (Galina et al. 1999, Wiese et al. 1999, Giese et al. 2005) or inside of the nuclei (Yanagisawa 2003), indicating the regulatory function of hexokinase.

The hexokinase signaling pathway interacts with ABA and ethylene signaling pathways (Rolland et al. 2005). Glucose feeding to the root of *A. thaliana* induces expressions of the genes related to the ABA biosynthesis pathway and the signaling components, and increases the ABA level in the plants (Cheng et al. 2002). Application of ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), to the wt plants inhibits the glucose effect on seedling growth (Zhou et al. 1998). An ethylene-insensitive mutant of *A. thaliana* (*ein*1-1) is hyper-sensitive to glucose. Rubisco content of the leaves in ethylene insensitive mutant of tobacco is lower than that of wt plants, suggesting the oversensitivity to the sugar in these mutants (Pierik et al. 2006, Tholen 2007). Glucose signaling also interacts with the cytokinin signaling pathway (Wingler et al. 1998, Hwang and Sheen 2001). These interactions between sugar and hormones complicate the clarification of sugar response in higher plants.

Pi limitation of photosynthesis in elevated CO_2 condition

When the plants are grown in elevated CO_2 or nutrient limited conditions, the growth rate relative to the photosynthate production becomes small. The carbon assimilation rate exceeds the carbohydrate export rate under these conditions and sucrose is

accumulated in the cytosol. Sugar-phosphates, which are the intermediates of the sucrose synthesis, also accumulate in the cytosol. Free Pi decreases when sugar-phosphates accumulate in the cytosol. Pi-TP translocator in the chloroplast envelope is essential for the import of Pi to the chloroplast to maintain the ATP synthesis in thylakoids. Limited Pi availability in the cytosol inhibits the Pi-TP translocation on the chloroplast envelope and limits the ATP synthesis and carbon assimilation in the stroma. The Pi limitation occurs especially under the elevated CO_2 condition (von Caemmerer and Furquhar 1981, Sawada et al. 1982, 1983, Stitt 1986, Sage and Sharkey 1987, Stitt and Quick 1989, von Caemmerer 2000). Pi limitation also represses the activities of chloroplastic F_o-F_1 ATPase in pea (Quick and Mills 1988), PPDK and PEPC in maize (Usuda and Shimogawara 1992), and Rubisco in soybean leaves (Sawada et al. 1990, 1992). However, Pi limitation would not occur when much starch is synthesized in the chloroplast, because the starch synthesis in the chloroplast releases Pi from sugar-phosphates. Pi also accumulates in the vacuole, and the cytoplasmic Pi concentration decreased only slightly even in the Pi deficient condition (Mimura et al. 1990). Thus, the Pi limitation of photosynthesis would occur only in the plants cultivated in elevated CO_2 and/or under severe Pi-limited conditions.

Starch accumulation and internal CO_2 conductance (g_i)

Cultivation in elevated CO_2 increases the carbohydrates content of the leaves, especially starch. The starch accumulation is also thought to repress the photosynthetic rate. Nakano et al. (2000) demonstrated that the repression of photosynthesis by long-term cultivation in elevated CO_2 was not caused by the decrease in the stomatal conductance, or the Rubisco content and the activity. They darkened bean plants, which grew under elevated CO_2, for two days to decrease the starch content of the leaves. The photosynthetic rate of the leaves in the darkened plant increased to the level of that grown in the ambient CO_2. Sawada et al. (2001) also reported similar results. These results indicate that the starch accumulation itself, but not the decrease in the contents of the photosynthetic components, represses photosynthesis. Starch is synthesized and accumulates in the chloroplast, and the

starch accumulation distorts the chloroplast shape (Cave et al. 1981). The distortion of the chloroplast shape enlarges the average distance from the intercellular space to the Rubisco catalytic site (Fig. 3). This distance affects the CO_2 conductance from the intercellular space to active sites of Rubisco protein (internal conductance, g_i), because the CO_2 conductance in the liquid phase is very low and 1/10000 of that in gaseous phase.

^{13}C discrimination examination revealed that g_i in many herbaceous and tree species are small (0.05-0.5 mmol m^{-2} s^{-1}, Hanba et al. 1999), and a major limiting factor of the photosynthetic carbon assimilation (Evans et al. 1986). Nafziger and Koller (1976) measured the g_i of soybean (*Glycine max* L.) leaves grown in the elevated CO_2 (2000 μl l^{-1}), ambient CO_2 (300 μl l^{-1}), and low CO_2 (50 μl l^{-1}) with the Gaastra's method (1959). The leaves grown under elevated CO_2 accumulated much starch and showed the low g_i. On the contrary, the leaves grown under low CO_2 accumulated less starch and showed the higher g_i than that grown under ambient CO_2. Their results suggest that the accumulation of starch in the leaf decreases the g_i and inhibits the photosynthesis of the leaves.

Developmental stages affect the effect of carbohydrates on photosynthesis

The carbohydrate accumulation represses photosynthesis, but the metabolism of carbohydrate depends on the leaf developmental stages. Mature source leaves with high photosynthetic activity export the surplus photosynthates to the sink organs. On the contrary, the young sink leaves import carbohydrates to construct their photosynthetic systems. With leaf senescence, the photosynthetic activity decreased. The carbohydrate repression of photosynthesis would not always occur, and the effect of carbohydrates on photosynthesis would change along the leaf developmental stage. Krapp et al. (1991) supplied 50 mM glucose solution to spinach leaves via the petioles, and measured the maximum photosynthetic rate per leaf area. In the source leaves, the photosynthetic rate of the glucose-treated source leaves was 70% lower than that of the control leaves to which distilled water was supplied. On the contrary, the photosynthetic rate of the glucose-treated leaves was not different from that of the control sink leaves. Nie et al. (1995) cultivated

wheat (*Triticum aestivum* L.) plants under elevated (550 μmol mol^{-1}) and ambient CO_2 (360 μmol mol^{-1}), and measured expression of the photosynthetic genes and the carbohydrate contents of the flag leaves. When the flag leaves were in the early expanding stage, mRNA levels of Rubisco genes (*rbcS* and *rbcL*) in the leaves grown under elevated CO_2 were lower than those of leaves grown under ambient CO_2. On the other hand, in the expanded leaves, the mRNA levels of Rubisco genes were not different between plants grown under elevated and ambient CO_2. These results suggest that the carbohydrate effects on photosynthesis would change along with the leaf developmental stages (Dickinson et al. 1991, Yoshida 2003).

Systemic regulation in plants

Plants do not have organ for long distance signaling like the nerve system. However, biotic and abiotic events occurring in a part of the plant affect the physiological characteristics of the other parts. This type of regulation is called “systemic regulation”. Recent studies revealed that the leaf morphology is also regulated systemically. Lake et al. (2001) treated the mature leaves with low light or elevated CO_2 without affecting the light and CO_2 environments of the young developing leaves in *A. thaliana*. The stomatal density of the young developing leaves decreases when the mature leaves are treated with low light or elevated CO_2, and the local environments of the young developing leaves did not affect the stomatal density. Thomas et al. (2004) and Miyazawa et al. (2006) also showed similar results. Yano and Terashima (2001) showed that the light environment of the mature leaves of *Chenopodium album* regulates the direction of cell division of the palisade tissue in the young leaves. These results clearly showed that the environments of the mature leaves affect the development of the young leaves.

Photosynthesis of the leaves is also affected by the other part of the plants. Pons and Pearcy (1994) shaded the first trifoliate leaves of soybean, while the other part of the plant remained in high light. The decrease in light saturated rate of photosynthesis was enhanced by this local shading treatmentcompared with the control plants grown in low light. When

individual leaves of *A. thaliana* are darkened, keeping the other parts exposed, the senescence of the covered leaves was accelerated. In contrast, when the whole plants are placed in darkness, the leaves show delayed senescence (Weaver and Amasino 2001). These results suggest that the photosynthesis and senescence of a leaf are regulated systemically. The systemic regulation of photosynthesis by the environment of the other leaves is important for maximizing the photosynthetic production by the whole plant individual. However, the mechanisms related to the regulation of photosynthetic activities by the environment of the other leaves have not been elucidated yet.

The light intensity that illuminated to the leaves is probably sensed with three mechanisms. Light receptors such as phytochrome, cryptochrome and phototropin received the light and affect seed germination, stomatal aperture, phototropism and chloroplast movement (Franklin et al. 2005). Carbohydrates and the redox state of photosystems also reflect the light environment around the leaves. Thus, these are the strong candidate of the regulation of photosynthesis and development of a leaf by the environments of the other leaves.

Photoreceptors

There are three types of photoreceptors (phytochromes, cryptochromes, phototropins) that function in plants. Phytochrome are red/far red photoreceptors, and 5 genes of phytochromes are found in *A. thaliana* (*phy*A~E). These phytochromes are red absorbing type (660 nm, P_R) in the dark condition. When the red light is illuminated to the plants, the phytochromes become active form that absorb far red light (730 nm, P_{fr}). P_{fr} induces the seed germination and phototropism, and reduces the hypocotyl elongation (Franklin et al. 2005). In dense stand, upper leaves absorb red light relative to far red right, and the R/FR ratio around the lower leaves become lower than that for the upper leaves. Some studies suggested that the low R/FR ratio induces the leaf senescence of the leaves through the phytochrome action (Rousseaux et al. 1996, 2000). They illuminated leaves of sunflower plants with low R/FR light and the plants grown in low R/FR showed faster leaf

senescence than those of the leaves illuminated with high R/FR light. However, application of far red right to the lower leaves would affect the excitation balance of photosystems and oxidize the plastoquinone pool. Leaf senescence of *phyB* mutant of *A. thaliana* shows similar response to low R/FR light as those of wt plants (Smith and Whitelam 1997). Analyses of mutants that defect in phytochrome chromophore synthesis are needed for the estimation of the roles of phytochrome in the systemic regulation of photosynthesis. The relationship between the phytochrome and the systemic regulation has not been clearly examined yet.

Cryptochrome is the homolog of the photolyase gene in prokaryote (Briggs and Huala 1999), and act as a blue light receptor in plants, and *A. thaliana* have two genes of cryptochrome (*cry*1, 2). Phototropin is also the blue light receptor in plants and *A. thaliana* have two genes of phototropin (*phot*1, 2). These photoreceptors affect the phototropism, stomatal aperture, hypocotyl elongation and chloroplast movements. There are few studies that suggest the relationship between these blue light photoreceptors and photosynthesis, and the signals activated by blue light receptors would not move from one cell to the other (Tlafka et al. 1999). Thus, the blue light receptors would not relate to the systemic regulation of photosynthesis.

Does carbohydrate of a leaf affect that the photosynthesis of the leaves in one plant?

As mentioned above, carbohydrates affect several physiological characteristics of the plants including photosynthesis of the leaves. Carbohydrate contents of the leaves are affected by many environmental conditions such as light, CO_2 concentration in the air, temperature, root salinity, water stress, and pathogen infection (Sasek et al. 1985, Everard et al. 1994, Lafta and Lorenzen 1995, Herbers et al. 2000). The carbohydrates content of the leaves reflects the environments of the leaves themselves. In addition, the environments and the carbohydrate metabolisms of the other part of leaves and other organs also affect the carbohydrate content of the leaves. For example, removal of sink organs, such as rice panicles and bean pods, increases the carbohydrate content of the leaves (Nakano et al.

1995, 2000). In a radish cultivar having a large storage root, the carbohydrate accumulation in the leaves is less pronounced even in the elevated CO_2 condition (Usuda and Shimogawara 1998). Nitrogen depletion causes the carbohydrate accumulation in the leaves in tomato (Groot et al. 2002). Leaves of nitrogen- and phosphorus-deficient cotton accumulate more starch than that of the well fertilized plants (Radin and Eidenbock 1986, Wong 1990). The fructan and starch content of the leaves increased during nitrogen deficiency in the seedlings of barley (Wang and Tillberg 1996). Feeding of a nutrient solution to the nitrogen deficient sunflower decreases the carbohydrate content of the leaves (Ono and Watanabe 1997). Accordingly, the carbohydrate content of the leaves is determined by the carbon assimilation of themselves, the demand for carbon by the sink organs, and the nutrient status of the plant. And thus, the carbohydrate would regulate the carbon assimilation balance among the leaves through the sugar repression of photosynthesis (Ono et al. 2001). Carbohydrate is also translocated to the other parts of the plant via phloem. Thus, carbohydrate is the strong candidate of the signaling molecules that is involved in the systemic regulation of leaf morphology and photosynthesis. Yano and Terashima (2001) and Coupe et al. (2006) also suggested the involvement of carbohydrate in the regulation of direction of cell division and stomatal density of the mature leaves. These mechanisms would be related to the maximization of the photosynthetic carbon assimilation in the plant, and contribute to the survival of the plant. Thus, the effects of carbohydrate accumulation on the photosynthetic properties should be examined not only at the single leaf level, but also at the whole plant level. However, these have not been sufficiently investigated yet.

Redox state of photosystems regulates gene expressions related to the photosynthesis

Redox state of the photosynthetic electron transport chain affects light acclimation of the photosystems via regulation of expression of the genes of the photosynthetic components (Huner et al. 1998). In *Synechocystis* PCC 6714, a cyanobacterium strain, the abundance of PSI is regulated by the redox state of cyt b_6/f complex (Murakami and Fujita

1991). In algae, high light condition reduces the redox state of the plastoquinone (PQ), and represses the expression of *lhcb* in *Dunaliella tertiolecta*. The transcription of *lhcb* is enhanced by oxidizing PQ with DCMU, whereas it is repressed by partially inhibiting the oxidation of PQ with dibromomethylisopropyl benzoquinone (DBMIB) (Escoubus et al. 1995). Culture at low temperaturse also reduces the PQ pool and suppresses the expression of *lhcb* gene in *Chlorella vulgaris* and *Danaliella salina* (Maxwell et al. 1995*ab*). Gene expressions of cytochrome b_6/f complex, thioredoxin, and genes related to the synthesis of chlorophyll precursors are regulated by the PQ redox state (Pearson et al. 1993, Danon and Mayfield 1994, Kropat et al. 1997).

Some studies suggested that this type of redox regulation of photosynthesis also occur in higher plants (Pfannschmidt 2003). Montane et al. (1998) showed that the cultivation of barley in low CO_2 and O_2 condition increased the amount of xanthophylls and induced the expression of early light inducible genes (ELIPs), which are the homologs of the LHCII genes. The redox state of photosystem II also regulates the PSII/PSI ratio in mustard (Pfannschmidt et al. 1999), expression of *lhcb*, and photophosphorylation of LHCII in ryegrass (Pursiheimo et al. 2001). PQ pool of a cytochrome b_6/f mutant of *Lemna perpusilla* is reduced even under the low light conditions, and fronds of the mutant have smaller amount of LHCII than those of wt plants (Yang et al. 2001). In this mutant, the LHCII protease always shows the high activity, although this protease is activated only in the high light in wt plants, suggesting the regulation of the protease activity by the PQ redox state.

On the other hand, some cultivars of ryegrass and wheat maintain the oxidized state of PQ even in high light condition by increasing the maximum photosynthetic rate, chlorophyll content and sucrose phosphate synthase (SPS) activity (Gray et al. 1996). Expression of LHCII gene is affected neither by DCMU treatment in tobacco plants (Petracek et al. 1997), nor by low temperature in barley leaves (Montane et al. 1997). Thus, the PQ redox state does not always affect the *lhcb* gene expression in all plant species. However, some molecules related to the redox state, such as thioredoxin, glutathione, and H_2O_2 act as regulators of gene expression and the activities of enzymes in higher plants

(Noctor et al. 2002, Baier and Dietz 2005). Reduced thioredoxin regulates the activities of Calvin cycle enzymes, such as fructose-1, 6-bisphosphatase, sedoheptulose-1, 7-bisphosphatase, and chloroplastic F_oF_1-ATPase (Schürmann and Jacquot 2000), and reduced glutathione regulates the expression of pathogenesis related protein (PR-1), cytosolic ascorbate peroxidase (APX), and *psbA* gene, which encode the D1 protein of photosystem II (Mullineaux and Rausch 2005). Thus, the redox regulation of photosynthesis and the gene expression is important not only in algae, but also in higher plants.

Signals of redox state of photosystems transfer from organ to the other organs

Redox state of the photosystems is also a candidate of the systemic signal in higher plants. Karpinski et al. (2001) illuminated a strong light (3000 μmol photon m^{-2} s^{-1}) to a part of leaves in *A. thaliana*. This treatment induced the ascorbate peroxidase (APX2) gene expression even in the unilluminated leaves. This suggests that the redox status of photosystems in the leaf affects the physiology of the other leaves. The signaling molecule would be hydrogen peroxide (H_2O_2, Slésak et al. 2007). H_2O_2 is a stable reactive oxygen species (ROS), and synthesized in chloroplasts, mitochondria, peroxisomes, and the apoplast. H_2O_2 is generated in the pathogen infected cells and induces the systemic acquired resistance (SAR) in the other part of plants (Neill et al. 2002, Alvarez et al. 1998, Capone et al. 2004). H_2O_2 also generates in the vascular cells of senesced leaves in *A. thaliana* (Fryer et al. 2003). Thus, H_2O_2 is a strong candidate for the signaling molecule in the systemic regulation.

Carbohydrate regulation of photosynthetic properties of a leaf

Based on these previous reports, accumulation of carbohydrates would repress photosynthesis, and the effects of carbohydrate on photosynthetic properties seem to change with leaf developmental stage. However, the detailed analyses of the changes in the

carbohydrate effect during leaf maturation have not been examined, and the contributions of three potential factors mentioned before, repression of photosynthetic genes, phosphate limitation, and decrease in g_i with starch accumulation are also unclear. In the study described in chapter 1, I fed sucrose solutions to the bean roots and increased the carbohydrate contents of the primary leaves. To investigate the feedback regulation of the photosynthesis at various leaf developmental stages, I compared the photosynthetic properties of the sugar-treated and control leaves at various developmental stages. I also examined involvement of three potential factors in relation to carbohydrate repression of photosynthesis.

Sugar signaling and nitrogen nutrition

Carbohydrate and nitrogen contents of the leaves would regulate the photosynthetic rate and the leaf senescence. For example, the limited nitrogen nutrition enhances the carbohydrate accumulation. Thus, the repression of photosynthesis by the carbohydrate accumulation should be discussed in relation to (with respect to) nitrogen nutrition, however, the relationship among CO_2 gas exchange of the leaves, nitrogen nutrition and the carbohydrate accumulation have not been well understood. In the chapter 2, I fed 20 mM sucrose solution to the root of bean plants, which were fed nutrient solution containing 0, 0.72 and 6 mM NO_3^-, and examined the effect of nitrogen nutrition on the feedback repression of the rate of CO_2 gas exchange of the primary leaves in bean plants.

Relationships between systemic regulation of photosynthesis and carbohydrates

There are some possibilities that the photosynthetic activities of leaves regulated systemically, however, detailed analyses of the systemic regulation of photosynthesis have not been reported yet. In the systemic regulation of photosynthesis, the carbohydrate and redox state of photosystems are the strong candidates of the signals.

In chapter 3, I examined the effect of local environment of the primary leaves of bean plants on the photosynthetic properties of the first trifoliate leaves, which emerged next to the primary leaves. To study the systemic regulation of photosynthesis, I constructed a local environment controlling system (Fig. 4). With this system, the CO_2 concentration in the air, humidity and light environments of the primary leaves can be controlled without affecting the environment of the other part of plants. I manipulated the CO_2 and light environment of the primary leaves, and measured the changes in photosynthetic properties of the trifoliate leaves.

The main purpose

The purposes of this study were (1) to examine the carbohydrate effects on photosynthetic properties of the leaves, (2) to elucidate the relationship between the carbohydrate effect and the nitrogen nutrition onleaf photosynthesis, and (3) to clarify the roles of carbohydrate and redox state of the chloroplast on the systemic regulation of leaf photosynthesis. Based on the results, I will discuss the roles of carbohydrate on the regulation of photosynthesis at the whole plant level.

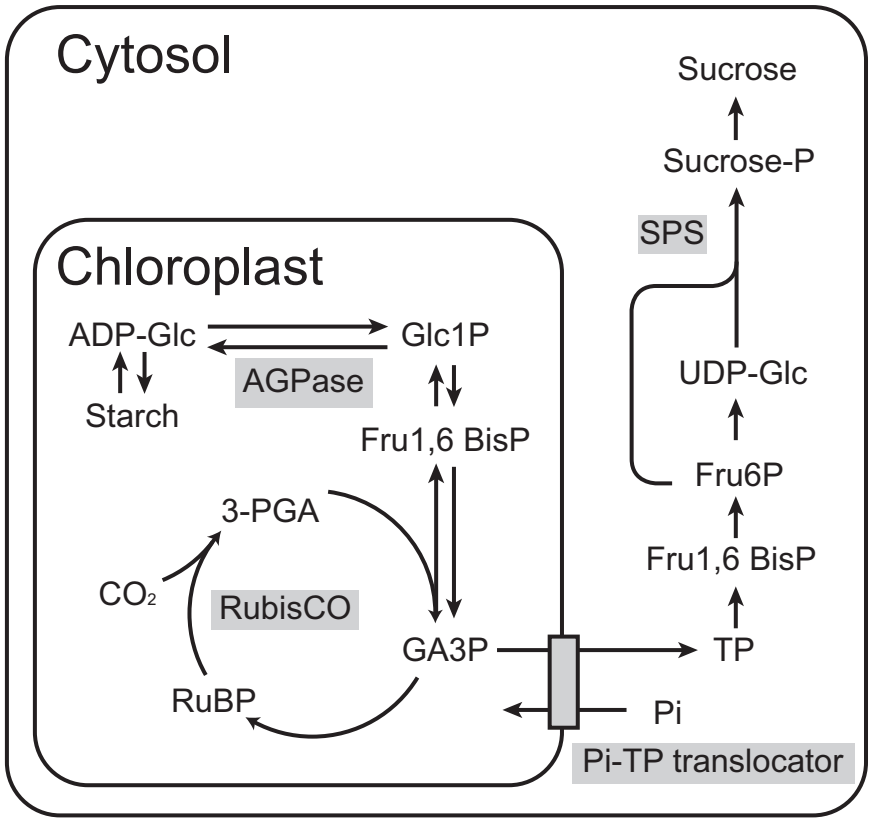

Fig. 1 Carbohydrate metabolism in plant cell

CO_2 is assimilated in the chloroplast and the two 3-phosphoglyceric acid (3-PGA) are synthesized from CO_2 and riburose-1, 5-bisphosphate (RuBP). 3-PGA is converted to the glyceraldehyde-3-phosphate (GA3P). GA3P is translocated from the chloroplast by Pi-TP translocator and sucrose is synthesized in the cytosol. Starch is also synthesized from the GA3P in the chloroplast. Fru1, 6BisP: fructose -1, 6-bisphosphate, Fru6P: fructose-6-phosphate, UDP-Glc: UDP-glucose, Sucrose-P: sucrose phosphate, Glc1P: glucose-1-phosphate, ADP-Glc: ADP-glucose, TP: triose phosphate, Rubisco: Riburose-1, 5-bisphosphate carboxylase / oxygenase, AGPase: ADP-glucose pyrophosphorylase, SPS: sucrose phosphate synthase.

