Full Length Research Paper

Changes in photosynthesis and activities of enzymes involved in carbon metabolism during exposure to low light in cucumber (Cucumis sativus) seedlings

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Two cucumber genotypes, S404 and S1 with low light-sensitivity and low light-tolerance, respectively were used to investigate the oxygen consumption rate of photosystem I, the oxygen evolution rate of photosystem II, cab transcript levels, and activities of enzymes involved in photosynthetic carbon reduction cycle. The results show that short term (24 h) / long term (five and 10 days) low light stress had significant effect on PSII activities while PSI's effect was not significant. Under the low light stress, S1 cab gene transcript levels were quickly recovered while S404 cab gene transcript levels were slowly recovered. The total dry mass and leaf area of S1 was lower than S404. Low light treatment decreased C₃ photosynthetic carbon cycle enzyme activities involved in ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) carboxylation and fructose-1,6-bisphosphatase (FBPase), and increased C₄ photosynthetic carbon cycle enzyme activities involved in nicotinamide adenine dinucleotide phosphate malate dehydrogenase (NADP-MDH). The NADP-MDH activity in S1 leaves increased significantly compared to S404. These observations suggest that S1 photosynthetic capacity is higher than S404 under low light conditions. Photosynthetic C₄-microcycle possibly would have played a role in low light stress. Therefore, the transcript levels of cab and the involvement of NADP-MDH in low light-resistance need further research.

Key words: Low light, oxygen consumption rate, oxygen evolution rate, cab gene, NADP-MDH.

INTRODUCTION

Photosynthesis is a process that converts light energy to oxygen and carbohydrates. In photosynthesis, a series of redox reactions occur in the electron transport system present in the chloroplast thylakoid membranes. Oxidation of water is catalyzed by photosystem II (PSII), a multi-subunit pigment protein complex located in the thylakoid membrane (Hillier and Babcock, 2001). Low light tolerance is a hereditary trait, where the plant viability under low light conditions corresponds with its ability to achieve high photosynthetic rates (Dymova and Golovko, 1998). Under low light conditions, plants must absorb and capture as much light energy to fix carbon dioxide and accumulate carbohydrates. The growth and development of plants are directly related to its light harvesting ability. The light-harvesting chlorophyll a/b pigment-protein (cab gene encoding) complexes of PSII (LHCII), which occupies approximately 50% of thylakoid chlorophyll, are the most abundant pigment proteins in the photosynthetic membrane. The primary function of LHCII is to harvest light energy and deliver it to the PSII reaction centre (Van Grondelle et al., 1994). Net photosynthetic rate descends with decreasing light intensity. Furthermore, the decreasing level of photosynthetic rate is correlated with tolerance to low light. Under low light conditions, plants having high tolerance
to low light, maintain correspondingly high photosynthetic rate (Ody, 1997; Dymova and Golovko, 1998).

Cucumber (Cucumis sativus) is a classical C₃ plant. Under normal conditions, cucumber follows Calvin-Benson cycle to process carbon assimilation. However, similar partitioning is not absolute for different carbon metabolism types (C₅, C₄, and Crassulacean acid metabolism (CAM)). The pathways involved in the photosynthetic carbon metabolism have adapted to the ecological environment in the course of evolution of plants (Wang and Zhang, 2000). Previous studies have indicated that the activities of photosynthetic carbon cycle enzymes involved in C₄ or CAM metabolism enhanced at various degrees in the C₃ plants, such as spinach, bean (low temperature stress, Holaday et al., 1992), wheatgrass (salt stress, Cushman, 1993), pigeon pea (cadmium and nickel, Sheoran et al., 1990). Casuarina equisetifolia (drought stress, Sánchez-Rodriguez et al., 1997), and pea (CO₂-free air, Harbinson and Foyer, 1991). Li et al. (2001) indicated that the cultivars, which had high enzyme activities involved in C₄ or CAM metabolism were selected and bred in order to improve the photosynthetic rate in C₃ plants. Therefore, in this study we attempted to detect key enzymes (C₃: ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) and fructose-1,6-bisphosphatase (FBPase), C₄: nicotinamide adenine dinucleotide phosphate malate dehydrogenase (NADP-MDH)) involved in low light stress, and investigate the differences in the photosynthetic carbon metabolism in two genotypes of cucumbers having low light sensitive and tolerant cultivars to study the role of C₄ photosynthesis under low light conditions.