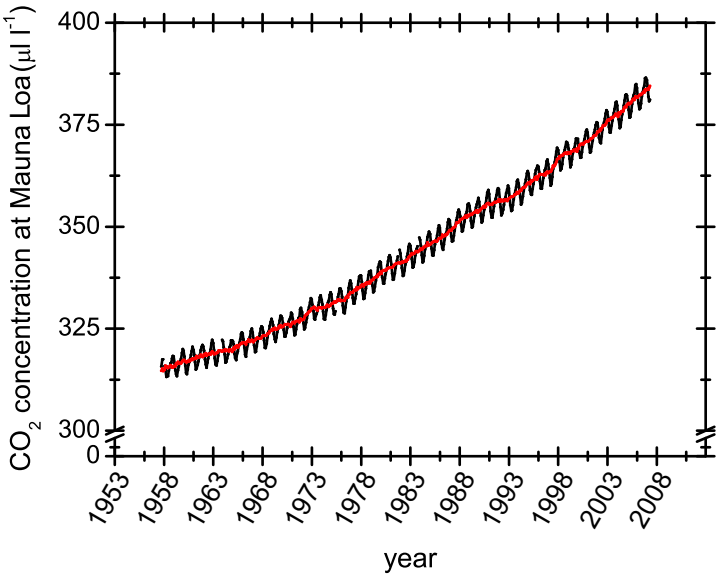

Fig. 2 Atmospheric carbon dioxide monthy mean mixing ratios.

Data prior to May 1974 are from the Scripps Institution of Oceanography (SIO), data since May 1974 are from the National Oceanic and Atmospheric Administration (NOAA). A long-term trend curve is fitted to the monthly mean values. The black line represents the monthly mean values, centered on the middle of each month. The red line represents the same, after correction for the average seasonal cycle.

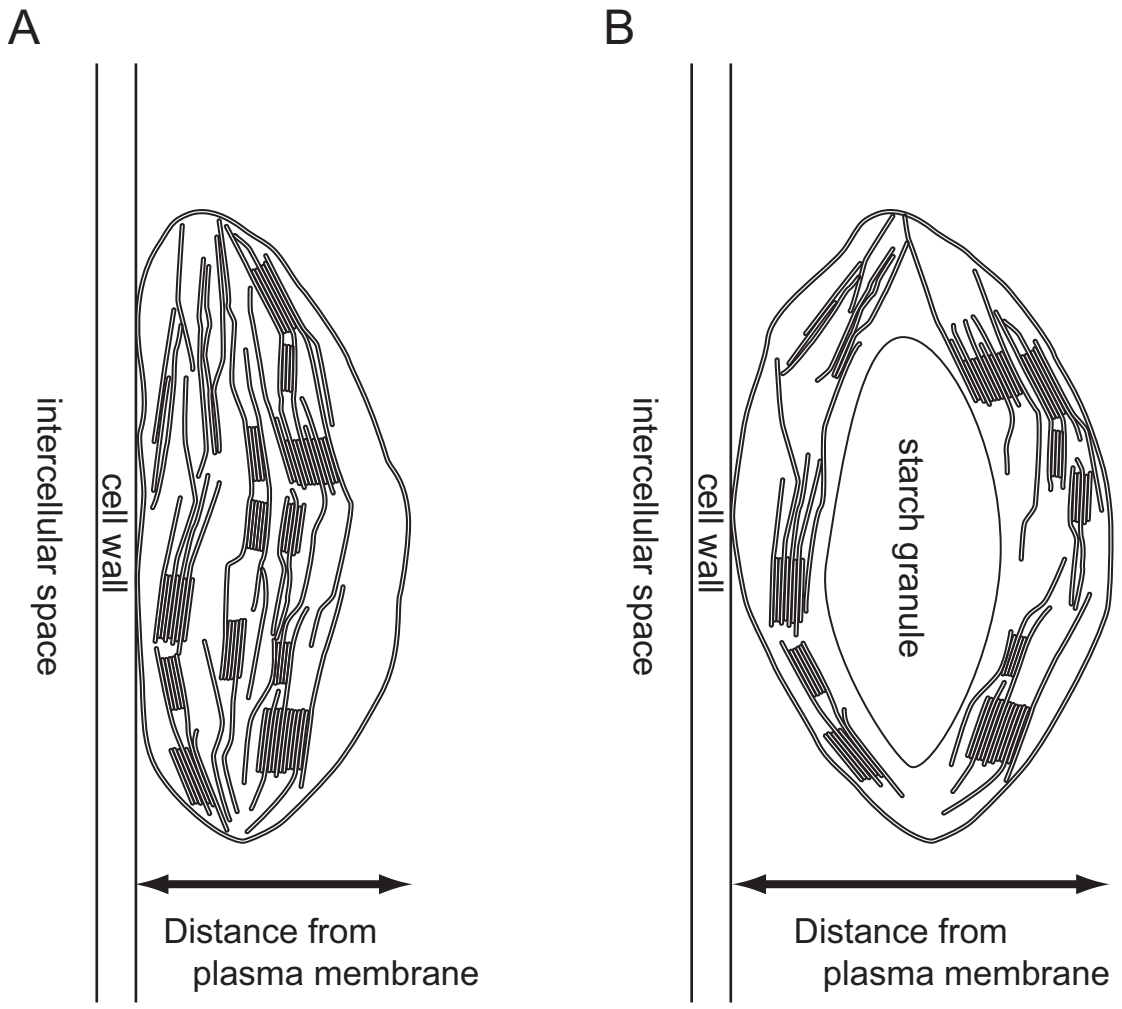

Fig. 3 The model of g_i decrease with starch accumulation in chloroplasts. A: a chloroplast without starch accumulation, B: a chloroplast with starch. When starch accumulates in the chloroplast, g_i may reduce, because of the increase in the distance of the chloroplasts from the plasma membrane.

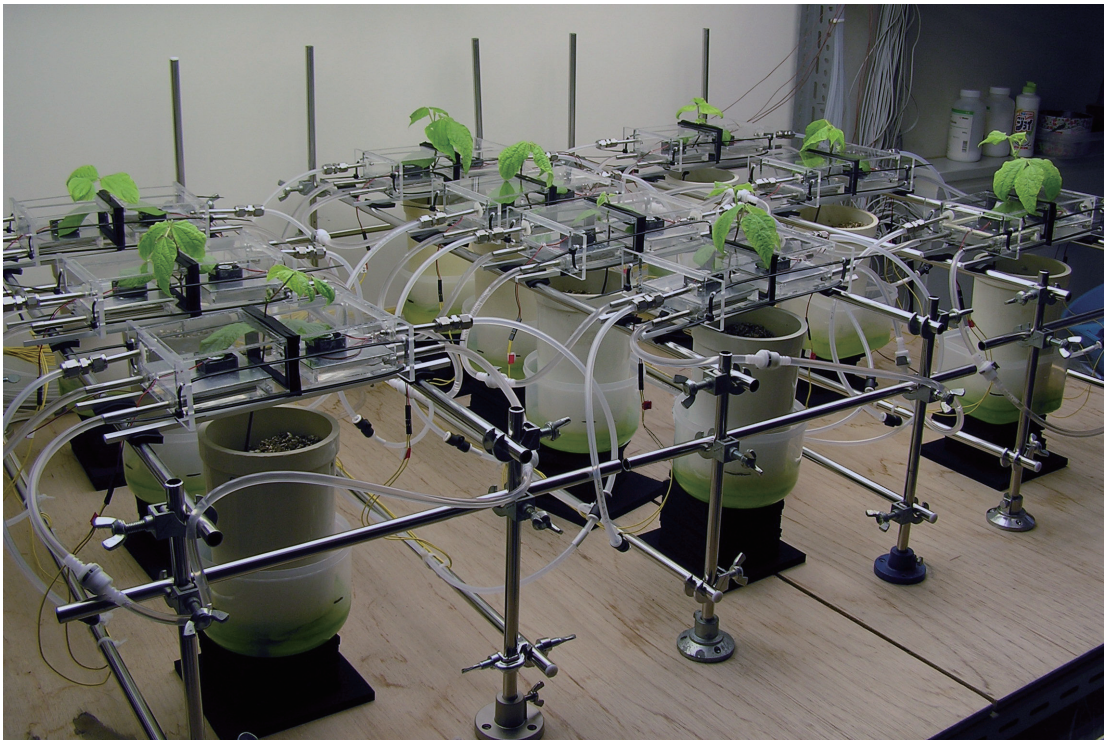

Fig. 4 Local environment controlling system

The primary leaves of bean (*Phaseolus vulgaris*) are covered with acrylic chambers, and treated with elevated or low CO_2 concentration without affecting the other parts of plants. ten bean plants can be treated at once.

Chapter 1

Effects of Carbohydrate Accumulation on Photosynthesis Differ between Sink and Source Leaves of *Phaseolus vulgaris* L.

Abstract

Accumulation of non-structural carbohydrate in leaves represses photosynthesis. However, the extent of repression should be different between sink leaves (sugar consumers) and source leaves (sugar exporters). We investigated effects of carbohydrate accumulation on photosynthesis in the primary leaves of bean (*Phaseolus vulgaris* L.) during the leaf expansion. To increase the carbohydrate content of the leaves, we supplied 20 mM sucrose solution to the roots for 5 days (sugar-treatment). The plants supplied only water and nutrients were used as controls. The carbohydrate contents, which are sum of glucose, sucrose, and starch, of the sugar-treated leaves, were 1.5-3 times of those of the control leaves at any developmental stage. In the young sink leaves, the photosynthetic rate at saturating light and at an ambient CO_2 concentration (A_{360}) did not differ between the sugar-treated and control leaves. A_{360} of sugar-treated source leaves gradually decreased relative to the control source leaves with the leaf expansion. The initial slope of the A-C_i curve, and Rubisco content per leaf area showed trends similar to that of A_{360}. Differences in Amax between the treatments were slightly smaller than those in A_{360}. These results indicate that the effect of carbohydrate accumulation on photosynthesis is significant in the source leaves, but not in the young sink leaves, and that the decrease in Rubisco content was the main cause of the carbohydrate repression of photosynthesis.

Keyword

Bean (*Phaseolus vulgaris L.*), Carbohydrate repression of photosynthesis, Depletion of Orthophosphate, Mesophyll conductance (g_i), Rubisco, Sugar-treatment.

Introduction

Accumulation of nonstructural carbohydrates in leaves often represses photosynthesis (Krapp et al. 1991, Krapp and Stitt 1995, Jeannette et al. 2000). For the carbohydrate repression of photosynthesis, three mechanisms have been proposed.

Accumulation of carbohydrate in leaves often causes feedback inhibition of sucrose synthesis and accumulation of sugar phosphates in the cytosol. The accumulation of sugar phosphates decreases orthophosphate concentration in the cytosol and thereby suppresses the antiport of triosephosphate / orthophosphate across the chloroplast envelope (Stitt and Quick 1989). Low availability of orthophosphate in the stroma suppresses ATP synthesis and reduction of PGA to triose phosphate, and thereby photosynthesis (Stitt 1986, Sharkey and Vanderveer 1989). The phosphate-limited photosynthesis is most likely to occur at saturating CO_2 concentrations (Sharkey 1985). Low orthophosphate concentrations in the stroma may also lower the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, Sawada et al. 1992). However, some mutants of *Arabidopsis thaliana* with impaired sucrose synthesis did not show the sign of reduction of photosynthesis due to the orthophosphate depletion, although they accumulated the phosphorylated intermediates (Strand et al. 2000, Chen et al. 2005). Thus, the phosphate limitation would not always occur in response to inhibition of sucrose synthesis.

Nafziger and Koller (1976) claimed that starch accumulation in chloroplasts causes deformation of the chloroplasts and thereby decreases the conductance for CO_2 diffusion from the intercellular spaces to the catalytic site of Rubisco (g_i). Nakano et al. (2000) also suggested that starch accumulation itself, not the decreased amounts of photosynthetic enzymes, caused the repression of photosynthesis in the leaves of bean plants with pods removed.

Expression of photosynthetic genes is suppressed by soluble sugars which would eventually repress photosynthesis (Sheen 1990, Pego et al. 2000). Long-term cultivation of plants at the elevated CO_2 increases the carbohydrate level in the leaves of many plants, and it has been often reported that expression of the photosynthetic genes is repressed (Nie et al.

1995, Miller et al. 1997, Chang et al. 1998).

The carbohydrate repression of photosynthesis, however, has been rarely examined in relation to leaf developmental stages, although metabolic roles of carbohydrates dramatically change depending on the leaf developmental stages. Young sink leaves import carbohydrates to construct their photosynthetic systems, while mature source leaves of high photosynthetic activities export photosynthates to sink organs. In one of such studies, Krapp et al. (1991) fed a 50 mM glucose solution to sink and source spinach leaves from their petioles via transpiration stream. The photosynthetic rate per leaf area of the glucose-fed source leaves was 30% of that of the control source leaves, while that of the glucose-fed sink leaves was not different from that of the control sink leaves. The results indicate that effect of carbohydrate accumulation on photosynthesis differs between the sink and source leaves.

In the present study, we investigated the effects of carbohydrate accumulation on photosynthesis in leaves of bean (*Phaseolus vulgaris* L.) at various developmental stages during the leaf expansion. The sink-source transition in the primary leaves of bean was examined previously (Miyazawa et al. 2003) and the transition occurred when the leaf area was about 40% of the fully expanded leaf area. We fed sucrose solution to the pot-grown plants with the primary leaves at various developmental stages to increase the carbohydrate contents in the leaves, and compared photosynthetic properties of sugar-treated and control leaves. The effects on the ribulose-1,5-bisphosphate (RuBP) regeneration capacity and those on the RuBP carboxylation capacity were separately evaluated. To identify mechanisms responsible for the decrease in these capacities, We examined photosynthetic parameters including the orthophosphate content, g_i, Rubisco content, in relation to the three potential mechanisms mentioned above. Based on these results, the significance of the developmental-stage dependent repression of photosynthesis is discussed.

Materials and Methods

Plant material

Seeds of *Phaseolus vulgaris* L. cv. Yamashiro-Kurosando were imbibed on wet paper for four days before sowing. The first day, when the seeds were imbibed, was called day 0. On day 4, germinated seedlings were planted in vermiculite in 12.7 cm-diameter pots (six seedlings per pot) and watered everyday. On day 10, five of them were thinned. The plants were grown in a light-controlled room with the fluorescent light (FPL36EX-W, Matsushita Electric Industrial Co., Ltd. Kadoma, Japan) at PPFD of 300 μmol m^{-2} s^{-1} during the light period of 14 h (6:00-20:00), relative humidity of 50 - 70%, and air temperatures of 25ºC. From day 7, the plants were fertilized everyday with 50 ml of the half strength Hoagland solution containing 2 mM KNO_3, 2 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.665 mM $NaHPO_4$, 25 μM Fe-EDTA, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.25 μM $NaMoO_4$, 50 μM NaCl, and 0.1 μM $CoSO_4$.

Sugar treatment

To increase the carbohydrate content of the primary leaf, we supplied 50 ml of 20 mM sucrose solution to the bean roots everyday for 5 days from days 7, 9, 11, 13, and 15 (Fig. 1). Sucrose solution was supplied between 15:00 and 17:00. Water and the nutrient solution were given at the same time. We call this treatment “sugar-treatment.” The bean plants that were supplied with only water and the nutrient solution were used as controls. After the sugar-treatment for 5 days, we measured various photosynthetic properties of the primary leaves: the measurements were thus conducted on days 12, 14, 16, 18, and 20.

Measurement of leaf nitrogen and carbon content.

Nitrogen and carbon contents of dried leaf discs were analyzed with a NC analyzer (vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

Measurements of photosynthesis

Rates of photosynthesis were measured with a portable infrared CO_2 gas analyzer (LI-6400, Li-Cor, Lincoln, NE, USA). The primary leaf was enclosed in the assimilation chamber in which the ambient CO_2 concentration (C_a) was kept at 360 μmol mol^{-1}. The leaf temperature was 25ºC. The leaf was illuminated at the PPFD of 1000 μmol m^{-2} s^{-1} for 20 min, and the rates of photosynthesis were measured at various C_a ranging from 50 to 1500 μmol mol^{-1}. The CO_2 concentration in the intercellular space (C_i) was calculated according to von Caemmerer and Farquhar (1981). The slope of the regression line of the A-C_i curve for C_i below 150 μmol mol^{-1} was calculated by the least squares method and regarded as the initial slope of the A-C_i curve.

Chlorophyll fluorescence and internal conductance for CO2 diffusion (gi)

Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM-101, Waltz, Effeltrich, Germany). The leaf was enclosed in the assimilation chamber of LI-6400 at the air temperature of 25ºC and C_a of 360 μmol mol^{-1}. After keeping the leaf in the dark for 20 min, maximum quantum yield of photosystem II (F_v / F_m) was measured.

We calculated the g_i according to the method of Terashima and Ono (2002). We measured the A-C_i curve and chlorophyll fluorescence at PPFD of 500 μmol m^{-2} s^{-1}. Leaf absorptance was assumed to be 85%.

After the measurements of the photosynthetic rate and chlorophyll fluorescence, leaf discs of 1 cm in diameter were sampled and quickly frozen in liquid N_2 at 17:00-20:00 for the subsequent analyses.

Measurement of chlorophyll content

The frozen leaf disc was well ground with a mortar and a pestle in liquid N_2. The chlorophyll was extracted with 80% acetone and centrifuged at 15000 × g. The absorbance of the supernatant was measured according to Porra et al. (1989).

Non-structural carbohydrate contents

Contents of glucose, sucrose and starch in the leaf were measured as described by Ono et al. (1996). Frozen leaf discs (1.57 cm^2) were ground in liquid N_2 to powder and carbohydrates were extracted with 80% ethanol. The suspension was incubated at 80ºC for 1 hour and centrifuged at 15000 × g for 10 min. The precipitations of these extracts were used for the estimation of starch. The supernatant was evaporated to remove ethanol with a centrifugal concentrator (CC-105, Tomy Seiko, Tokyo, Japan). The same volumes of distilled water and chloroform were added to the concentrated supernatant and mixed well. The mixture was centrifuged at 15000 × g and the upper clear phase was used for the estimation of glucose and sucrose.

Orthophosphate content

The orthophosphate concentration was measured as described by Sawada et al. (1992) and Saheki et al. (1985). The frozen leaf disc (0.785 cm^2) was ground in liquid N_2 and suspended with 1 ml of 1% perchloric acid. The extract was centrifuged at 750 × g and the supernatant was diluted 10 times with 1% perchloric acid. 400 μl of this diluted extract was mixed with 400 μl molybdate reagent containing 15 mM ammonium molybdate, 100 mM zinc acetate (pH 5.0), 0.25% (w/v) SDS, and 2.5% (w/v) ascorbic acid (pH 5.0). After incubation for 15 minutes at 30 ºC, A_{850} was measured. KH_2PO_4 solution was used as the standard.

Contents of Rubisco large subunit and the light harvesting complex II

The frozen leaf discs (2.355 cm^2) were ground in liquid N_2. The soluble protein was extracted with a 300 μl protein extraction buffer containing 100 mM Na-phosphate buffer (pH 7.5), 50 mM 1,4-dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 tablet / 50 ml complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 1% (w/v) polyvinylpyrrolidone, 0.7% (w/v) polyethylene glycol, and 5% (w/v) triton-X 100. The extract was centrifuged at 750 × g and the supernatant was used for analyses by SDS-PAGE.

Rubisco large subunit (LSU) and the light harvesting complex II (LHCII) contents were measured according to Makino et al. (1986) with some modifications. The gel was stained with Coomassie brilliant blue (CBB) R-250. The 47 kDa band of LSU and the 27 kDa band of LHCII were cut out, and the CBB R-250 of these blocks was eluted with formamide at 55 ºC for 5 hour. A_{595} of these elutes were measured. BSA was used for the standard.

Statistical analyses

The difference between the treatments was analyzed with Student's *t*-test.

Results

We designated the day when we started imbibition of the bean seeds as day 0 (Fig. 1). The juvenile bean plants were planted in the vermiculite on day 4 and watered everyday. The half strength of the Hoagland solution was supplied everyday from day 7. The primary leaves emerged on day 7-9 and fully expanded on day 23. According to Miyazawa et al. (2003), the sink-source transition of the primary leaves of the same bean plants occurred when the leaf area attained 40% of the fully expanded area. In the present study, the leaf area reached 40% around day 10. The daily net photosynthesis calculated from the dark respiration rate and the photosynthetic rate at growth PPFD of 300 μmol m^{-2} s^{-1} and CO_2 concentration of 360 μmol mol^{-1} increased gradually with leaf development and turned to be positive around day 10 (data not shown). We fed 50 ml of 20 mM sucrose solution to the pot everyday for 5 days before the measurement of photosynthesis and sampling. We call this treatment "sugar-treatment". The leaves collected on day 12 were treated with sucrose solution for five days when the leaves were mainly in the sink phase. With leaf expansion, the sugar-treated period was shifted from the sink to source phases, and we were able to see the effect of sugar-treatment on the photosynthetic properties of the leaves at various stages in terms of the sink-source transition.

Carbohydrates contents

Changes in carbohydrate contents per leaf area with leaf development are shown in Fig. 2. We sampled the leaves towards the end of the day. Thus, the carbohydrate contents in Fig. 2 would be regarded the maximum values within the diurnal cycle. Glucose contents in the control leaves were high when the leaves were young, and gradually decreased with leaf development (Fig. 2A). On the contrary, the glucose contents of the sugar-treated leaves decreased only slightly. The glucose contents of the sugar-treated leaves were higher

than those of the control leaves, but their differences on day 12 were small. The differences between the treatments on days 14 and 16 were statistically significant.

Sucrose contents of the sugar-treated leaves were higher than those of the control ones at any stages (Fig. 2B). The difference in the sucrose content between the control and sugar-treated leaves was relatively small on day 12, and increased with leaf development.

Starch contents were relatively low on days 12 and 14 in both of sugar-treated and control leaves, and increased with leaf expansion (Fig. 2C). Starch contents of the sugar-treated leaves were 1.5-3 times higher than those of the control leaves. Total carbohydrate content, the sum of the glucose, sucrose and starch contents, also showed the trends similar to the starch contents (Fig. 2D).

LMA

In the control leaves, the LMA was almost constant throughout the experimental periods (Table 1). The LMA did not differ between the treatments on day 12 or 14. The LMA of the sugar-treated leaves was slightly higher than that of the control leaves on day 16, 18 and 20. The increase in the LMA in the sugar-treated leaves was mostly attributed to the increase in the carbohydrate contents (Fig. 2D).

Photosynthesis

Changes in the photosynthetic rate on a unit leaf area basis at PPFD of 1000 μmol m^{-2} s^{-1} and at C_a of 360 μmol mol^{-1} (A_{360}) are shown in Fig. 3A. A_{360} gradually decreased with the developmental stage. The A_{360} was not different between the sugar-treated and control leaves on day 12, but was lower in the sugar-treated leaves than in the control leaves on other days. The difference in A_{360} between the treatments gradually increased with leaf expansion, and the differences were statistically significant on days 18 and 20. The initial slope of the A-C_i curve, the indicator of the Rubisco carboxylation capacity *in vivo*, showed a trend similar to that of A_{360} (Fig. 3B).

As the indicator of RuBP regeneration capacity *in vivo*, I measured the rate of photosynthesis at 1000 μmol photon m^{-2} s^{-1} and at a saturating ambient CO_2 of 1500 μmol mol^{-1} (Amax) (Fig. 3C). The Amax of the control leaves was almost constant after day 14. On the contrary, the Amax of sugar-treated leaves gradually decreased, and the Amax values of sugar-treated leaves were significantly lower than those of the control leaves on days 18 and 20.

Chlorophyll content and chlorophyll fluorescence

Chlorophyll contents per leaf area decreased with leaf development, and chlorophyll *a*/*b* ratios did not change during the experimental periods (Table 1). There was no difference in the chlorophyll contents and *a/b* ratio between the treatments, except for the chlorophyll *a*/*b* ratio on day 20. F_v / F_m measured just after the dark pretreatment for 20 min at the ambient CO_2 concentration of 360 μmol mol^{-1}, was unchanged during the leaf development (Table 1). Though the F_v / F_m values in the sugar-treated leaves were slightly higher than those of the control leaves, they did not statistically differ between the treatments.

Rubisco large subunit (LSU) and light-harvesting chlorophyll binding protein II (LHCII) content

Changes in Rubisco LSU and the LHCII contents per leaf area are shown in Fig. 4. The Rubisco LSU contents decreased with leaf development (Fig. 4A). The decrease in Rubisco LSU content showed a trend similar to those of the A_{360} and the initial slope of A-C_i curve (Fig. 3A, B), though the decrease in Rubisco LSU contents was slightly faster than the decreases in A_{360} and the initial slope. While, the Rubisco LSU content did not differ between the sugar-treated and control leaves on day 12, Rubisco LSU content of the sugar-treated leaves were lower than those of the control leaves after day 14. The difference between the treatments gradually increased with leaf age. LHCII content also decreased

with leaf development (Fig. 4B). There was no difference in LHCII content between the treatments.

Nitrogen and carbon content

The nitrogen content of the sugar-treated leaves was slightly lower than that of the control leaves on day 12, and the difference between the treatments increased with leaf development (Table 2). The carbon content showed a trend similar to that of LMA (data not shown). The nitrogen / carbon (N/C) ratio of the sugar-treated leaves was always lower than that of the control leaves, and the difference between the treatments were greater at the later stages of leaf development.

Orthophosphate content

The orthophosphate contents gradually increased with leaf development (Fig. 5A). On day 12, the orthophosphate contents did not differ between the treatments. On the contrary, the orthophosphate content of the sugar-treated leaves were slightly lower than that of the control leaves after day 14, and the difference between the treatments gradually increased with leaf development.

Internal conductance for CO_2 diffusion

Changes in the internal conductance for CO_2 diffusion from the intercellular space to the Rubisco catalytic site, g_i, with leaf development are shown in Fig. 5B. g_i of the control leaves gradually deceased with leaf development. On the contrary, g_i of the sugar-treated leaves on day 12 was slightly higher than those on other days. From day 14, the g_i of the sugar-treated leaves was almost constant.

Discussion

In the present study, we divide the leaf expansion into three stages. The leaves examined on day 12 were treated with sugar when they were mainly in the sink phase, and thus are called “sink leaves” (see the first paragraph of results, see also Miyazawa et al. 2003). The leaves examined in day 14 were treated when the leaves were in the sink-source transition and thus are called “transitional leaves”. The leaves examined after day 16 are called “source leaves”.

Although the carbohydrate contents of the sugar-treated sink leaves examined on day 12 were about twice of that in the control sink leaves (Fig. 2D), the photosynthetic rates were not affected by the sugar-treatment (Fig. 3). However, the carbohydrate contents, even in the sugar-treated leaves, were lower than the levels in the transitional and source leaves. Thus, it would be probable that the carbohydrate levels in the sink leaves were lower than the threshold that induces the sugar effect on photosynthesis. The low photosynthetic rates and high growth respiration rate in the sink leaves especially before the day 10 (data not shown), would keep the carbohydrate content low in the primary leaves. In the separate experiments, we fed the sucrose solution at 60 or 120 mM to the pots. However, the total carbohydrate contents of such “sink” primary leaves were not significantly different from that with the plant fed in the “sink” primary leaves in the 20 mM sucrose solution. The feeding of higher concentrations of sucrose solution also caused shrinkage of the primary leaves. Thus, we used the 20 mM solution. In the study by Krapp et al. (1991), the sugar-fed sink leaves also contained lower amounts of carbohydrates than the sugar-fed source leaves. Except for the extreme supplement of soluble sugars, which were used in some studies (Jang and Sheen 1997), the carbohydrate would not accumulate to the levels that are enough to induce the sugar-repression of photosynthesis in the sink-leaves.

In the sink leaves, however, glucose levels, which repress the photosynthetic genes (Jang and Sheen 1997), were highest in the experimental periods. Thus, it is also probable that the glucose repression of photosynthetic genes does not occur in the sink-leaves.

In the transitional leaves, the soluble sugar contents were enhanced by the sugar-treatment, although the starch content of the leaves did not increase markedly. The A_{360} of the sugar-treated transitional leaves was slightly lower than that of the control transitional leaves (Fig. 3A). The initial slope of A-C_i curve was also decreased in the sugar treated leaves, but Amax was not affected by the sugar treatment (Fig. 3B, C). Thus, the decrease in A_{360} was attributed to the decrease in the initial slope of A-C_i curve, which reflect the RuBP carboxylation capacity of the leaf, but not to that in Amax.

The initial slope of A-C_i curves, which reflects the RuBP carboxylation capacity of Rubisco (Farquhar et al. 1980), is mainly determined by the content and the properties of Rubisco and g_i. However, in the transitional leaves, the starch accumulation, which is thought to cause the decrease in g_i (Nafziger and Koller 1976), was not marked. Moreover, g_i of the sugar-treated transitional leaves was even slightly greater than that of the control leaves (Fig. 5B). Thus, the decrease in g_i with the starch accumulation did not occur in the transitional leaves.

On the other hand, the Rubisco LSU contents of the sugar-treated transitional-leaves slightly decreased (Fig. 4A). Thus, the decrease in the initial slope of A-C_i curve in the sugar-treated transitional leaves was mainly attributed to the smaller amount of Rubisco. The decrease in Rubisco content was probably due to the sugar repression of expression of Rubisco genes (Koch 1996). In rice, Rubisco protein is mainly synthesized during the leaf expansion (Mae et al. 1983). Thus, the decrease in the Rubisco content in sugar-treated transitional leaves is probably caused by the decrease in de novo synthesis of Rubisco. The nitrogen content of the sugar-treated leaves also slightly decreased in the transitional stage (Table 1). This decrease in nitrogen content would be partly due to the decrease in Rubisco content (Fig. 4A). Because the chlorophyll content, chlorophyll *a*/*b* ratio or LHCII content were not different between the treatments (Table 1, Fig 4B), probably the contents of chlorophyll proteins did not respond to the sugar treatment. Thus, the decrease in nitrogen content in the sugar-treated leaves did not cause the general decrease in the protein contents.

In the source leaves, both of the soluble sugar and starch content were increased by the sugar-treatment (Fig. 2). These increases also affect the LMA of the sugar-treated leaves (Table 1). The A_{360} of the sugar-treated source leaves were lower than those of control leaves (Fig. 3A). The decrease in A_{360} would be due to the decrease in both the RuBP carboxylation and regeneration capacity, because both of the Amax, the indicator of RuBP regeneration capacity, and initial slope of A-C_i curve decreased in the sugar-treated leaves (Fig. 3B, C). Also, A-C_i analysis suggested that the A_{360} was co-limited by the RuBP carboxylation and regeneration capacities (data not shown). The Rubisco contents of the source leaves were decreased by the sugar-treatment (Fig. 4A). On the contrary, g_i of the source leaves were not affected by the sugar-treatment (Fig. 5B), although the starch accumulated markedly in the sugar-treated leaves. Thus, as was the case with the transitional leaves, the decrease in the RuBP carboxylation capacity would be the result of the sugar-repression of photosynthetic genes due to the soluble sugar accumulation. The decrease in g_i with starch accumulation was not apparent in this study.

Amax was decreased by the sugar-treatment only in the source leaves (Fig. 3C). Also, the marked and significant starch accumulation was observed only in the source-leaves (Fig. 2C). These results suggest some possibilities that the starch accumulation decreases the Amax, although starch per se is solid and biochemically inactive. The relationship between the starch accumulation and the decrease in Amax is unclear, but the glucose, which affects the photosynthetic gene expression through the hexokinase (Jang and Sheen 1994, 1997), is generated by the starch degradation in the night. This glucose generation by starch degradation is suggested to affect the photosynthetic gene expression level (Cheng et al. 1998). Also, the components of photosynthetic electron transport chain, which affect the Amax, are more stable than Rubisco in the senesced leaves (Hidema et al. 1991, Mae et al. 1993). The stability partially explains the fact that significant decreases in Amax occur only in source leaves.

Amax is thought to be limited by the photosynthetic electron transport rate. The chlorophyll content, chlorophyll *a*/*b* ratio, and LHCII were not influenced by the sugar treatment of the source leaves (Table 1, Fig. 6B). Thus, the chlorophyll protein contents

would be unaffected by the carbohydrate accumulation. F_v / F_m, which represents the activity of PSII, also did not change with sugar-treatment. These results suggest that the decreases in the amounts or the activities of other electron transport chain components such as plastocyanin (Dijkwel et al. 1996), and H^+-ATPase (Krapp et al. 1993) by the carbohydrate accumulation. The gene of LHCII (*cab*) is known to be down-regulated by soluble sugars (Dijkwel et al. 1996), but the effect was not observed in the present study. Some studies suggested that the LHCII protein content was little affected by the mRNA level of *cab* gene (Flachmann and Kühlbrandt 1995).

The orthophosphate contents of the leaves, which also affect the photosynthetic rate at saturated CO_2 concentration, slightly decreased by the sugar-treatment (Fig. 5A). However, the orthophosphate content of the leaves increased with leaf development. Thus, the orthophosphate would not be depleted at least in source leaves, and the low level of orthophosphate content in the sugar-treated source leaves would not totally explain the decrease in Amax. In addition, because the orthophosphate is released in starch synthesis (Stitt, von Schaewen and Willmitzer 1990), the extreme starch accumulation by the sugar-treatment (Fig. 2C) might cause considerable release of orthophosphate. Therefore, it is most likely that the orthophosphate content would not be depleted in the chloroplasts in the present experiment.

In the present study, we demonstrated that the sucrose supply to the bean roots influenced the photosynthesis in the source leaves, but not in the sink leaves. It is most probable that the accumulation of soluble sugars in the sugar-treated leaves at the transitional and source stage caused the decrease in Rubisco content of the leaves.

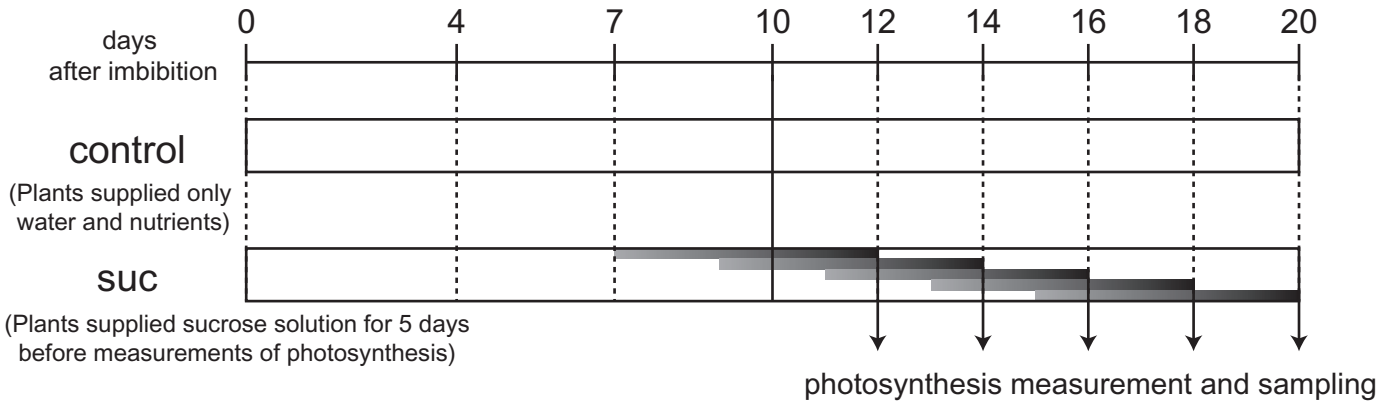

Fig. 1

Schedule of sugar treatment. The day 0 was the first day when the seed of bean was imbibed. The bean plants were planted at day 4 and watered everyday. Six plants were planted in one pod, and they were thinned at day 10. 50 ml of the half-strength of Hoagland solution was supplied everyday from day 7. The black box shows the periods, when 50 ml of 20 mM sucrose solution was supplied to the bean roots. The day 10, which is drafted with solid line, is the sink-source transition point forecasted from Miyazawa and Terashima (2003) and the daily photosynthetic rate.

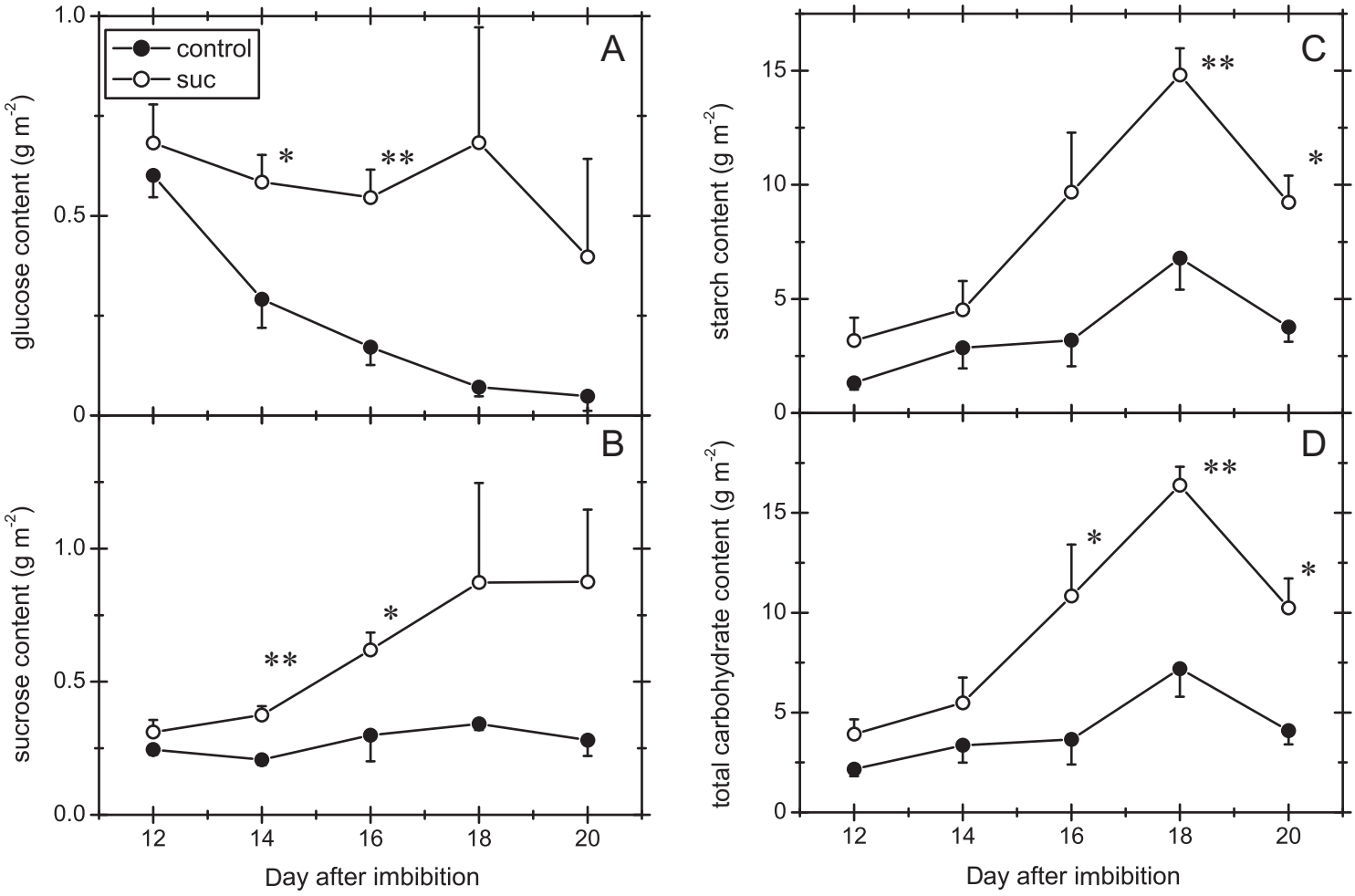

Fig. 2

Changes in carbohydrate contents on the leaf area basis in the primary leaves of bean. A, glucose; B, sucrose; C, starch; and D, total carbohydrate. Total carbohydrate is sum of the glucose, sucrose and starch contents. ●: control leaves, ○: sugar-treated leaves. Error bars denote SE (n = 3-6). * and ** means the significant difference at $p < 0.05$ and $p < 0.01$ with the Student's *t*-test, respectively.

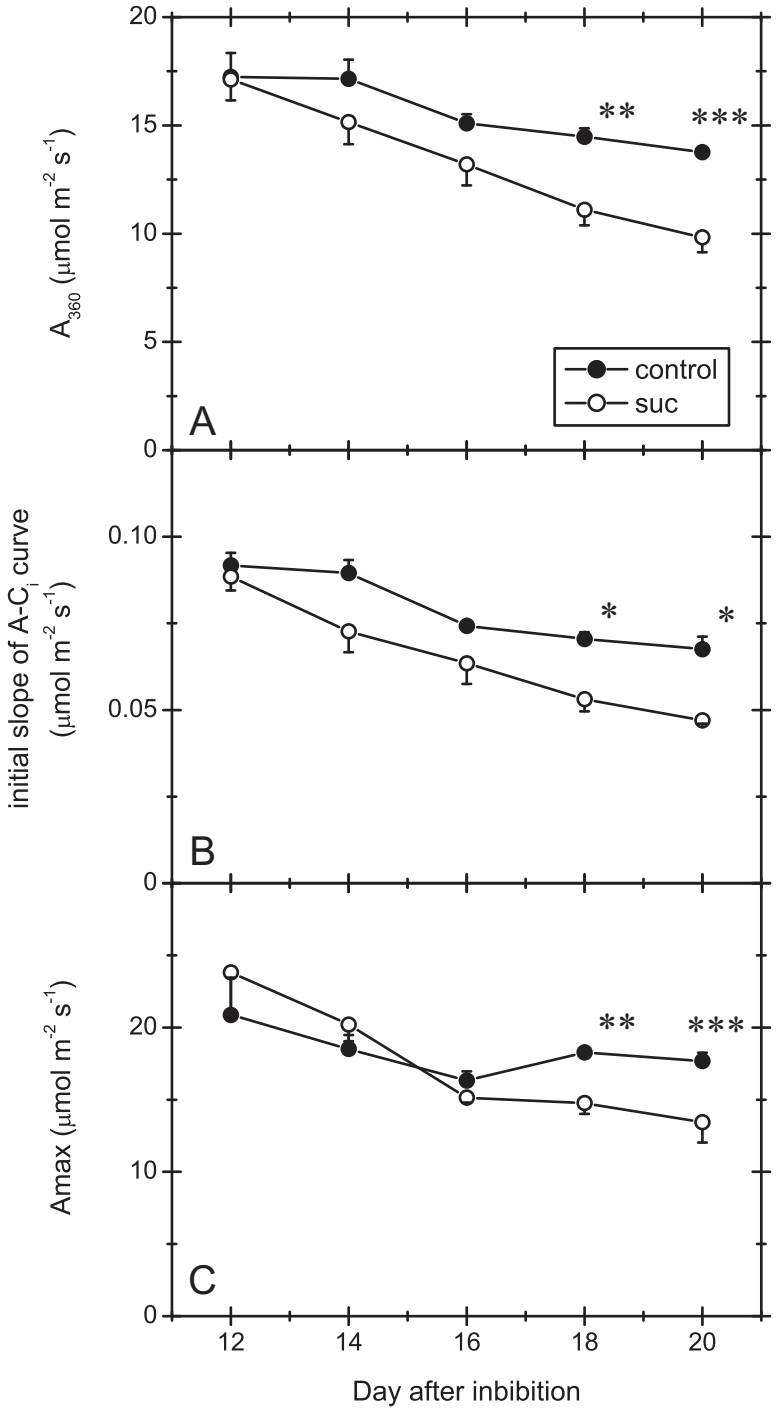

Fig. 3 Changes in photosynthetic rate on the leaf area basis in the primary leaves of bean. A, photosynthetic rate at PPFD of 1000 μmol m^{-2} s^{-1} and CO_2 concentration of 360 μmol mol^{-1} (A_{360}); B, a slope of the regression line of the A-Ci curve for the range of Ci below 150 μmol mol^{-1} measured at PPFD of 1000 μmol m^{-2} s^{-1}; and C, photosynthetic rate at PPFD of 1000 μmol m^{-2} s^{-1} and CO_2 concentration of 1500 μmol mol^{-1} (A_{max}). ●: control leaves, ○: sugar-treated leaves. Error bars denote SE (n = 3-6). * , ** and *** mean the significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ with the Student's *t*-test, respectively.