Materials and Methods

Plants growth and stress treatment

Cucumber (C. sativus L.cv. S1 and S404) seeds were germinated and grown in controlled growth chamber on substrate containing 50% turf and 50% vermiculite in day/night light temperature at 25±18°C, relative humidity at 50/60%, with day/night regime of 10/14 h and a photon flux density at 800 µmol m² s⁻¹. The nutrient solution (Vinit-Dunand et al., 2002) containing 2.5 mM Ca(NO₃)₂, 4H₂O, 1.0 mM NH₄NO₃, 0.5 mM KH₂PO₄, 0.5 mM MgSO₄·7H₂O, 2.5 mM KCl, 0.064 mM NaFeEDTA, 0.025 mM H₂BO₃, 10.0 mM MnCl₂·4H₂O, 1.5 mM ZnCl₂, 0.5 mM CuCl₂·2H₂O, 0.2 mM Na₂MoO₄, pH 5.2, was supplemented every four days. Stress treatments were carried out after the emergence of three primary leaves. 100 µmol photons m⁻² s⁻¹ was provided by a reflector-type sodium lamp (E40, Mengkai Xia Company, China), at the same time, controls were treated under 800 µmol photons m⁻² s⁻¹.

Short term low light stress

After treatment for 4 h under 800 µmol photons m⁻² s⁻¹, the seedlings were subjected to low light treatment (100 µmol photons m⁻² s⁻¹). Mature leaves (the third leaf under growing point) were excised at 0, 8, 16 and 24 h after the seedlings were exposed to low light illumination, and prepared for thylakoid membrane isolation and total RNA extraction.

Long term low light stress

Mature adult leaves were sampled after 0, five, 10, 15 and 20 days exposure to either 100 µmol m⁻² s⁻¹ photon flux density (PFD) or 800 µmol photons m⁻² s⁻¹. The leaf samples were used for the isolation of thylakoid membrane and measurement of dry weight, leaf area, and activities of enzymes involved in the photosynthetic carbon cycle.

Isolation of thylakoid membranes and determination of chlorophyll concentration

Thylakoid membranes were isolated under dark or green light conditions following a modified method adapted from Yang et al. (2004). Briefly, 10 g of leaf samples were homogenized in 15 mL of grinding buffer (0.4 mM succrose, 2 mM EDTA, 20 mM Tris–HCl and pH 7.8) in a chilled kitchen blender under 4°C. Homogenate was filtered through two layers of Miracloth and the dark green filtrate centrifuged at 3000 rpm for 5 min under 4°C. The supernatant solution was discarded, and the dark green pellet was homogenized in grinding buffer and centrifuged at 3000 rpm for 5 min. The resulting pellet was suspended in suspending buffer (20 mM NaCl, 2 mM (N-morpholino)ethanesulfonic acid (MES), 0.4 M sucrose and pH 6.5). Thylakoid membrane aliquots were frozen in liquid nitrogen, and stored at -80°C for further analysis.

Chlorophyll concentrations in the thylakoid membrane preparations were measured in 80% acetone based on the method described by Arnon (1994).

Measurement of PSI oxygen consumption rates

The oxygen consumption rates of PSI were measured at 25°C using oxygen electrode unit (Hansatech light source power supply Oxy-Lab). The assays were conducted in 800 µmol photons m⁻² s⁻¹ light at 25°C, and in 1 mL of reaction solution containing reaction buffer (50 mM Tricine-NaOH pH 7.5, 0.4 M Sucrose, 10 mM NaCl, 5 mM MgCl₂), freshly added reagents (1 mM NaNO₂, 0.5 mM MV, 10 µM DCMU, 1 mM ascorbic acid, and 200 µM DCPIP), and thylakoid membranes (20 µg chlorophyll ml⁻¹).

Measurement of PSII oxygen evolution rates

The oxygen evolution rates of PSII were measured at 25°C using oxygen electrode unit (Hansatech light source power supply Oxy-Lab). The assays were conducted in 800 µmol photons m⁻² s⁻¹ light at 25°C, and in 1 mL of reaction solution containing reaction buffer (50 mM MES-NaOH pH 6.5, 0.4 M Sucrose, 50 mM CaCl₂), freshly added reagents (0.2 mM 2,6 dichlorobenzoniquinone (DCBQ)), and thylakoid membranes (30 µg chlorophyll ml⁻¹).