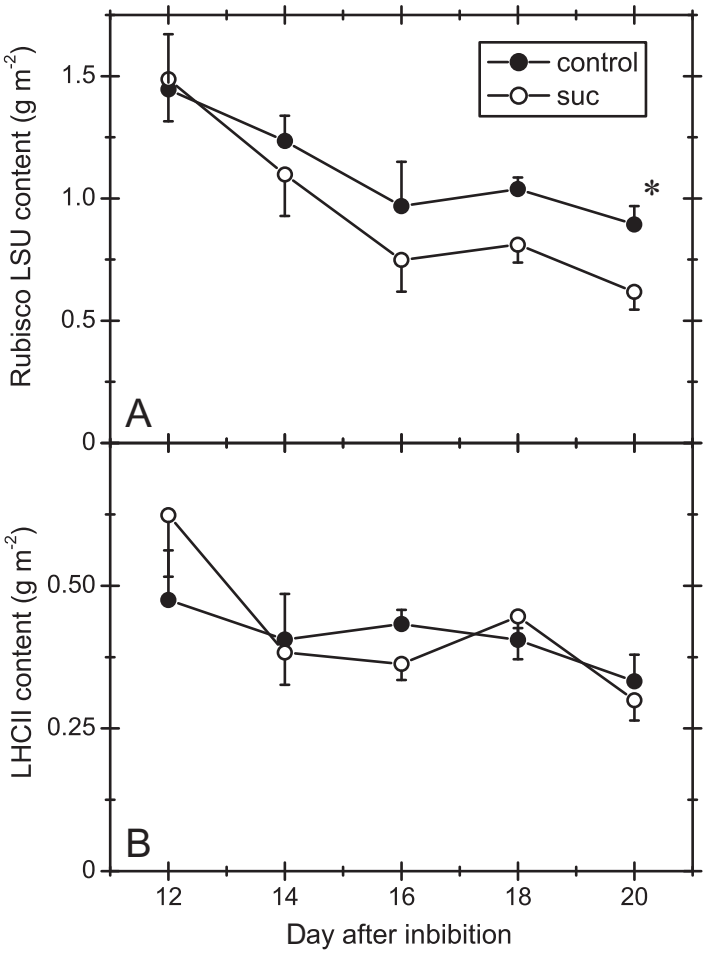

Fig. 4

Changes in Rubisco large subunit content (A) and light harvesting complex II content (B) of the primary leaves of bean. These contents are shown on leaf area basis. ●: control leaves,○: sugar-treated leaves. Error bars denote SE (n = 3-6). * means the significant difference at $p < 0.05$ with the Student's *t*-test.

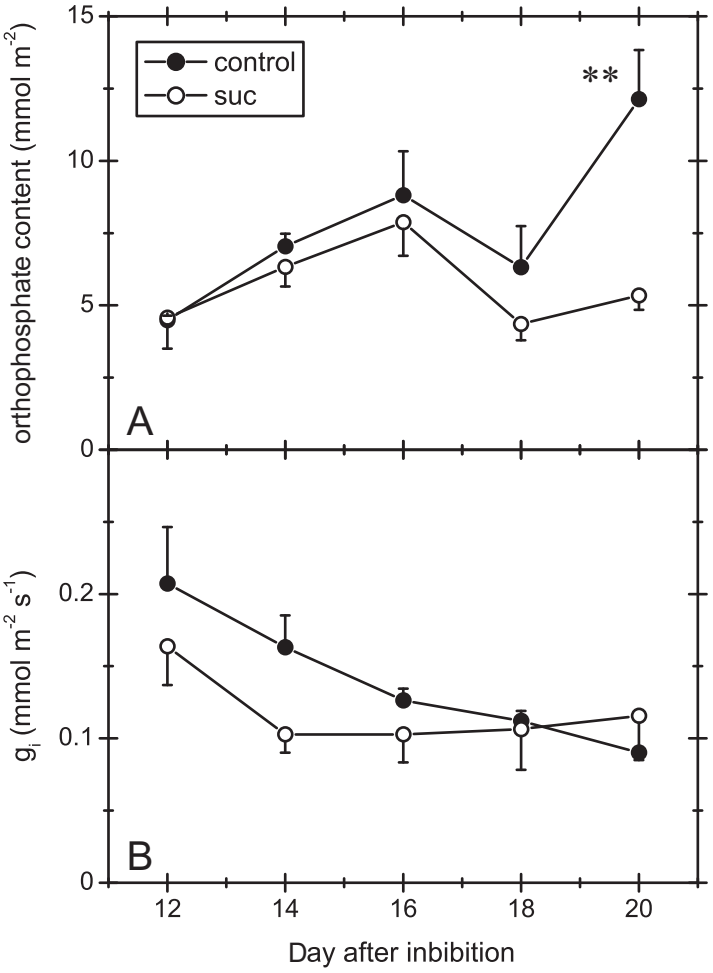

Fig. 5

Changes in the orthophosphate content (A) and internal conductance (gi, B) on the leaf area basis of the leaves. ●: control leaves, ○: sugar-treated leaves. Error bars denote SE (n = 3-6 except for gi of the control leaves at day 20 [n = 2]). ** means the significant difference at $p < 0.01$ with the Student's *t*-test.

Table 1 LMA, nitrogen content per leaf area, nitrogen / carbon (N/C) ratio, Chlorophyll (chl) content, chlorophyll *a* / *b* ratio, and F_v / F_m of the primary leaves of bean. Control and suc denote control and sugar-treated leaves, respectively. Means ± SE, n = 3-6. * and ** shows the statistical significance at $p < 0.05$ and $p < 0.01$ with Student's *t* -test in each developmental stage, respectively.

		Days after imbibition				
		12	14	16	18	20
LMA (g m^{-2})	control	24.0 ± 1.57	24.3 ± 1.45	24.3 ± 1.13**	28.9 ± 1.63	25.8 ± 3.12
	suc	26.5 ± 0.65	25.5 ± 1.96	32.1 ± 1.74	33.9 ± 2.59	30.8 ± 2.88
Nitrogen content (g m^{-2})	control	1.74 ± 0.11	1.34 ± 0.09	1.23 ± 0.08	1.17 ± 0.03**	1.35 ± 0.09
	suc	1.68 ± 0.08	1.23 ± 0.13	1.07 ± 0.09	0.90 ± 0.05	0.89 ± 0.13
N/C ratio	control	0.150 ± 0.006	0.121 ± 0.011	0.116 ± 0.011*	0.097 ± 0.006*	0.125 ± 0.010**
	suc	0.131 ± 0.006	0.107 ± 0.017	0.076 ± 0.010	0.061 ± 0.008	0.065 ± 0.004
chl *a* + *b* (mmol m^{-2})	control	0.45 ± 0.01	0.38 ± 0.04	0.33 ± 0.02	0.30 ± 0.01	0.23 ± 0.01
	suc	0.44 ± 0.03	0.40 ± 0.01	0.34 ± 0.02	0.34 ± 0.03	0.21 ± 0.02
chl *a* / *b* (mol mol^{-1})	control	3.29 ± 0.08	3.55 ± 0.08	3.41 ± 0.03	3.47 ± 0.01	3.22 ± 0.05*
	suc	3.45 ± 0.05	3.49 ± 0.04	3.36 ± 0.06	3.50 ± 0.09	3.34 ± 0.02
F_v / F_m	control	0.66 ± 0.01	0.73 ± 0.02	0.68 ± 0.02	0.71 ± 0.02	0.70 ± 0.03
	suc	0.74 ± 0.02	0.77 ± 0.01	0.72 ± 0.01	0.70 ± 0.06	0.72 ± 0.02

Chapter 2

Effect of Nitrogen Nutrition on the Carbohydrate Repression of Photosynthesis during Leaf Development in *Phaseolus valgaris* L.

Abstract

In this study, I examined the effect of sucrose feeding to the roots of nitrogen limited bean (*Phaseolus vulgaris* L.) plants on the photosynthetic CO_2 exchange of the young and mature primary leaves. When the nitrogen fertilization was limited, the nitrogen content and the photosynthetic rate decreased, and the carbohydrate contents increased in the mature primary leaves relative to those of well fertilized plants. Sucrose feeding to the plants increased the carbohydrate content and decreased the photosynthetic rate of the mature leaves. On the contrary, the nitrogen and carbohydrate content of the young leaves were less affected by the nitrogen nutrition and the sugar feeding than those of mature leaves. The effect of sucrose feeding on photosynthesis was not enhanced by the nitrogen limitation. These results suggest that the effect of carbohydrate accumulation on photosynthesis is effective only in the mature leaves but not in the young leaves. It is probably that that the decrease in the photosynthetic rate under the nitrogen limited condition may be due to the carbohydrate accumulation in the leaves.

Keywords: Nitrogen, Carbohydrate, Photosynthesis, A-C_i curve, carbohydrate repression of photosynthesis, Chlorophyll fluorescence, Bean (*Phaseolus vulgaris* L.)

Introduction

In leaves accumulating carbohydrates, the expression of photosynthetic genes such as Rubisco small subunit (*rbcS*) and light harvesting complex of PSII (*lhcb*) is repressed (Sheen et al. 1990, Krapp and Stitt 1995, Koch 1996, Ono et al. 2001). Accumulation of carbohydrates in the leaves also accelerates the leaf senescence (Wingler et al. 1998, Yoshida 2003, Parrott et al. 2005). I have reported that the carbohydrate contents of the primary leaves increased and the photosynthetic rate decreased in the bean (*Phaseolus vulgaris* L.) plants, which were applied sucrose solution exogenously from the root (Araya et al. 2006). Such decreases in the photosynthetic rate occurred only in the mature, source leaves, but not in the young, sink leaves. These results suggest that carbohydrate accumulation in the leaves decreases the photosynthetic rate only in the mature leaves and leads to the leaf senescence.

The effects of carbohydrate accumulation on photosynthesis are enhanced in nitrogen deficient plants (Paul et al. 1997). Sugar feeding to roots of *A. thaliana* accelerated the decrease in the maximum quantum yield of PSII (F_v / F_m) during the leaf senescence in the nitrogen deficient plants, while the decrease was not observed in the nitrogen sufficient plants (Wingler et al. 2004, 2006). Furthermore, a senescence associated gene, *sag12*, was induced by the sugar feeding only in the nitrogen deficient *A. thaliana* (Pourtau et al. 2006). Although these results suggest that carbohydrate accumulation decreases the photosynthetic capacity of the leaves especially in nitrogen deficient plants, the effect of carbohydrate accumulation on the gas exchange was only examined with an oxygen electrode in the saturating CO_2. Thus, the direct effects of carbohydrate accumulation on the photosynthetic gas exchange in the nitrogen deficient plant have not been examined. The purpose of this study is to clarify the effect of nitrogen nutrition on the carbohydrate repression of the photosynthetic gas exchange during the leaf maturation.

To increase the carbohydrate content of primary leaves, I fed 50 mM sucrose solution for 5 days to the roots of bean plants grown with the nutrients solution containing 0 or 0.72

mM NO^{3-} as described previously (Araya et al. 2006), and measured the photosynthetic rate and carbohydrate contents.

Materials and Methods

Plant material

Bean (*Phaseolus vulgaris* L. cv. Yamashiro-Kurosando) was grown as described previously (Araya et al. 2006). The day, on which the bean seeds were placed on wet paper, was defined as day 0. On day 4, the germinated seedlings were planted on 12.7 cm diameter pots filled with vermiculite. From day 7, the plants were fertilized every day with 50 ml of half-strength of Hoagland's solution (2 mM K^+, 2 mM Ca^{2+}, 0.75 mM $MgSO_4$, 0.665 mM $NaHPO_4$, 25 μM Fe-EDTA, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.25 μM $NaMoO_4$, 50 μM NaCl, and 0.1 μM $CoSO_4$) that contained either 0 or 0.72 mM of nitrate. They were called "0 mM" and "0.72 mM" plants, respectively. NO_3^- of the 0 and 0.72 mM Hoagland's solution was replaced by Cl^- (Hikosaka 1996).

Sugar treatment

To increase the carbohydrate contents of the primary leaves, I supplied 50 ml of 20 mM sucrose solution to the bean roots everyday for 5 days from days 7 or 15 as described previously (Araya et al. 2006). I call this treatment "sugar-treatment", and the sucrose-fed plants were called 'sugar-treated' plants. After the sugar-treatment for 5 days, I measured various photosynthetic properties of the primary leaves: the measurements were therefore conducted on the day 12 (young leaf) or 20 (mature leaf). The sink-source transition of the primary leaves occurred around day 10 (Araya et al. 2006), and thus the young leaves was sugar-treated when the leaf became from sink to source, and the mature leaves have already become source at the first day of the sugar-treatment. Plants that were fed only water and nutrient solution are called 'control' plants. For example, I denote a plant, which was fed sucrose and nutrient solution containing 0.72 mM NO^{3-}, as "0.72 mM sugar-treated" plant. To compare the effects on photosynthesis between limited nutrition plants and well fertilized plants, some of the data for the plants grown with a nutrient

solution containing 6 mM NO_3^- nutrition (6 mM plants), published in Araya et al. (2006), are shown in figures.

Photosynthetic rate

Photosynthetic rates were measured with a portable infrared CO_2 gas analyzer (LI-6400, Li-Cor, Lincoln, NE, USA) as described previously (Araya et al. 2006). The slope of the regression line of A-C_i curve for the range of C_i below 150 μL L^{-1} was calculated by the least squares method and regarded as an initial slope of A-C_i curve. Initial slope of the A-C_i curve is thought to reflect the RuBP carboxylation capacity, and the maximum photosynthetic rate (A_{max}), the rate of photosynthesis at 1000 μmol photon m^{-2} s^{-1} and at a CO_2 of 1500 μL L^{-1}, represents the RuBP oxygenation capacity (Furquhar and von Caemmerer 1980).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured with a pulse amplitude modulation fluorometer (PAM-101, Waltz, Effeltrich, Germany). The leaf was enclosed in an assimilation chamber of LI-6400 at the air temperature of 25ºC and C_a of 360 μL L^{-1}. After keeping a leaf in the dark for 20 min, maximum quantum yield of photosystem II (F_v / F_m) was measured. After the measurement of F_v / F_m, the leaf was illuminated at a PPFD of 500 μmol m^{-2} s^{-1} and at the CO_2 concentration of 1500 μL L^{-1} measured the quantum yield of photosystem II in the light (Φ_{PSII}'). qP, which reflect the redox state of plastoquinone, and F_v' / F_m', which reflect the quantum yield of 'open' photosystemII, were calculated according to Oxborough (1997) without measuring F_o'. After the measurements of the photosynthetic rate and the chlorophyll fluorescence, leaf discs of 1 cm in diameter were sampled and quickly frozen in liquid N_2 at 17:00-20:00 for subsequent analyses. Some leaf discs were dried at 80ºC for a week for measurements of nitrogen and carbon contents.

Nitrogen contents

Nitrogen contents of dried leaf discs were analyzed with an NC analyzer (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany).

Non-structural carbohydrate contents

Contents of glucose, sucrose and starch in the leaf were measured as described previously (Araya et al. 2006)

Statistical analyses

The effects of sugar-treatment and the nitrogen fertilization were analyzed with 2-way analysis of variance (ANOVA) in young and mature leaves, respectively. The *p*-value for the sugar-treatment was shown above the column in the figure when the difference between sugar-treated and control leaves was significant. In addition, significant differences ($p < 0.05$) among the nitrogen fertilizations with the Tukey's multiple tests were shown as the different alphabets in the figures. When the interaction was significant, these data were analyzed with the Tukey's multiple tests, and the significant differences among treatments were also shown above the column in the figures.

Results

The day, on which the bean seeds were placed on wet paper, was defined as day 0. From day 7, the plants were fertilized every day with 50 ml of half-strength of Hoagland's solution that contained either 0 or 0.72 mM of nitrate. They were called “0 mM” and “0.72 mM” plants, respectively. To increase the carbohydrate contents of the primary leaves, I supplied sucrose solution to the bean roots everyday for 5 days as described previously (Araya et al. 2006). I call this treatment “sugar-treatment”, and the sucrose-fed plants were called ‘sugar-treated’ plants. I measured various photosynthetic properties of the primary leaves on the day 12 (young leaf) or 20 (mature leaf). Plants that were fed only water and nutrient solution are called ‘control’ plants. To compare the effects on photosynthesis between limited nutrition plants and well fertilized plants, the data for the plants grown with 6 mM NO_3^- nutrition (6 mM plants), published in Araya et al. (2006), are shown in figures.

Carbohydrate contents (Fig. 1)

Glucose contents were higher at the young stage than at the mature stage (Fig. 1A). The nitrogen nutrition did not obviously affect the glucose contents at the young or mature stage. Sugar-treatment slightly increased the glucose contents at the young and mature stages, but the differences were not statistically significant.

Nitrogen nutrition did not affect the sucrose content at both of young or mature stage (Fig. 1B). At the young stage, sugar-treatment increased the sucrose content in the 0 mM leaves, but not in the 0.72 or 6 mM leaves. On the other hand, sugar-treatment increased sucrose contents at the mature stage.

Starch contents were not affected by the nitrogen nutrition or the sugar-treatment in 0 mM leaves, but sugar treatment increased the starch content in 0.72 and 6 mM leaves at the young stage (Fig. 1C). At the mature stage, however, the starch contents in the 0 and

0.72 mM leaves were higher than that in the 6 mM leaves. Sugar-treatment increased the starch content at the mature stage.

Nitrogen contents (Fig. 2)

The nitrogen contents of the young leaves were not affected by the nitrogen nutrition (Fig. 2). Sugar-treatment slightly decreased the nitrogen contents of the 0 and 0.72 mM leaves, but these decreases were not statistically significant. At the mature stage, the nitrogen content of the 6 mM control leaves was higher than those of the other treatments. Sugar-treatment decreased the nitrogen contents in the 0.72 and 6 mM leaves.

Photosynthesis (Fig. 3)

The photosynthetic rate at a PPFD of 1000 μmol m^{-2} s^{-1} and at the 360 μL L^{-1} CO_2 (A_{360}) was shown in Fig. 3A. At the young stage, A_{360} of the 0 mM leaves was lower than that of the 6 mM leaves. Sugar-treatment decreased the A_{360} in the 0 mM leaves at the young stage. At the mature stage, A_{360} of the 0 and 0.72 mM leaves was lower than that of the 6 mM leaves. Sugar-treatment decreased the A_{360} at the mature stage. However, the decrease in A_{360} by the sugar-treatment was less pronounced in the 0 mM plants than in the nitrogen supplied plants at the mature stage.

At the young stage, the initial slope of A-C_i curve was lower in the 0 mM leaves than in the 6 mM leaves, and sugar-treatment slightly decreased the initial slopes in 0.72 and 6 mM leaves (Fig. 3B). At the mature stage, the initial slopes of A-C_i curve showed the trends similar to the A_{360} of the mature leaves.

A_{max} was not affected by the nitrogen nutrition at the young stage (Fig. 3C). The sugar-treatment increased the A_{max} at the young stage, although these differences were not statistically significant. At the mature stage, A_{max} of the 0 and 0.72 mM leaves were lower than that of the 6 mM leaves, and the sugar-treatment significantly decreased the A_{max}.

The nitrogen nutrition did not affect the stomatal density of the young leaves (Fig. 3D). Sugar treatment decreased the stomatal conductance of the 0 mM young leaves. The stomatal conductance of the 0.72 and 6 mM young leaves were not affected by the sugar treatment. At the mature stage, higher nitrogen nutrition increased the stomatal density in control leaves. The sugar treatment decreased the stomatal density of the 0.72 and 6 mM mature leaves, but the differences were not statistically different.

Chlorophyll fluorescence (Fig. 4)

F_v / F_m were greater in the 0 mM leaves than in the 6 mM leaves at the young stage (Fig. 4A). F_v / F_m of the mature leaves were higher in the 0 and 0.72 mM leaves than in the 6 mM leaves. The sugar-treatment did not affect F_v / F_m.

Φ_{PSII}' was measured at 500 μmol photon m^{-2} s^{-1}, and thus the parameter did not directly reflect the status of the photosystem at the A_{max}, however, this parameter showed almost same trends as the maximum electron transport rate, which is calculated from the A-C_i curve (data not shown). Φ_{PSII}' did not differ among the treatments at the young stage (Fig. 4B). At the mature stage, sugar-treatment decreased Φ_{PSII}', and the Φ_{PSII}' of the 6 mM leaves was higher than those of the 0 and 0.72 mM treatments.

At the young stage, qP was higher in the 6 mM leaves and 0.72 mM sugar-treated leaves than those of 0 mM leaves and 0.72 mM control leaves (Fig. 4C). On the other hand, the sugar-treatment decreased qP at the mature stage. qP of the 6 mM leaves were higher than those of the 0 and 0.72 mM leaves. Sugar-treatment did not affect the F_v' / F_m' at both of young and mature stages (Fig. 4D).

Discussion

In this study, I examined the effect of nitrogen nutrition and carbohydrate accumulation on photosynthesis in the young and mature leaves. Both of the low nitrogen nutrition and sugar-treatment induced the carbohydrate accumulation and the decrease in photosynthetic rate only in the mature leaves but not in the young leaves. Limited nitrogen nutrition did not enhance the decrease in photosynthetic rate by the carbohydrate accumulation in the leaves.

The effect of nitrogen nutrition and the sugar-treatment on the carbohydrate and nitrogen contents of the leaves

The carbohydrate contents, which are sum of the glucose, sucrose and starch contents, of the young leaves were much lower than those in the mature leaves, and were affected little by the sugar and nitrogen nutrition (Fig. 1). Ainsworth et al. (2006) also reported that, even in elevated CO_2, carbohydrate accumulation did not occur in the young leaves. Limited nitrogen fertilization certainly enhanced carbohydrate accumulation in the mature leaves, but not in the young leaves. On day 12, the plants have only two primary leaves, and thus the sink size was large relative to the source leaves. On the contrary, the mature leaves accumulated much carbohydrate when the plants were supplied with limited or no nitrogen nutrition. When the primary leaves became mature, some other young leaves emerged and source organs became large relative to the sink organs. This changes in sink / source balance in a whole plant would also be responsible for the carbohydrate accumulation in the mature leaves (Ono et al. 1996, 2001).

Sugar-treatment decreased photosynthetic rate only in the mature leaves.

In the young leaves, the A_{360} was not affected by the sugar-treatment except for the 0 mM leaves (Fig. 3A). The low A_{360} in the 0 mM sugar-treated leaves was due to the low

stomatal conductance (Fig. 3D). On the contrary, sugar-treatment decreased the A_{360} in the mature leaves. Our previous study also showed that the carbohydrate repression of photosynthesis was more effective in the mature leaves than in the young leaves (Araya et al. 2006). Thus, the carbohydrate repression of photosynthesis in the young leaves was not detected in the study. However, the carbohydrate accumulation in the young sink leaves would not occur in vivo even in the nitrogen deficient condition. Thus, the carbohydrate repression of photosynthesis is not related to the construction of photosystems in the young leaves.

Nitrogen depletion in the present study did not enhance the decrease in the photosynthetic rate by the sugar-treatment even in the mature leaves (Fig. 3), although the nitrogen depletion was thought to reinforce the carbohydrate effects on photosynthesis (Paul et al. 1997, Wingler et al. 2006). The carbohydrate accumulation of the mature leaves except for 6 mM control leaves was high, and there are small differences among them. Thus, there are some possibilities that the previously reported effects of nitrogen depletion on the carbohydrate repression was due to the carbohydrate accumulation, and the additional carbohydrate supplement for the nitrogen deficient plant may have little impact on the carbohydrate content of the leaves, and photosynthesis. Price et al. (2004) showed that the global transcription profiling in nitrogen deficient plants of *A. thaliana*, cultured in the MS medium containing 3% glucose, were little affected after 3h of the nitrate feeding. This result suggested the importance of carbohydrate accumulation in the effects of nitrogen on the gene expression profiles. However, long-term effects of nitrate feeding on gene expression were not examined in Price et al. (2004), and further studies are needed for the elucidation of nitrogen effect on carbohydrate repression of photosynthesis. For example, the research about the effects of nitrogen nutrition on photosynthesis in the mutants that defect the nitrogen-specific signaling pathways reveals the carbohydrate roles in the regulation of photosynthesis by nitrogen fertilization.

Carbohydrate repression affects the redox state of plastoquinone.

Carbohydrate accumulation in the mature leaves decreased the initial slope of A-C_i curve and A_{max} (Fig. 3B, C). In the previous study, I reported that the decrease in the initial slope of A-C_i curve, which would represent the RuBP carboxylation activity, was caused by the decrease in the Rubisco content (Araya et al. 2006). On the other hand, the reason for the decrease in A_{max}, which represents the RuBP regeneration activity, was not explained yet. In the young leaves, the Φ_{PSII}' did not differ among treatments (Fig. 4B). On the other hand, the Φ_{PSII}' decreased in the sugar-treated mature leaves. These changes in Φ_{PSII}' were attributed to the decrease in qP, but not in F_v' / F_m' (Fig. 4C, D). These results suggested that the nitrogen limitation and the sugar-treatment decreased the RuBP regeneration rate by the limitation on the reducing side of plastoquinone. The plastocyanin gene, *petE*, is repressed by the sugar (Oswald et al. 2001), and plastocyanin act as a major regulator of photosynthetic electron transport rate (Burkey et al. 1996, Schöttler et al. 2004). Thus, the carbohydrate repression of the plastocyanin gene would be a factor of the decrease in RuBP regeneration rate.

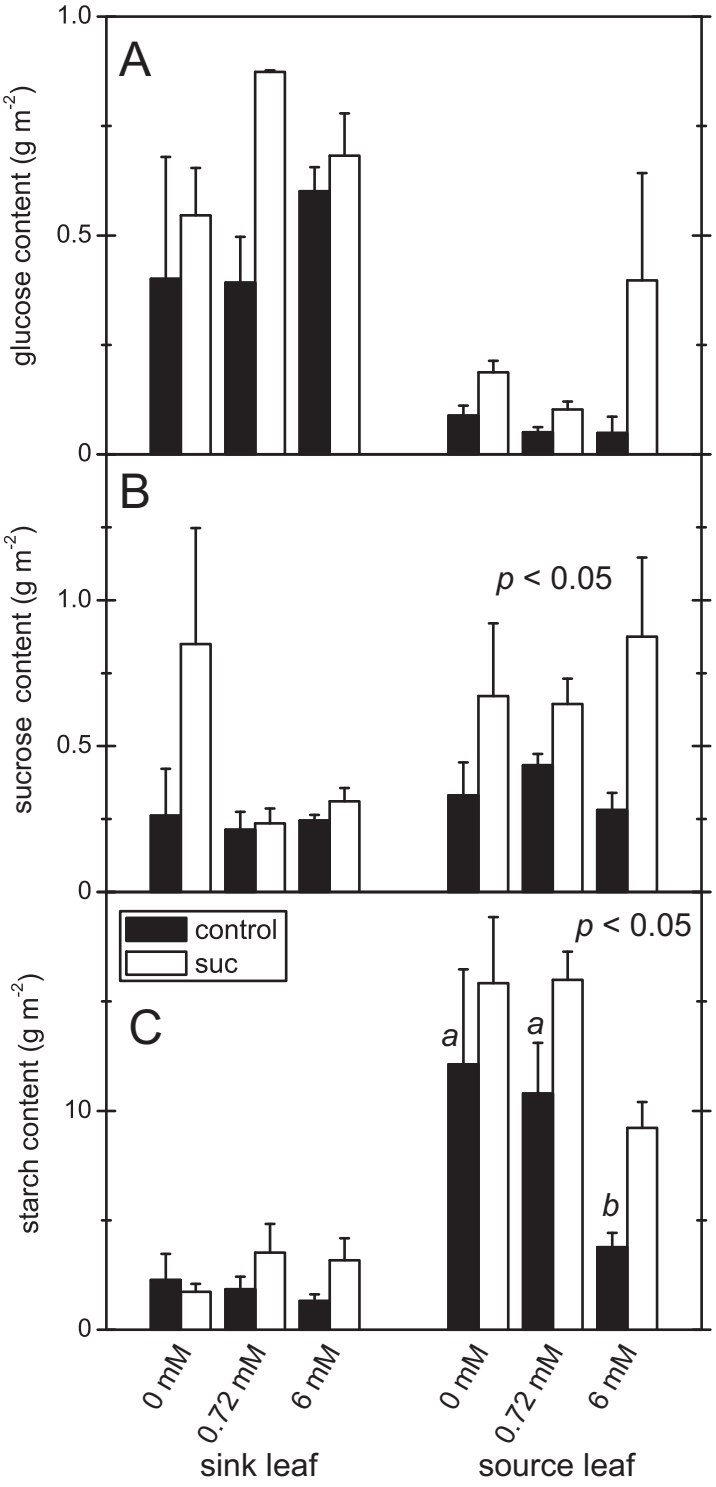

Fig. 1

Carbohydrate contents on the leaf area basis. A, glucose; B, sucrose; and C, starch contents. The *p*-value above the column means the significant differences between control and sugar-treated leaves with the 2-way ANOVA. Alphabets, which is shown in the figures, means the significant differences among the nitrogen nutrition at $p < 0.05$ with the Tukey's multiple test. Error bars denote SE (n = 4).

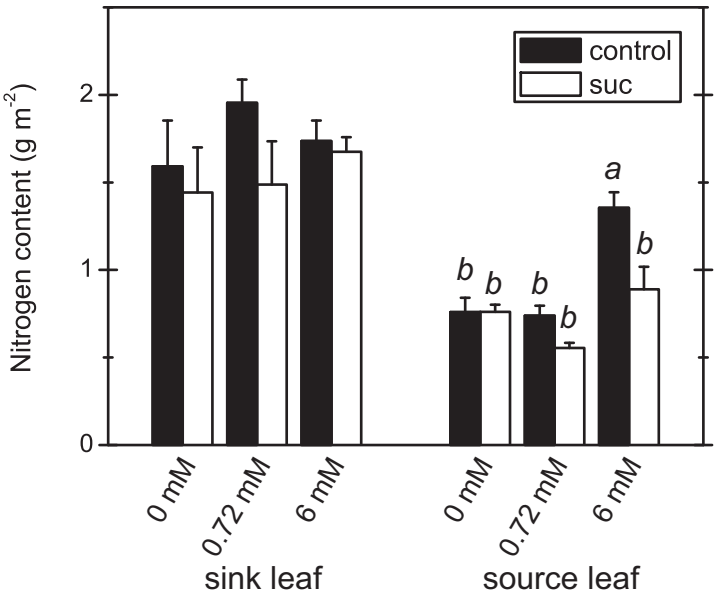

Fig. 2

Nitrogen content on the leaf area basis. The alphabets are as in Fig. 1. Error bars denote SE (n = 4).

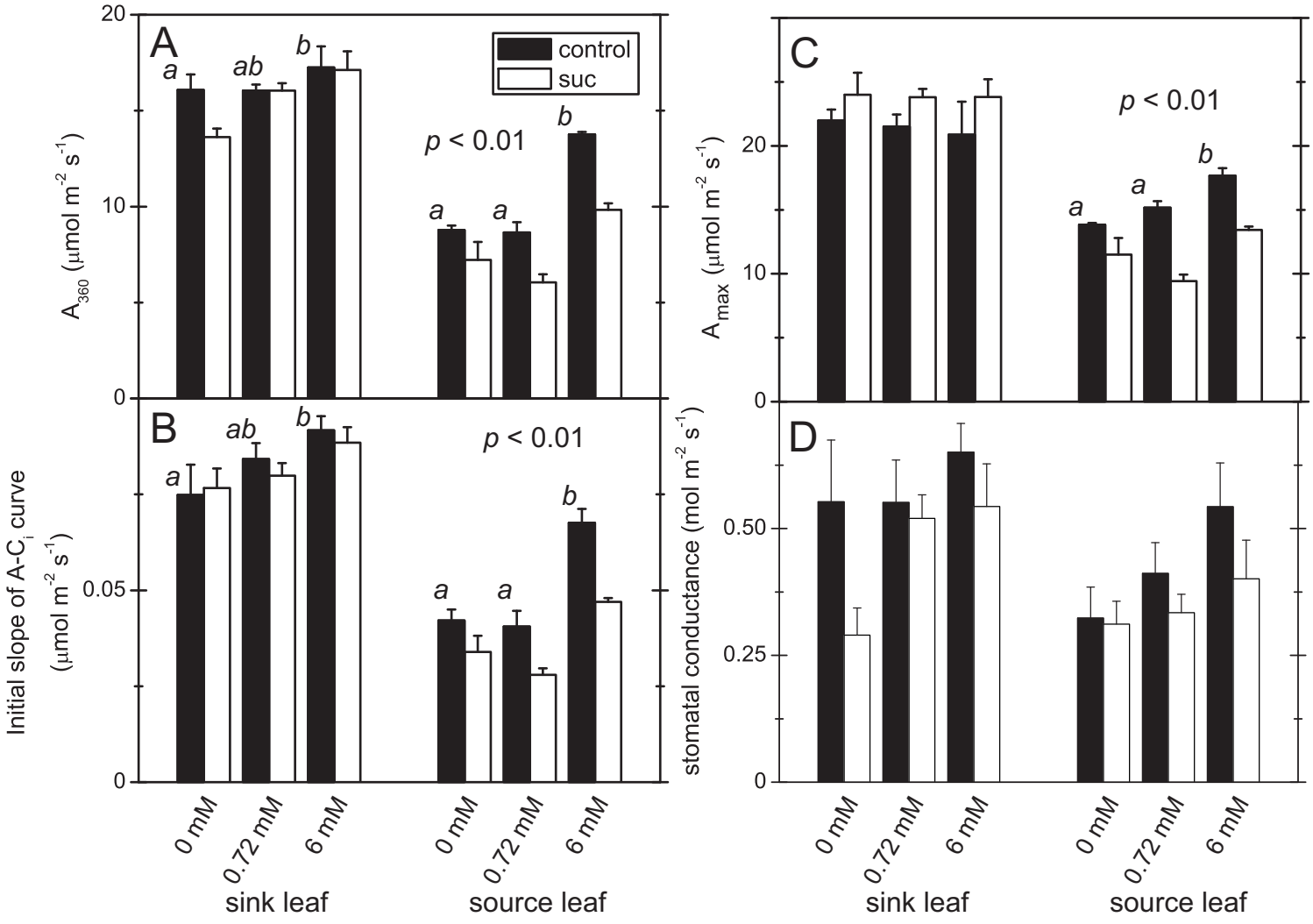

Fig. 3

Changes in photosynthetic rate on the leaf area basis. A, A_{360}; B, an initial slope of the regression line of the A-Ci curve; and C, Amax. The p-value and alphabets are as in Fig. 1. Error bars denote SE (n = 4).

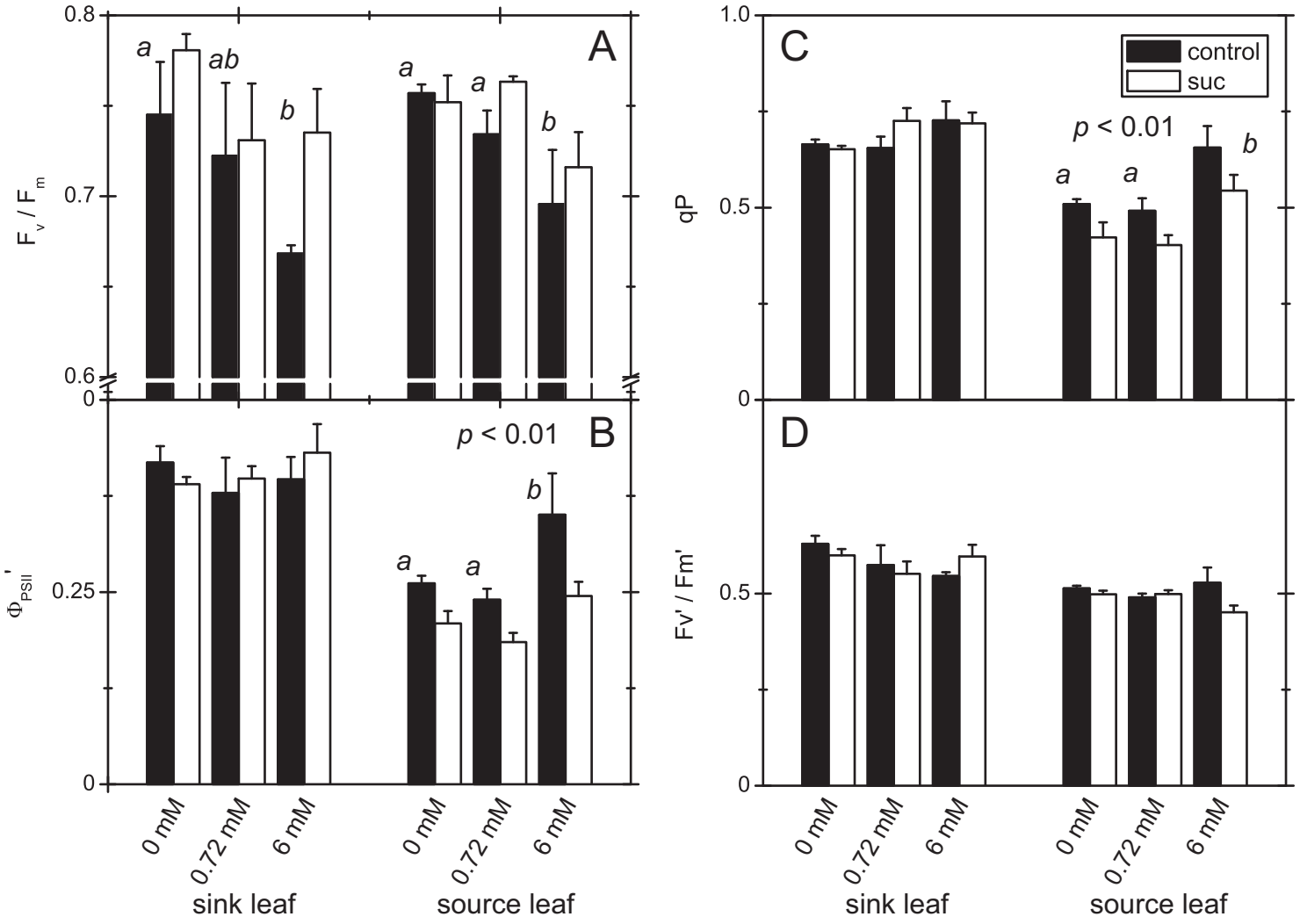

Fig. 4

Changes in the chlorophyll fluorescence parameters. A; maximum quantum yield of PSII (F_v/F_m), B; quantum yield of PSII at a PPFD of 500 μmol m^{-2} s^{-1} and CO_2 concentration of 1500 μL L^{-1} (Φ_{PSII}'), C; parameter related to the redox state of plastoquinone (qP), and D; quantum yield of 'open' PSII (F_v'/F_m'). The *p*-value and alphabets are as in Fig. 1. Error bars denote SE (n = 4).

Chapter 3

Manipulation of light and CO_2 environments of the primary leaves of bean (*Phaseolus vulgaris* L.) affects photosynthesis in both the primary and the first trifoliate leaves: Involvement of systemic regulation.

Abstract

Possible involvement of systemic regulation of the photosynthetic properties of young leaves by the local environments and/or photosynthate production of the mature leaves were examined using *Phaseolus vulgaris* plants. When primary leaves (PL) were treated with air containing 150 μl CO_2 l^{-1} with the other plant parts in ambient air at a photosynthetic photon flux density (PPFD) of 300 μmol photon m^{-2} s^{-1}, decreases in the photosynthetic rate measured at 360 μl CO_2 l^{-1} and a PPFD of 300 μmol photon m^{-2} s^{-1} (A_{360}) were markedly retarded in both PL and the first trifoliate leaves (TL) as compared to plants treated with 400 CO_2 μl l^{-1}. Conversely, when PL were treated with 1000 μl CO_2 l^{-1}, decreases in A_{360} were accelerated in both PL and TL. Shading of PL accelerated the decrease in PL A_{360}, and delayed the decrease in TL. In the CO_2 treatments, changes in A_{360} in TL were mainly attributed to the changes in RuBP carboxylation rate, while the shading of PL caused increases in both the RuBP carboxylation and regeneration rates in TL. The Rubisco activity on chlorophyll basis, an indicator of sun/shade acclimation, differed both among PL and among TL in accordance with the redox state of photosystem II in PL. Although carbohydrate contents of TL were not affected by any manipulation of PL, changes in the photosynthetic capacities of TL acted to compensate for changes in PL photosynthesis. These results clearly indicate that the CO_2- and shade-treatments of PL not only affect photosynthetic properties of PL themselves but also systemically affected photosynthetic properties of TL. Possible roles of the redox state and photosynthate concentration in PL in regulation of photosynthesis in PL and TL are discussed.

Keywords: A-C_i curve, Bean (*Phaseolus vulgaris* L.), Carbohydrates, Local environment control system, Photosynthesis, Redox state, Systemic signaling

Introduction

Recent studies suggest that the physiology and development of young leaves can be significantly influenced by the environmental conditions experienced by mature leaves. Yano and Terashima (2001) showed that the light environment of mature leaves regulates direction of cell division of the palisade tissue cells in developing *Chenopodium album* leaves. Light and CO_2 environments of mature leaves also affect stomatal density of the developing leaves in *Arabidopsis thaliana* (Lake *et al*. 2001; Coupe *et al*. 2006), tobacco (*Nicotiana tabacum* L., Thomas et al. 2004) and poplar (*Populus trichocarpa* × *P. deltoids*, Miyazawa et al. 2006). These results suggest the existence of a signaling mechanism with the ability to modify developmental processes of new leaves.

Using *A. thaliana* plants, Weaver and Amasino (2001) showed that shading of a particular leaf, with the other leaves exposed, caused rapid senescence of the shaded leaf. Conversely, when whole plants were shaded, all the leaves senesced slowly (Hikosaka 1996; Weaver and Amasino 2001). In bean (*Phaseolus vulgaris* L.), senescence of the primary leaves was markedly delayed when the young developing leaves were shaded (Ono *et al*. 2001). Delayed senescence of unshaded leaves would contribute to maintenance of the supply of photosynthates at the whole plant level. All these results indicate that senescence of a given leaf is determined by the environments of other leaves or the status of the leaf relative to other leaves within the plant.

Mechanisms involved in such the long-distance or systemic regulation of leaf photosynthetic properties are, however, unknown. Lake et al. (2002) suggested that jasmonate, ethylene, reactive oxygen species and carbohydrates as candidates for the systemic regulation of stomatal density in newly developing leaves. Among them, the carbohydrates are known to regulate photosynthetic genes (Sheen 1990; Koch 1996), and some studies suggest that leaf carbohydrate status affects senescence rates (Dai *et al*. 1999; Miller *et al*. 2000; Ono *et al*. 2001; Yoshida 2003; Pourtau *et al*. 2004; Parrott *et al*. 2005; Wingler *et al*. 2006). Carbohydrates produced in one leaf can be translocated to the other leaves via the phloem (Joy 1964, Lattanzi et al. 2005, Carvalho *et al*. 2006). Coupe et al.

(2006) showed that elevated CO_2 around mature leaves of *A. thaliana* plants increased the carbohydrate content of young, sink leaves, and suggested the involvement of carbohydrate in the systemic signaling.

Sims et al. (1998) exposed two out of three leaflets of a trifoliate soybean leaf to a CO_2 concentration of 1000 μl l^{-1}, while keeping the remaining leaflet and the other parts of the plant at 250 μl CO_2 l^{-1}, and vice versa. The carbohydrate contents of the high CO_2 leaflets were significantly higher than the low CO_2 leaves. However, the Rubisco activity of the high CO_2 leaflets was not affected by the carbohydrate content but by the CO_2 concentration that the rest of the plant was exposed to. They suggested that the repression of photosynthesis under elevated CO_2 is not due to carbohydrate accumulation in young, recently expanded leaves. The leaves studied in their study maintained almost constant, or even showed increased photosynthetic capacity for 10 days after the onset of the treatment. In our previous study (Araya et al. 2006), I showed that the degree of suppression of photosynthesis by carbohydrates changes with leaf age. Photosynthetic repression by carbohydrates was more effective in mature, senescing leaves than in young leaves. Sims *et al*. (1998) only showed changes in the Rubisco contents and in the maximum carboxylation rate (V_{cmax}) calculated from gas exchange data. Thus, effects of carbohydrate accumulation on photosynthesis have not been fully clarified yet, and detailed analyses of effects of local environment on the photosynthetic properties and senescence are needed.

Information of the redox state of the photosynthetic apparatus in leaves can be transferred to the other leaves. Karpinski *et al*. (1999) showed that the illumination of the mature leaves of *A. thaliana* with strong light (3000 μmol photon m^{-2} s^{-1}) induced expression of *APX2* in other leaves that were kept in the dark. The redox state of the photosynthetic apparatus regulates the expression of photosynthetic components (Pfannschmidt 2003), such as LHCII (Pursiheimo *et al*. 2001; Yang *et al*. 2001), and thereby underlies acclimation of the photosynthetic apparatus to light environments. Thus, the systemic signaling of the redox state of the leaves would also contribute to regulation of the photosynthetic properties of the other leaves.

In this study, I explored effects of CO_2 and light level of particular leaves on photosynthetic organization of both the treated leaves themselves, and untreated leaves in the same plant, and also examining possible involvement of the carbohydrates and/or redox signal in the regulation. In particular, I paid attention to the systemic regulation of photosynthesis. I constructed a local environment control system (Fig. 1), allowing control of the CO_2 concentration and photon flux density of the primary leaves of bean (*Phaseolus valgaris* L.) plants separately from those of the rest of the plants, and examined photosynthetic properties, redox state of PSII and carbohydrate contents of the primary and the first trifoliate leaves.

Materials and Methods

Plant materials

Bean seeds (*Phaseolus vulgaris* L. cv. Yamashiro-Kurosando) were imbibed on wet paper for four days. Four days after the start of the treatment (day 4), the germinated seedlings were planted in 12.7 cm-diameter pots filled with vermiculite (three to six seedlings per pot). The pots, 20 cm in height, were dipped in water to a depth of five cm. Plants were grown in an environment-controlled room at a PPFD of 300 μmol photons m^{-2} s^{-1} (light / dark = 14h / 10h), at 25ºC, with a CO_2 concentration of 400 μl l^{-1}. Atmospheric CO_2 concentration was monitored with a portable infrared gas analyzer (IRGA, GM70, Vaisala, Helsinki, Finland). From day seven, the plants were fertilized everyday with 50 ml of a half strength Hoagland solution containing 2 mM KNO_3, 2 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.665 mM $NaHPO_4$, 25 μM Fe-EDTA, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.25 μM $NaMoO_4$, 50 μM NaCl, and 0.1 μM $CoSO_4$. On day 10, the seedlings were thinned to one plant per pot.

Leaf chambers (Fig. 1A, B)

The chamber consisted of two 10 × 10 × 5 cm acrylate boxes. One side of the box was open. The rim of the open side was covered with foam polyurethane, 5 mm thick, to minimize gas leakage and damage to the stem (See Fig. 1A). The two primary leaves were enclosed, one within each box, and the boxes were secured with elastic plastic bands.

Local environment control system

Room air was passed through a soda lime column to remove CO_2, and pumped to the system. The flow rate of the CO_2-free air was regulated with a rotormeter with a needle

valve (KOFLOC, Kyoto, Japan). The CO_2-free air and 100% CO_2 gas were mixed with a mass flow controller (model 3610, KOFLOC) and the mixed gas was bubbled through water to humidify. The humidified gas was passed through a Liebig condenser maintained at 18ºC to regulate the dew point of the gas, maintaining a vapor pressure deficit (VPD) at around 1.1 kPa. The gas was introduced into each of the 10 chambers at a flow rate of 1 L min^{-1}. The CO_2 concentration of the outlet gas from each of these chambers was measured alternately with an IRGA (LI-840, Li-Cor, Lincoln, NE, USA).

CO_2- and shade-treatments

On day 17,primary leaves (PL) of bean plants were enclosed with the chambers and subjected to CO_2 concentrations of 150, 400, or 1000 μl l^{-1} (hereafter 150-, 400- or 1000-treatments). PL of some plants were subject to 400 μl CO_2 l^{-1} and a PPFD of 50 rather than 300 μmol photons m^{-2} s^{-1} by covering the chambers with a layer of black shade cloth (shade-treatment). During the experiment, 50 ml of half strength of the Hoagland's solution was given to each plant every second day. On days 19, 23 and 27, photosynthetic activities of PL and the first trifoliate leaves (TL) were measured for the same plants. I refer to the plants, PL and TL of the 150-treatment as 150-plants, 150-PL, and 150-TL, respectively. The plants and leaves of 400-, 1000- and shade-treatments were called in the similar manner.

Photosynthesis and chlorophyll fluorescence

Photosynthesis and dark respiration rates of PL and TL were measured with a portable IRGA (LI-6400, Li-Cor). Leaf fluorescence parameters were measured using a pulse amplitude modulation fluorometer (PAM-101, Waltz, Effeltrich, Germany). The leaf was enclosed in the LI-6400 6 cm^2 transparent top leaf cuvette with an air temperature of 25ºC, an atmospheric CO_2 concentration (C_a) of 360 μl l^{-1} and the VPD of approximately 1.4 kPa. After dark pretreatment of the leaf for 20 min, the dark respiration rate and

maximum quantum yield of photosystem II (F_v / F_m) were measured. The leaf was illuminated with white light from a metal halide lamp (PCS-UMX250, Nippon P. I., Tokyo, Japan) at a PPFD of 300 μmol photons m^{-2} s^{-1} for 20 min to measure the rate of photosynthesis (A_{360}) and stomatal conductance. Subsequently, CO_2 concentration and light intensity were changed to those of the growth environments (150, 400 or 1000 μl CO_2 l^{-1} and PPFD at 300 μmol photons m^{-2} s^{-1} or at 50 μmol photons m^{-2} s^{-1}), and the photosynthetic rate (A_{growth}), steady state quantum yield of PSII (Φ_{PSII}'), reduction state of plastoquinone (1-qP) and the stomatal conductance were measured. 1-qP was obtained without measuring F_o', according to Oxborough and Baker (1997). After the measurements of the A_{growth}, the leaf was illuminated at the PPFD of 1000 μmol photons m^{-2} s^{-1}, and an A-C_a curve performed using C_a of 50, 100, 150, 200, 250, 360, 750, 1000, 1250, and 1500 μl CO_2 l^{-1}. The CO_2 concentration in the intercellular space (C_i) was calculated according to von Caemmerer and Farquhar (1981). The slope of the regression line of the A-C_i curve for C_i below 200 μl CO_2 l^{-1} was calculated by the least squares method and regarded as the initial slope of the A-C_i curve. The maximum photosynthetic rate (A_{max}) was measured at a PPFD of 1000 μmol photons m^{-2} s^{-1} and a C_a of 1500 μl CO_2 l^{-1}.

After the photosynthetic measurements, leaf discs of 1 cm in diameter were sampled and snap frozen in liquid N_2 for subsequent analysis. The leaves were frozen between 17:00 and 20:00 hours. Five leaf discs were dried at 80ºC for a week allowing measurements of leaf dry mass per area (LMA) and nitrogen concentration (vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany). Leaf area was estimated using a scanner and software, image J (National Institute of Mental Health, Bethesda, MD, USA).

Rubisco activity and soluble protein content

Rubisco activity was measured according to Lilley and Walker (1974) and Sawada *et al*. (1990) with some modifications. The frozen leaf discs (2.235 cm^2) were ground with a mortar and pestle in liquid N_2. The soluble protein was extracted in 1 ml of 100 mM Hepes-KOH buffer (pH 7.8), 5 mM DTT, 10 mM $MgCl_2$, 1mM EDTA, 1 tablet / 50 ml

complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and 2 mM phenylmethylsulfonyl fluoride. The extract was centrifuged at 15000 × g for 20 seconds, and 100 μl of the supernatant was immediately suspended with 1860 μl of an assay medium containing 90 mM Bicine-KOH (pH 8.2), 18 mM $NaHCO_3$, 4.5 mM $MgCl_2$, 0.9 mM ATP, 4.5 mM creatine phosphate, 10 unit creatine kinase, 10 unit 3-phosphoglycerate kinase, 25 unit glyceraldehyde-3-phosphate dehydrogenase, 65 μM NADH, and 0.65 mM RuBP, and the change in the absorbance at 340 nm was measured for 90 s (initial activity). The same procedures with the assay medium without RuBP were conducted as the control. For the total activity, 200 μl of the supernatant of the extract was mixed with 50 μl of a pre-incubation medium containing 450 mM Hepes-KOH (pH 7.8), 5 mM $MgCl_2$, and 50 mM $NaHCO_3$ to activate the Rubisco. The mixture was kept on ice for 20 min. Then, 100 μl of the mixture was mixed with 1860 μl of the assay medium and the change in absorbance at 340 nm was measured (total activity). Activation state was calculated as the ratio of the initial activity to the total activity. The protein content in the supernatant was measured with Coomassie plus protein assay reagent (Pierce, Rockford, IL, USA).

Non-structural carbohydrate and chlorophyll content

Glucose, sucrose, starch and chlorophyll content of the leaves were measured with the frozen leaf discs as described previously (Araya *et al*. 2006).

Statistical analyses

The differences among CO_2-treatments were analyzed using one-way ANOVA for each day. When significant differences among the treatments were detected, Tukey's multiple tests were conducted, and significant differences at $p < 0.05$ are indicated by different letters in the figures. The differences between 400- and shade-treatments were analyzed with the Student's *t*-tests for each day, and the significant differences at $p < 0.05$,

0.01 and 0.001 were shown as *, **, and ***, respectively. I also conducted two-way ANOVA (for the results of the two-way ANOVA, data not shown).

Results

Leaf area and LMA

In this study, the treatments started on day 17 when the leaf areas of PL and a leaflet of TL were approximately 47 and 20 cm^2. PL was already fully expanded on day 17 while the area of TL were about two third of the full expansion.

LMA of the 1000-PL tended to be greater, and those of the 150-PL and shade-PL tended to be smaller than those of 400-PL (Table 1). Neither the CO_2- nor shade-treatment affected the LMA of TL.

Photosynthetic activities of PL under treatment conditions

The photosynthetic rates measured under their treatment conditions (A_{growth}) are shown in Fig. 2A. The A_{growth} of the 400-PL and 1000-PL decreased through time, while the changes in A_{growth} of the 150-PL and shade-PL were small during the treatments. A_{growth} of the 1000-PL were significantly higher than those of 400-PL on days 17 and 19, but not different on days 23 and 27. A_{growth} of the 150-PL and shade-PL were lower than those of 400-PL and 1000-PL during the treatment. I also continuously monitored CO_2 concentrations in the chambers with an IRGA, and the rates of photosynthesis calculated from the monitoring data of CO_2 concentration in the chamber were largely consistent with the rates shown in Fig. 2A (data not shown). Φ_{PSII}' of the 400-PL were higher than those of the 150-PL except on day 27 (Fig. 2B). Φ_{PSII}' of the 1000-PL was lower than that of both the 400-PL and 150-PL on day 23. Shade-treated plants exhibited consistently high Φ_{PSII}'. 1-qP of the 150-PL were higher than those of the other treatments on days 17 and 19 (Fig. 2C). The 1-qP of the 400-PL were significantly lower than those of the 150-PL at each time point except the final one. The 1-qP of the 1000-PL was significantly higher than that of the 400-PL on day 23, but not significantly different on any other days. The 1-qP of the shade-PL was consistently approximately 25% of the 400-treatments. The stomatal

conductance of the 1000-PL and shade-PL tended to be lower, and those of the 150-PL tended to be higher than that of the 400-PL (Fig. 2D).

Changes in photosynthetic properties of PL

The photosynthetic rates at a CO_2 concentration of 360 μl l^{-1} and a PPFD of 300 μmol photons m^{-2} s^{-1} (A_{360}) of PL are shown in Fig. 3A. A_{360} of the 150-PL tended to be higher than those of the 400-PL during the treatment, although this was not significant. The A_{360} of the 1000-PL and the shade-PL were similar to that of the 400-PL on day 19, but significantly lower on days 23 and 27. Stomatal conductance at 360 μl CO_2 l^{-1} and a PPFD of 300 μmol photons m^{-2} s^{-1} tended to decrease during the treatment (Fig. 3B). There were no statistically significant differences in the stomatal conductance of PL among the treatments except for that between the shade-PL and 400-PL on day 27. A_{max} showed the trends similar to those of the A_{360} for the CO_2-treatments (Fig. 3C), except that the A_{max} of the shade-PL were similar to those of the 400-PL during the treatments. The initial slopes of the A-C_i curves of the 150-PL were always higher, and the initial slopes of the 1000-PL and shade-PL were always lower than those of the 400-PL (Fig. 3D). The initial activities of the Rubisco showed similar trends (Fig. 4A). The initial activities of 1000-PL were lower than those of the 150-PL and 400-PL. Initial activities of the 150-PL were significantly higher than those of the 400-PL and 1000-PL on day 27. The shade-treatment significantly decreased the initial activity of PL. Total activity also showed the trends similar to those of the initial activity (Fig. 4B), and the activation state of Rubisco was high and almost the same among the treatments (Fig. 4C). Dark respiration rates of PL measured at 360 μl CO_2 l^{-1} were not affected by the CO_2-treatments, whereas those of the shade-PL were approximately half that of the other treatments (Table 1). F_v / F_m of PL subject to CO_2-treatments decreased with increasing CO_2 over the course of the experiment. Shading treatments caused significantly higher F_v / F_m than un-shaded plants. On day 27, F_v / F_m was highest in 150-PL followed by 400-PL and 1000-PL (Table 1). F_v / F_m was always higher in the shade-PL than in 400-PL.

Changes in the photosynthetic properties of TL

Among the CO_2-treatments, A_{360} was highest in the 150-TL and followed by those of the 400-TL and 1000-TL on days 23 and 27 (Fig. 3E). The A_{360} of the 150-TL were higher than those of the 1000-TL by 15-60 %. A_{360} of the shade-TL were always higher than those of the 400-TL. The stomatal conductance of the 150-TL was initially higher than those of the 400-TL and 1000-TL (Fig. 3F). On day 23 and 27, there were no statistical differences in the stomatal conductance among the CO_2-treatments. A_{max} of TL did not differ statistically among the CO_2-treatments (Fig. 3G). Shade-treatment decelerated the decrease in A_{max}, and a statistical difference in A_{max} between the 400-TL and shade-TL on day 23 was noted. The initial slopes showed trends similar to the A_{360}, but the relative differences among treatments were smaller than those of the A_{360} (Fig. 3H). The initial activities of Rubisco showed tendencies similar to those of A_{360} and the initial slope, and the initial activity of the 1000-TL were lower than those of the 150-TL and 400-TL (Fig. 4D). The initial activities of the shade-TL tended to be higher than those of the 400-TL on days 23 and 27. Total activity also showed the trends similar to those of the initial activity (Fig. 4E). The activation state of Rubisco was almost identical across the treatments and three measurements (Fig. 4F). CO_2- or shade-treatments did not bring about statistically significant differences in the dark respiration rate of TL (Table 1). The CO_2-treatments did not cause any statistical difference in F_v / F_m (Table 1). The F_v / F_m of the shade-TL tended to be greater than those of the 400-treatments, and the difference was statistically significant on day 23.

Carbohydrates, nitrogen and proteins

The glucose and sucrose contents were greatest in the 1000-PL followed by those of the 400-PL and 150-PL in this order on days 23 and 27 (Fig. 5A-C). The starch contents of the 1000-PL were always significantly higher than those of the other treatments.

Conversely, the carbohydrates were always low in the shade-PL, although neither of the differences between the shade-PL and 400-PL was statistically significant except for sucrose content on days 23 and 27. The carbohydrate contents of TL did not differ among the treatments and remained lower than those of the 400-PL (Fig. 5D-F)

The soluble protein content or nitrogen content of PL did not differ significantly among the CO_2-treatments (Table 2). The shade-treatment exhibited significantly lower soluble protein content in PL. The nitrogen contents of the shade-PL were also generally lower than those of the 400-PL, although this was only significant on day 19. Neither soluble protein nor nitrogen content of TL differed among the treatments.

Chlorophyll contents and Rubisco total activity / chlorophyll ratio

Neither chlorophyll contents of PL nor those of TL differed among the CO_2-treatments (Table 2). However, chlorophyll contents of the shade-PL and shade-TL were higher than those of the 400-PL and 400-TL, respectively. Rubisco total activity / chlorophyll ratios, which are known to decrease with shade acclimation (Björkman 1981), were lower in the 1000-PL and 1000-TL than those of the 400-PL and 400-TL, respectively (Fig. 6A, B). The total activity / chlorophyll ratio of the 150-PL increased during the treatment, and the values were higher than those of the 400-PL on days 23 and 27. The total activity / chlorophyll ratios of the 150-TL were also higher than those of the 400-TL. The total activity / chlorophyll ratios of the shade-PL and shade-TL were always lower than those of the 400-PL and 400-TL, respectively.

Discussion

In this study, I demonstrated that light and CO_2 environments of PL affected photosynthetic capacity and light acclimation in both PL and TL. These results suggest that not only local environments, but the systemic signaling from the other leaves affects photosynthesis of the young leaves. Moreover, the light and CO_2 environment of PL had different effects on photosynthetic capacity of TL.

Effect of CO_2 and light environment of PL on their photosynthesis

A_{360} of the 1000-PL was lower, and that of the 150-PL was higher than that of the 400-PL (Fig. 3A). The carbohydrate contents increased with increasing CO_2 concentration (Fig. 5A-C). When accumulated in the leaf, carbohydrates repress expression of the photosynthetic genes (Koch 1996) and accelerate leaf senescence (Dai *et al*. 1999; Ono *et al*. 2001). Thus, the differences in the decrement of A_{360} depending on the CO_2-treatments may be due to the different levels of the carbohydrates in the leaves.

Both of the initial slope of A-C_i curve and the value of A_{max}, representing the RuBP carboxylation and regeneration capacities, respectively (von Caemmerer and Farquhar 1981), declined most rapidly in the 1000-PL, and most slowly in the 150-PL (Fig. 3C, D). I attribute the changes in the initial slope of the A-C_i curve to changes in Rubisco activity (Fig. 4A, B). The decrease in A_{max} would be also attributed to the sugar repression of expression of the components involved in RuBP regeneration capacity such as plastocyanin (Oswald *et al*. 2001).

Our results show the strong effects of carbohydrates on leaf photosynthesis, are inconsistent with the results of Sims *et al*. (1998). However, they used young leaves while I examined senescing PL, and the degree of the carbohydrate repression changes with the developmental stages of the leaves and is strongest during senescence (Krapp et al. 1991; Araya *et al*. 2006; Wingler *et al*. 2006). Thus, the differences in leaf age may explain the differences between our results and those of Sims *et al*. (1998).

Rubisco total activity / chlorophyll ratios of the 1000-PL were lower than those of the 400-PL (Fig. 6A). In the 150-PL, the ratios increased during the treatment and became higher than those of the 400-PL. Taking account of that the Rubisco / chlorophyll ratio is generally high when the leaves were exposed to sunny conditions (Björkman 1981; Hidema *et al*. 1991; Hikosaka 1996; Hikosaka and Terashima 1996; Makino *et al*. 1997; Okada and Katoh 1998), the photosynthetic apparatus of the 150-PL was more sun-type, while that of the 1000-PL was more shade-type, respectively, than the 400-PL plants. Since 1-qP was greatest in the 150-PL on days 17 and 19 (Fig. 2C), the redox state could be related to these sun / shade acclimations (Huner et al. 1998).

The shade-treatment accelerated the decrease in A_{360} of PL (Fig. 3A). In general, the photosynthetic rate decreases when the leaves are shaded (Terashima and Evans 1988, Hikosaka 1996, Hikosaka and Terashima 1996, Bailey et al. 2001). The shade-treatment decreased the initial slope of A-C_i curves of PL (Fig. 3D), which was attributable to the faster decrease in the Rubisco activity (Fig. 4A, B, A significant age × treatment interaction was detected by two-way ANOVA, data not shown). However, the shade-treatment did not affect the A_{max} (Fig. 3C). Previous studies suggest that the amount of light harvesting complex II (LHCII) is regulated by the redox state of photosystem II (PSII) (Escubus et al. 1995, Maxwell et al. 1995, Yang et al. 2001, Pursiheimo et al. 2001). Probably, the shade-treatment led to an increase in the amount of LHCII in response to the low 1-qP (Fig. 2C). The low total Rubisco activity / chlorophyll ratio (Fig. 6A) in the shade-PL would be explained by the decrease in the Rubisco content and increase in the LHCII content.

Systemic regulation of photosynthetic capacity of TL by the environment and / or activity of PL

A_{360} of 1000-TL was lower, and those of 150- and shade-TL was higher than that of the 400-TL (Fig. 3E), clearly indicating that the CO_2 and light environments of PL affected the photosynthetic properties of TL.

The CO_2-treatments of PL affected the initial slope of A-C_i curve, but not A_{max}, of TL (Fig. 3G, H), suggesting that changes in A_{360} of TL are the result of changes in RuBP carboxylation capacity. Rubisco activity showed a trend similar to A_{360} among the CO_2-treatments (Fig. 4D, E), and the calculated effect of the stomatal conductance on A_{360} was small (Fig. 3F). The Rubisco activity / chlorophyll ratio of the 150-TL was higher, and that of the 1000-TL was lower than that of the 400-TL (Fig. 6B), indicating that the light acclimation of the photosynthetic apparatus in TL was also affected by the environment of the primary leaves.

Conversely, the shade-treatment of the primary leaves enhanced both the initial slope of A-C_i curve and the A_{max} of TL (Fig. 3G, H). The chlorophyll content increased, and the total Rubisco activity / chlorophyll ratio decreased in the shade-TL (Table 2, Fig. 6B). In the shade-PL, the 1-qP during the treatment was very low (Fig. 2C), suggesting that the redox state of PL has a systemic effect on the light acclimation of TL. The signaling mechanism of PL redox state to the TL is unknown, although leaf-to-leaf transfer of hydrogen peroxide represents a good candidate (Karpinski et al. 1999, Ślesak et al. 2007). Further experiments are required to confirm the effects of the redox state of PL in regulation of photosynthetic properties and/or senescence in TL, and to identify the signaling molecules (Coupe et al. 2006).

The roles of carbohydrates in the photosynthetic regulation by the systemic signaling

The carbohydrate contents of PL increased in the 1000-treatment and decreased by the 150- or shade-treatments (Fig. 5A-C), relative to the 400-treatment. Conversely, the carbohydrate contents of TL were not affected by the CO_2- or shade-treatments of PL (Fig. 5D-F). Thus, the environment of PL did not affect the carbohydrate content of TL. However, it appeared that alterations in the photosynthetic capacity of TL acted to compensate for changes in PL photosynthesis (Fig. 3), suggesting that the photosynthetic capacity of TL was regulated by the demand for photosynthate by the whole plant, i.e. by the supply of the photosynthates from the other leaves. I only measured carbohydrate

contents in leaves sampled in the late afternoon, but carbohydrates are also translocated overnight (Kaiser and Bassham 1979ab). Thus, detailed analyses of the C translocation are required for understanding the role of carbohydrate on the long-distance signaling. The correlation of the redox state of PSII in PL and photosynthetic properties of TL and the consistent implication of adjustment of leaf photosynthesis contributing to maintenance of whole plant photosynthesis urge us to clarify molecular mechanisms of those features in systemic regulation of photosynthesis.

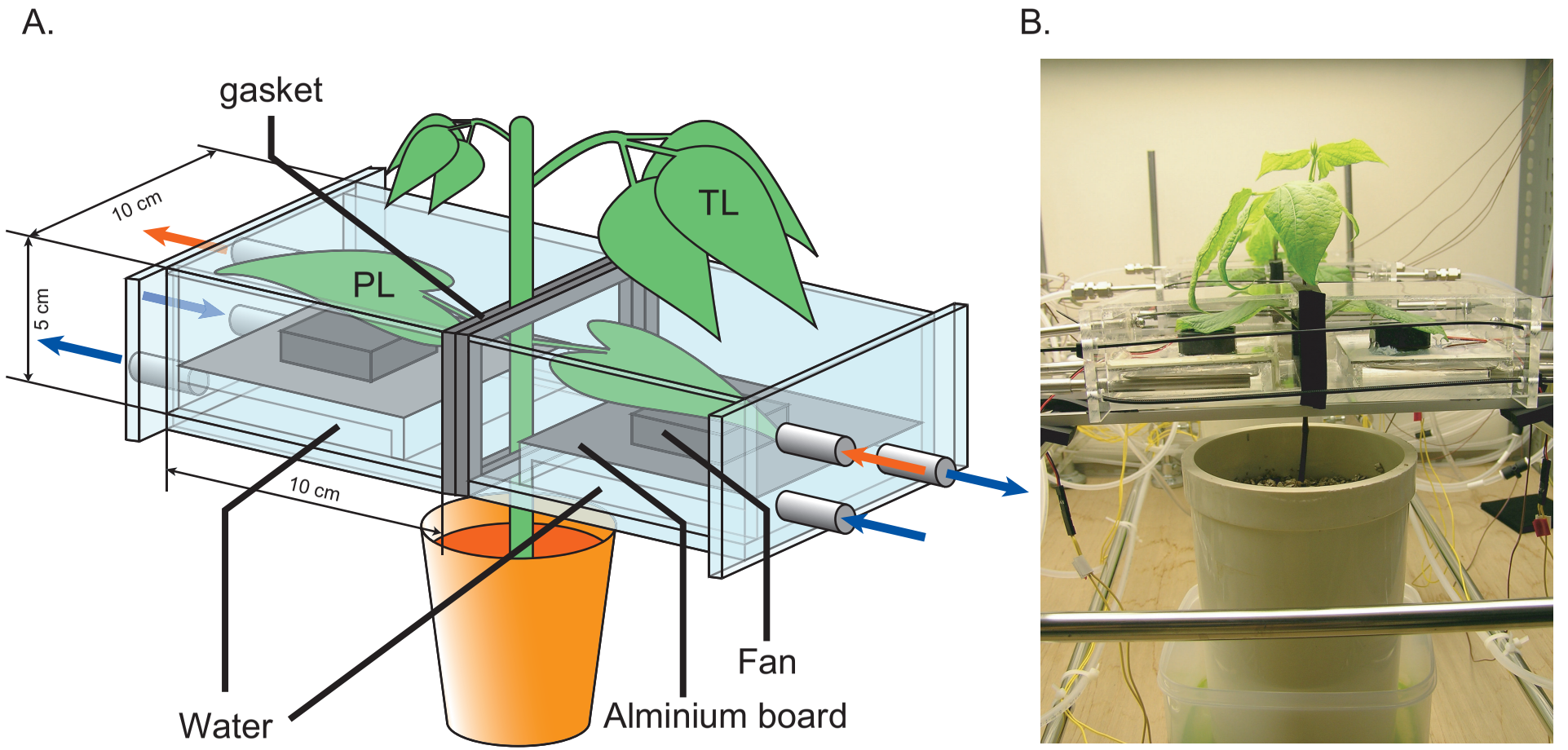

Fig. 1

A; Leaf chamber. Water at 25 ºC was circulated in the lower parts of the half chambers (blue arrows). The air (orange symbols) was agitated by two 5 V computer fans. B; A bean plant during the treatment.

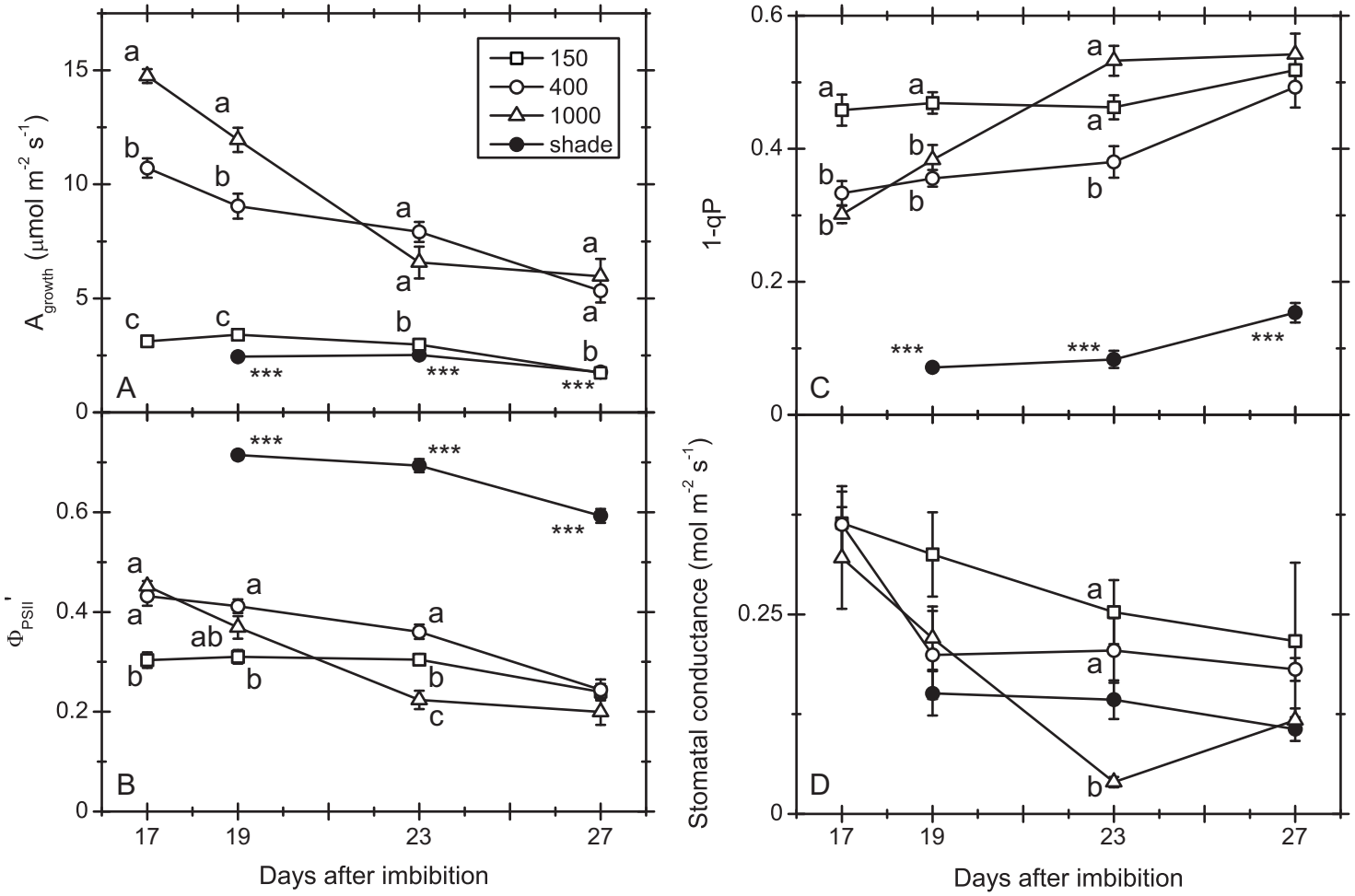

Fig. 2

Changes in the photosynthetic properties of the primary leaves (PL) under the conditions that mimicked the treatments. A, photosynthetic rate on leaf area basis (A_{growth}); B, quantum yield of PSII at light (Φ_{PSII}'); C, 1-qP; and D, stomatal conductance on leaf area basis. Open squares, 150-treatment; open circles, 400-treatment; open triangles, 1000-treatment; and closed circles, shade-treatment. Error bars denote SE (n = 5 - 11). Significant differences at $p < 0.05$ with the Tukey' s multiple tests among CO_2 treatments in each day are indicated by letters. The significant differences between 400- and shade-treatments with the Student' s *t*-test at $p < 0.05$, 0.01 and 0.001 were shown as *, **, and ***, respectively.

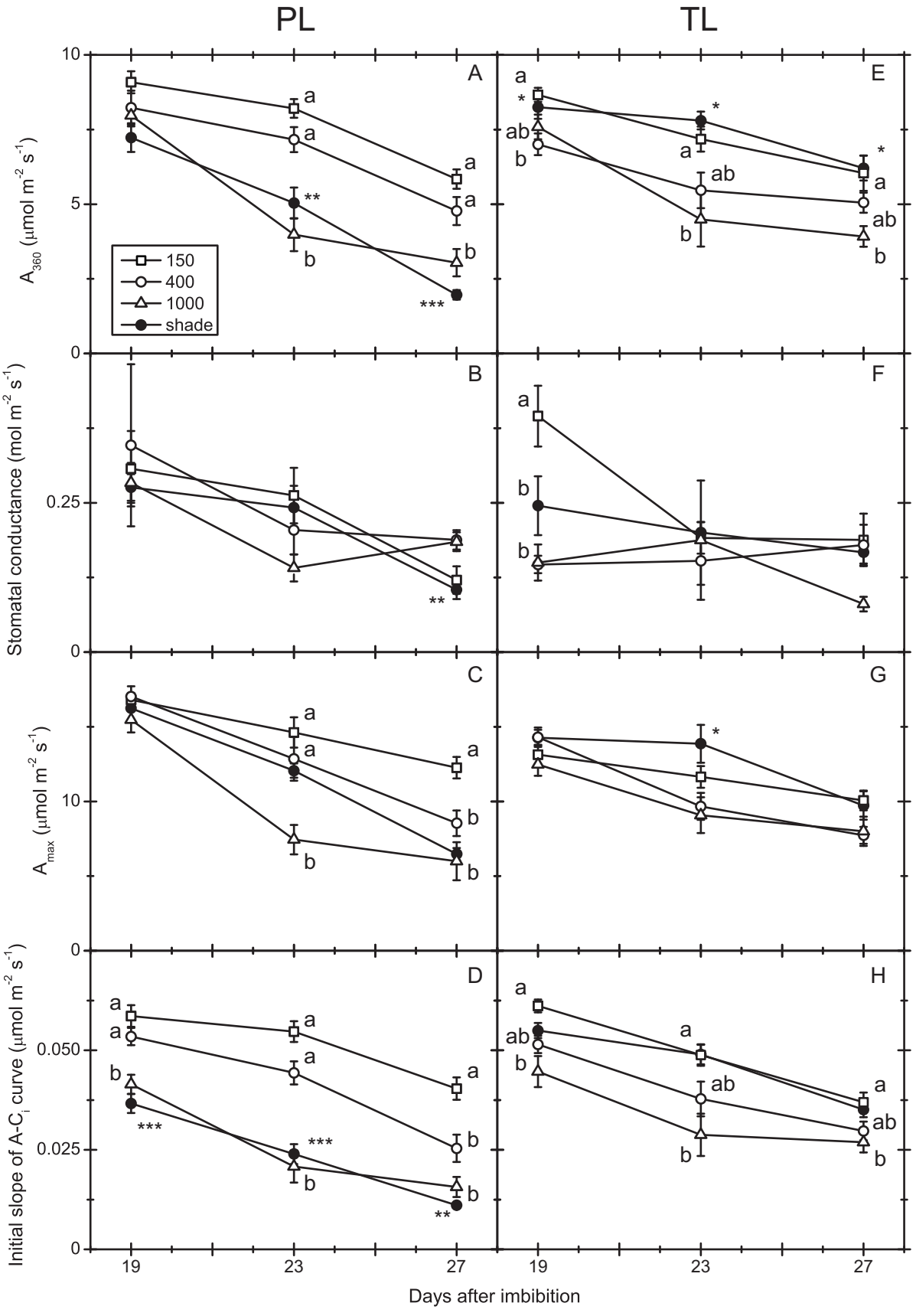

Fig. 3

Changes in the photosynthetic rate on leaf area basis. A, E, photosynthetic rate at a PPFD of 300 μmol photons m^{-2} s^{-1} and a CO_2 concentration of 360 μl l^{-1} (A_{360}) ; B, F, stomatal conductance at a PPFD of 300 μmol photons m^{-2} s^{-1} and a CO_2 concentration of 360 μl l-1; C, G, photosynthetic rate at a PPFD of 1000 μmol photons m^{-2} s^{-1} and a CO_2 concentration of 1500 μl l^{-1} (A_{max}) and D, H, a slope of regression line of the A-Ci curve for the range of Ci below 200 μl l^{-1} measured at a PPFD of 1000 μmol photons m^{-2} s^{-1}; A-D, primary leaves (PL) and E-H trifoliate leaves (TL). Symbols and alphabets are as in Fig. 2. Error bars denote SE (n = 5 - 11).

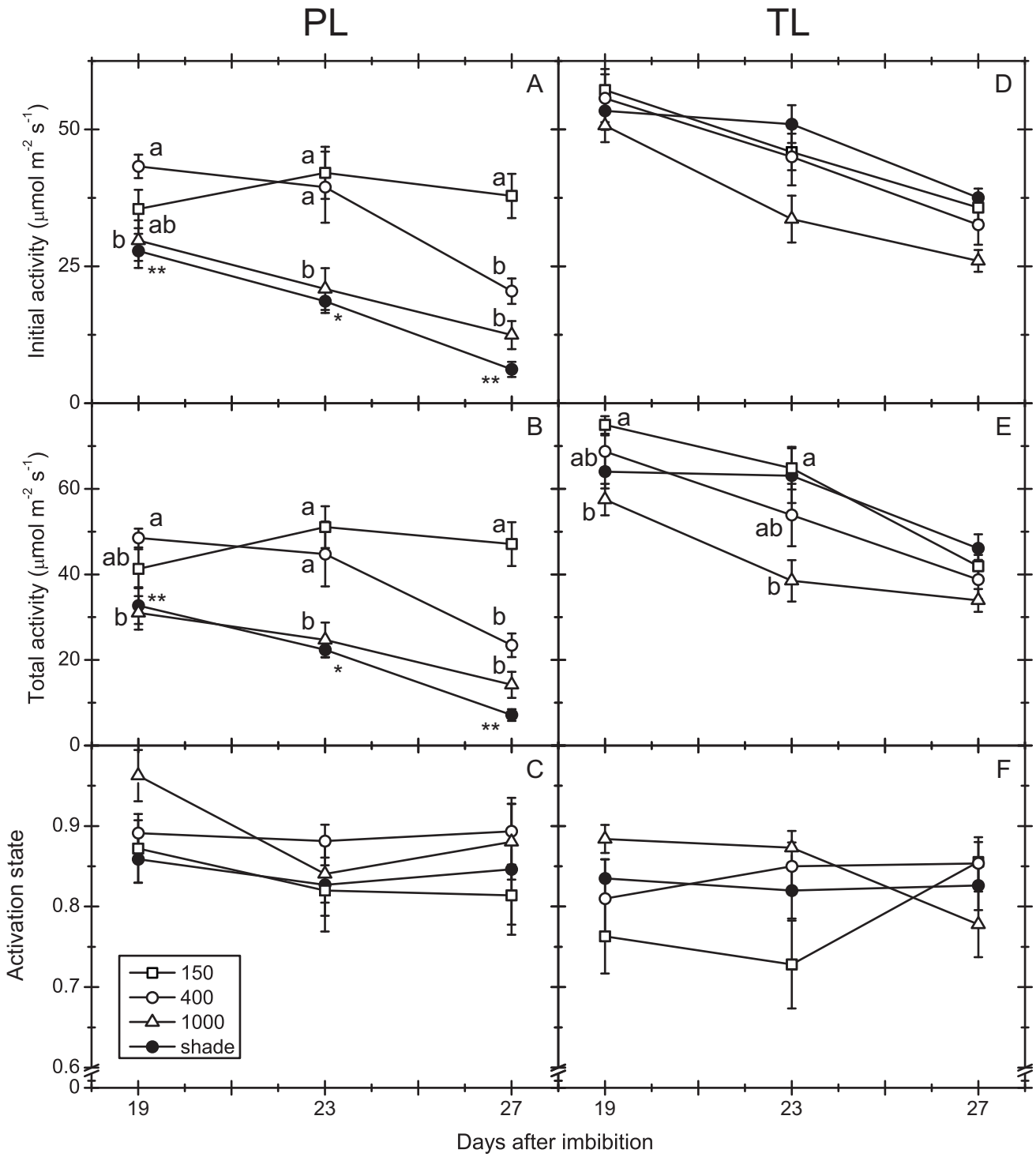

Fig. 4

Changes in the Rubisco activity of the primary (PL) and trifoliate leaves (TL) on leaf area basis. A, D, initial activity; B, E, total activity and C, F, activation state. A-C, PL and D-F, TL. Symbols and alphabets are as in Fig. 2. Error bars denote SE (n = 4 - 10).

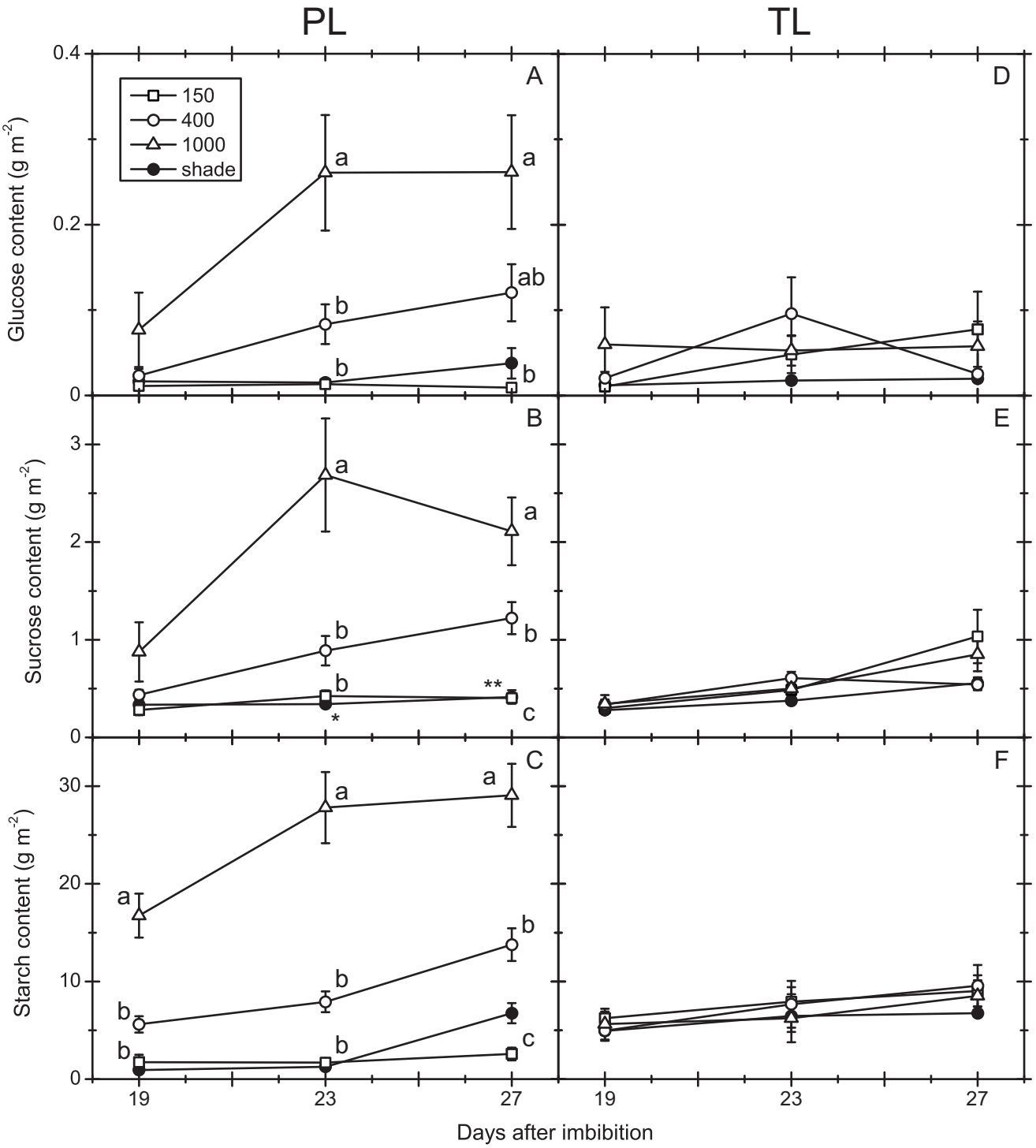

Fig. 5

Changes in the carbohydrate content of the primary (PL) and trifoliate leaves (TL) on leaf area basis. A, D, glucose content; B, E, sucrose content and C, F, starch content. A-C, PL and D-F, TL. Symbols and alphabets are as in Fig. 2. Error bars denote SE (n = 5 - 11).

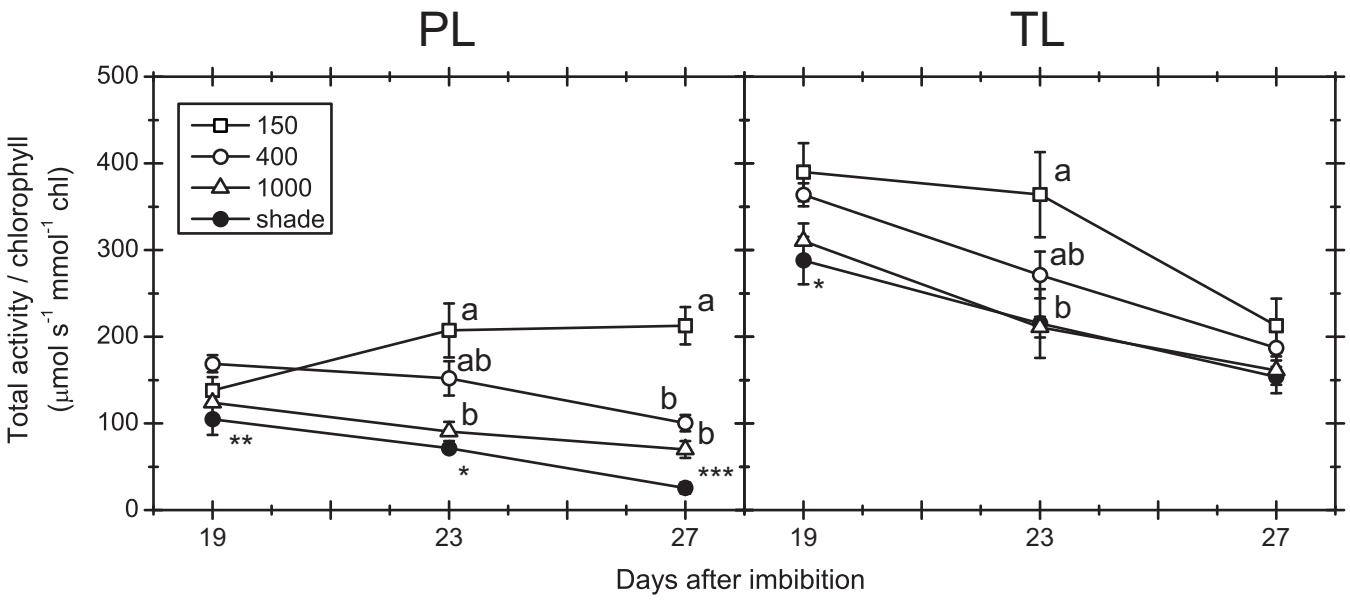

Fig. 6

Changes in the Rubisco total activity / chlorophyll ratio of the primary (PL) and trifoliate leaves (TL). Symbols and alphabets are as in Fig. 2. Error bars denote SE (n = 4 - 11).

Table 1 Changes in leaf mass per area (LMA), maximum quantum yield of PSII (F_v / F_m) and dark respiration rate of the primary (PL) and trifoliate leaves (TL) during treatments. These data denotes average ± SE (n = 5 - 11). Significant differences at $p < 0.05$ with the Tukey's multiple tests among CO_2 treatments in each day are indicated by letters. The significant differences between 400- and shade-treatment with the Student's *t* -test at $p < 0.05$, 0.01 and 0.001 were shown as *, **, and ***, respectively.

		PL			TL		
	treatments	19d	23d	27d	19d	23d	27d
LMA (g m^{-2})	150 ppm	17.0 ± 0.9^{a}	20.9 ± 1.1^{a}	22.0 ± 0.8^{a}	24.0 ± 1.1	29.2 ± 1.2	29.2 ± 1.4
	400 ppm	22.1 ± 1.0^{a}	27.2 ± 2.0^{a}	35.7 ± 1.8^{b}	25.5 ± 0.5	31.6 ± 3.2	31.7 ± 2.1
	1000 ppm	32.9 ± 2.6^{b}	48.6 ± 2.7^{b}	51.7 ± 4.2^{c}	24.7 ± 1.3	28.1 ± 2.4	32.1 ± 1.2
	shading	16.8 ± 0.8**	18.3 ± 0.8**	20.6 ± 0.9**	23.7 ± 1.9	24.8 ± 2.5	29.1 ± 1.7
F_v / F_m	150 ppm	0.760 ± 0.005	0.709 ± 0.011	0.684 ± 0.009^{a}	0.762 ± 0.004	0.744 ± 0.007	0.743 ± 0.010
	400 ppm	0.749 ± 0.008	0.688 ± 0.019	0.656 ± 0.014^{ab}	0.760 ± 0.004	0.731 ± 0.018	0.725 ± 0.018
	1000 ppm	0.746 ± 0.009	0.674 ± 0.017	0.604 ± 0.029^{b}	0.752 ± 0.012	0.702 ± 0.022	0.710 ± 0.028
	shading	0.806 ± 0.003***	0.785 ± 0.006**	0.774 ± 0.007***	0.772 ± 0.008	0.783 ± 0.007*	0.780 ± 0.005
Dark respiration rate (μmol m^{-2} s^{-1})	150 ppm	1.01 ± 0.10	1.12 ± 0.06	1.08 ± 0.10	1.47 ± 0.09	1.25 ± 0.07	1.00 ± 0.06
	400 ppm	1.12 ± 0.08	1.27 ± 0.08	1.06 ± 0.07	1.42 ± 0.10	0.94 ± 0.05	0.96 ± 0.08
	1000 ppm	1.07 ± 0.05	1.32 ± 0.06	1.33 ± 0.11	1.25 ± 0.12	0.99 ± 0.18	0.95 ± 0.10
	shading	0.50 ± 0.04***	0.39 ± 0.05***	0.52 ± 0.08***	1.37 ± 0.16	0.91 ± 0.14	0.85 ± 0.03

Table 2 Changes in chlorophyll content, soluble protein content, and nitrogen content of the primary (PL) and trifoliate leaves (TL) during treatments. These data denotes average ± SE (n = 5 - 11). The significant differences between 400- and shade-treatment with the Student's *t* -test at $p < 0.05$, 0.01 and 0.001 were shown as *, **, and ***, respectively.

		PL			TL		
	treatments	19d	23d	27d	19d	23d	27d
chlorophyll content (mmol m^{-2})	150 ppm	0.301 ± 0.015	0.263 ± 0.013	0.224 ± 0.014	0.201 ± 0.013	0.198 ± 0.023	0.225 ± 0.027
	400 ppm	0.290 ± 0.011	0.286 ± 0.013	0.230 ± 0.017	0.188 ± 0.008	0.196 ± 0.010	0.208 ± 0.016
	1000 ppm	0.256 ± 0.014	0.257 ± 0.016	0.194 ± 0.025	0.186 ± 0.010	0.186 ± 0.026	0.233 ± 0.026
	shading	0.327 ± 0.022	0.318 ± 0.036	0.319 ± 0.037*	0.229 ± 0.018*	0.316 ± 0.035**	0.311 ± 0.026**
soluble protein content (g m^{-2})	150 ppm	2.07 ± 0.10	1.90 ± 0.15	1.94 ± 0.21	2.85 ± 0.09	2.31 ± 0.15	2.05 ± 0.17
	400 ppm	1.91 ± 0.07	2.11 ± 0.28	1.53 ± 0.13	2.61 ± 0.10	2.64 ± 0.23	2.04 ± 0.14
	1000 ppm	1.97 ± 0.11	1.75 ± 0.14	1.40 ± 0.14	2.58 ± 0.15	2.06 ± 0.12	1.75 ± 0.10
	shading	1.52 ± 0.12*	1.17 ± 0.07*	0.85 ± 0.05***	2.40 ± 0.16	2.43 ± 0.25	2.06 ± 0.08
nitrogen content (g m^{-2})	150 ppm	0.790 ± 0.036	0.832 ± 0.048	0.692 ± 0.052	1.12 ± 0.015	1.09 ± 0.083	0.846 ± 0.040
	400 ppm	0.778 ± 0.026	0.786 ± 0.101	0.568 ± 0.066	1.18 ± 0.037	1.03 ± 0.084	0.762 ± 0.087
	1000 ppm	0.698 ± 0.035	0.750 ± 0.072	0.552 ± 0.041	1.09 ± 0.043	1.04 ± 0.107	0.909 ± 0.052
	shading	0.666 ± 0.031*	0.583 ± 0.046	0.414 ± 0.021	1.08 ± 0.073	1.03 ± 0.029	0.820 ± 0.071

General Discussion

Repression of expression of photosynthetic genes is the main mechanism of feedback regulation of photosynthesis.

In Chapter 1, I showed that the feedback regulation of photosynthesis was significant in mature leaves, but not in young sink leaves. The main factor of the feedback regulation of photosynthesis was the decrease in Rubisco content. The decrease in Rubisco content would be due to the repression of Rubisco gene expression in the sugar-treated leaves, because the Rubisco content of the leaves is regulated by the abundance of mRNA of *rbcS* and *rbcL* (Irving and Robinson 2007). Neither Pi limitation of photosynthesis nor the decrease in g_i in the carbohydrate accumulated leaves occurred.

The repression of the gene expression of photosynthetic genes occurs drastically in several hours (1.5-5 h) in the protoplasts that were fed sucrose or glucose solution (Sheen 1990, Krapp et al. 1993), although the repression of photosynthesis in the intact leaves after the sugar feeding occurred in several days (2-7 days, Krapp et al. 1991, Krapp and Stitt 1995). These differences would be attributed to the time course of the carbohydrate accumulation and/or to the form of the carbohydrates that accumulate in the leaves.

When the sugar solutions were fed to the plants via the petioles or the roots, the carbohydrate accumulation of the leaves occurred slowly (2-7 days), and the accumulated carbohydrates were mainly sucrose or starch (Krapp et al. 1991). On the contrary, the sugar feeding to the protoplast would immediately change the sugar concentration in the cytosol, and, in particular, when glucose is fed, it is is immediately catalyzed by hexokinase. Because of the presence of invertase, effects of sucrose are also quick. Thus, the sugar feeding to the protoplasts affects the photosynthetic gene expressions rapidly, but that to the plants via petiole or roots affects slowly.

The carbohydrate accumulation in the leaves of the plants fed with sugar via roots is slow, and the some intermediates of the carbohydrate metabolism also accumulate in the leaves. These intermediates may also affect the gene expression of the photosynthetic

components of the leaves. There are few studies examining the effects of intermediates of carbohydrate metabolism on the gene expression in plant cells. Metabolome, transcriptome, and proteome analyses in both of the protoplasts and the leaves are needed for elucidation of the detailed mechanisms of the long-term effects of carbohydrate accumulation on photosynthesis of the leaves.

How does starch accumulation in the leaves affect gene expression of the photosynthetic components?

The carbohydrates content of the leaves is high in daytime, and low in night. However, the accumulated carbohydrate in daytime is, in many cases, starch (see Chapter 1-3). Starch is not a physiologically active form of carbohydrate, and the starch seems not to affect gene expression of Rubisco directly. Moore et al. (1998) suggested that the "sucrose cycling" occurs in the starch accumulated leaves, and this would involve in the sugar repression of photosynthetic genes. In the night, the starch is hydrolyzed and turns into sucrose, and sucrose is transported to other sink organs. A part of sucrose is transported to the vacuole for storage. When vacuolar invertase activity is high, sucrose is readily hydrolyzed (Goldschmidt and Huber 1992). The products of sucrose hydrolysis, glucose and fructose, are transported to the cytosol, and are phosphorylated by the hexokinase. The hexokinase is thought to a glucose sensor, and the catalysis of hexose may have some roles in glucose sensing. This "sucrose cycling" would be involved in the sugar repression of photosynthetic gene in the starch-accumulated plants, which suggests importance of sugar repression in the night for the regulation of Rubisco content. Some studies suggest that mRNA levels of *rbcS* and *rbcL* are fluctuating according to the circadian clock (Pilgrim and McClung 1993). Irving and Robinson (2007) suggested that regulation of the Rubisco content is regulated by the gene expression of *rbcS* and *rbcL* according to the data of Suzuki et al. (2001). However, the Rubisco gene expression that was shown in Suzuki et al. (2001) was measured in the leaves that corrected at noon (11:00~13:00), although the peaks of *rbcS* and *rbcL* mRNA levels were found in the night

(Pilgrim and McClung 1993). Cheng et al. (1998) showed that the gene expression of *rbcS* and *rbcL* was decreasedby the elevated CO_2 in *A. thaliana*, and the decrease in gene expression was more significant in the night than in the daytime. They also showed that, in the elevated CO_2, much starch was present throughout the day, although the accumulation of sucrose occurred only in the daytime. These studies suggest that the accumulation of carbohydrate itself does not directly affect the gene expression of the photosynthetic components, but the metabolic flow of carbohydrates may act as important signals in the plants. The relationship between the metabolic flux of the carbohydrates and the gene expression of the photosynthetic genes should be studied to elucidate the essential step of the carbohydrate repression of photosynthesis.

Effects of leaf developmental stages on the feedback regulation of photosynthesis

In Chapters 1 and 2, I have described that the feedback regulation of photosynthesis was significant in mature source leaves, but not in young sink leaves. The carbohydrate content of a leaf would reflect the balance between the photosynthetic production of the leaf and the demand for the produced carbohydrate by the other organs. In the sink leaves, amounts of carbohydrates that are consumed by the leaves themselves for their construction are high relative to their carbohydrate production. Carbohydrate is also translocated from the other leaves, and the demand for the carbohydrate produced in the sink leaves by the other organs is very small. Thus, the carbohydrate content of the sink leaves would not reflect the balance between the photosynthetic production and the demand for carbohydrate, and regulation of photosynthetic characteristics by the carbohydrate accumulation in the sink leaves may not work properly for the optimizing the leaf photosynthesis in relation to the demand for carbohydrates. The extreme carbohydrate accumulation in the sink leaves is rarely observed, because of their high carbohydrate consumption rates and low photosynthetic rates. On the other hand, the carbohydrate content of the source leaves fairly reflects the balance between the photosynthetic production of the leaves and the demand for carbohydrates by the other organs. In general, the nitrogen content of the leaves is well

correlated with the photosynthetic rate of the leaves (Evans 1989), and the nitrogen nutrition is often limited in nature. Thus, the carbohydrate accumulation in mature leaves indicates the excess activity of the photosynthesis relative to the carbohydrate demand by the other organs, and thereby the excess investment of nitrogen to the leaves, as well. Carbohydrate regulation of photosynthesis may contribute to maximizing the photosynthetic production of the plant and increasing fitness of the plant via optimizing the nitrogen investments to each leaves in the plant.

Nitrogen nutrition affects the carbohydrate content of the leaves, and the photosynthetic activity of the leaves.

Nitrogen nutrition of the plants affects the plant growth and the demands for carbohydrate of the plant by affecting their growth. Limited nitrogen nutrition slows the plant growth and decreases the demand for carbohydrates by the growing organs, and induces the carbohydrate accumulation in the leaves. In Chapter 2, the nitrogen limitation decreased the photosynthetic activity in the primary leaves of bean plants. The carbohydrate accumulation in the leaves was induced by the low nitrogen nutrition, and the photosynthetic rate of the leaves was well correlated with the carbohydrate contents of the leaves. These results suggest that the nitrogen limitation represses photosynthesis via the carbohydrate accumulation in the leaves. On the other hand, some studies showed that the nitrogen fertilization to the nitrogen limited plants immediately induces the gene expressions of photosynthetic components and enhances the photosynthetic rate of the leaves (Ono et al. 1997, Wang et al. 2000). These results suggest that the nitrogen fertilization itself induces the gene expression of photosynthetic components, and carbohydrate is not the intermediate signaling components in induction of photosynthesis by nitrogen. The global induction of gene expression by the nitrogen nutrition only occurs when the glucose is contained in the growth medium in *A. thaliana* (Price et al. 2004). As mentioned in Discussion of Chapter 2, Price et al. (2004) only showed the rapid response (~3h) of gene expression to the nitrogen fertilization. The nitrogen signal transduction is

emerged not only by the nitrate or ammonium (Miao et al. 1991, Scheible et al. 1997), but the glutamine, 2-OG and cytokinin also play the signaling roles in plants (Sugiharto et al. 1992, Sakakibara et al. 1998). These signaling molecules would be synthesized and affect photosynthesis of the leaves more slowly than the nitrate and ammonium signals. These signals would interact with carbohydrate metabolism and determine the plant response to the changing nitrogen environments.

The systemic effect of carbohydrate accumulation in the leaf on the photosynthesis of the other leaves

In Chapter 3, I showed the effects of light or CO_2 environments of the primary leaves of bean on the photosynthesis of the trifoliate leaves. Shading and the 1000-treatments decreased, and the 150-treatment enhanced the photosynthetic rate of the primary leaves. On the other hand, the 1000-treatment decreased, and the shade- and 150-treatments enhanced the photosynthetic rate of the trifoliate leaves. Carbohydrate contents and the redox state of the primary leaves of the respective treatments are summarized in Fig. 1. The photosynthetic rate of the primary leaves was low when the carbohydrate accumulated or the photosystems were oxidized. Thus, both of the low carbohydrate content and the reduced photosystems are needed for the sustaining the high photosynthetic activity of the primary leaves. On the other hand, the photosynthetic rate of the trifoliate leaves was high when the carbohydrate accumulation in the primary leaves was low.

In this study, the carbohydrate did not directly transfer from the primary leaves to the other leaves (Chapter 3, Fig. 5). Thus, there are no evidences for the systemic regulation of photosynthesis by the carbohydrates. However, this type of regulation seems to maintain the carbohydrate production of the plants at a steady level. For example, elevated CO_2 around the primary leaves enhances the carbohydrate production of the primary leaves, and the carbohydrate production of the leaves become greater than the carbohydrate

consumption in the plants. Under these conditions, the carbon / nitrogen (C/N) ratio of the plant increased, and plants appear to be avoid the excess increase in the C/N ratio (Makino and Osmond 1991, Noguchi and Terashima 2006). To keep the C/N ratio of the plants low, the photosynthetic activity of the other leaves would be repressed by the carbohydrate accumulation in the primary leaves. Thus, if carbohydrate accumulation in the leaf affects the photosynthetic rate of the other leaves, this would contribute to maintenance of the C/N ratio of the plant. The carbohydrate accumulation induces ABA synthesis (Cheng et al. 2002). Such intermediate signaling molecules would be transferred from the carbohydrate accumulated leaves to the other leaves, and affects the photosynthetic activities of the other leaves.

When the photosystems in the primary leaves were reduced or oxidized, the chloroplasts in the trifoliate leaves became sun-type or shade-type, respectively. Thus, the redox state of photosystems of the primary leaves would affect the light acclimation of the other leaves. However, these situations are not often found in the natural condition. In general, lower leaves are in low irradiance levels than the upper leaves. If the light environment of the lower leaves affect the light acclimation of the young leaves, the young leaves would never have the sun-type photosystems. Thus, the signals of the redox state of photosystems should be generated from young mature leaves, but not the senesced leaves. There are few studies examining generation of the signaling molecules during leaf maturation and senescence. Localization of the generating point of the signals should be prerequisite for determination of the signaling molecules related to the systemic regulation of photosynthesis and those of development of the leaves.

Conclusion

Environmental conditions such as high light, elevated CO_2 and low nitrogen nutrition induce the carbohydrate accumulation in the leaves. This carbohydrate accumulation regulates the photosynthetic activities of the leaves in accordance with the redox state of the photosystems. The carbohydrate and the redox state of photosystems also

induce some intermediate signaling molecules, and these molecules transferred from the leaves to the other leaves in the plant. These signaling molecules repress the photosynthetic activity of the other leaves, regulate the light acclimation of chloroplasts, and maintain the steady carbohydrate production of the plant. These regulations would keep the C/N ratio of the plants at a constant level and thus increase the plant fitness in nature.

treatments	carbohydrate content of primary leaves	redox state of photosystem of the primary leaves	photosynthetic activity of the primary leaves	photosynthetic activity of the trifoliate leaves	light acclimation of the trifoliate leaves
shading-treatment	Low	Oxydized	Low	High	shade-type
1000-treatment	High	intermediate	Low	Low	shade-type
150-treatment	Low	Reduced	High	High	sun-type

Fig. 1 The summery of the results of chapter 3

This table shows the effects of each treatment in chapter 3 on carbohydrate content, redox state of photosystem, and photosynthetic activity of the primary leaves and the photosynthetic acticity of the trifoliate leavse, respectively.

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