Preparation of cab probes

Total RNA was extracted from 200 mg fresh cucumber leaves using Trizol reagent (Gibco, BRL, USA) followed by synthesis of single-strand cDNA using Reverse Transcription System Kit (TIANGEN,
Beijing, China). Single-strand cDNA was subjected to PCR amplification. Primers were designed using published consensus sequence of cucumber *cab*: 5’ GAGAGTCCCCGGTCTGATCTAC- GGGTTG 3’ as the forward primer and 5’AACCTCAACTCA- GCCAAATGCCCT 3’ as the reverse primer. The PCR mixture (50 μl) contained 1 μl template cDNA, 0.2 mM of each dNTP, 1 mM MgSO4, 10 μM of each primer and 1 unit of KOD Plus (TOYOBO, Japan). After pre-denaturing at 94°C for 2 min, PCR amplification was accomplished in 30 cycles at the following temperatures: denaturing at 94°C for 15 s, annealing at 60°C for 30 s and 30 s at 68°C for elongation. At last elongating at 68°C for 10 min. PCR products were cloned into pGEM®-T Easy vector (Promega, USA) and were sequenced.

**Northern blot analysis**

Total RNA for each treatment was extracted from 5 g frozen leaves of the samples using the hot phenol method (Kay et al., 1987). Isolated leaf RNA from low light stressed at different time points was used for RNA gel blot analysis. For Northern blot analysis, total RNA (25 μg) was denatured with 50% formamide and 6.3% formaldehyde and separated on denaturing agarose gels (Sambrook et al., 1989). The RNA was transferred onto Hybond-N+ nylon membrane (Millipore Corporation, USA) and fixed by baking at 80°C (2 h). The cap probes were purified from PCR-amplified fragments of selected clones and labeled with [32P] dCTP using Amersham Rediprime™ II Random Prime Labelling Kit (GE Healthcare, USA) according to the manufacturer's protocol. The membrane (Immobilon™-Ny manual, Millipore, USA) was hybridized with the probe in hybridization buffer (7% SDS, 500 mM sodium-phosphate, pH7.2, 1 mM EDTA, pH 8.0) for 24 h at 65°C. Membranes were then washed four times with 2×SSC and 0.1% (w/v) SDS at room temperature for 15 min, followed by two washes in 0.2× SSC and 0.1% (w/v) SDS at 65°C for 15 min. For detection of the radioactive signals, the filters were exposed to a Fuji medical X-ray film (Fujifilm, Japan). Molecular dynamics scanner was used to detect and quantify signal.

**Growth analysis**

The seedlings were harvested at 0, five, 10, 15 and 20 days after treatment. In all cases, the seedlings were divided into shoots and roots. Immediately after sampling the roots were carefully washed in three successive baths and dried in an oven at 60°C for 48 h and weighed. The leaf area was measured using an automatic leaf area meter (LI-3000, LI-COR, USA).

**Enzyme assays**

NADP-MDH, FBPase, rubisco activities were measured in leaf disc extracts in which metabolism had been stopped by freezing and stored in liquid nitrogen. The leaves were pulsed in liquid nitrogen and the leaf powder was resuspended either in 0.1 M Tricine-KOH buffer (pH 8.0) containing 1 mM dithiothreitol (DTT), 10 mM MgCl2, 1 mM EDTA, and 1% Triton X-100 (v/v) in the NADP-MDH and FBPase extraction buffer described by Harbinson and Foyer (1991). For rubisco assays extraction buffer was 100 mM Bicine-NaOH (pH 7.8) containing 10 mM MgCl2, 10 mM β-thioglycerol glycol, 2% PVP (w/v), 1% BSA (w/v) and 1% Triton X-100 (v/v). An aliquot of the whole extract was taken to determine chlorophyll contents (Arnon, 1954).

NADP-MDH and FBPase were measured as described by Harbinson and Foyer (1991). The assay for rubisco was adapted from that of Wang (2006).

**Statistical analysis**

All the dates were statistically analyzed by Excel and SPSS Software. All treatments are carried with three entire plants and repeated three times.

**RESULTS**

**Growth analysis**

Total dry mass and leaf area of the two cucumber genotypes under control and low light stress conditions were measured and shown in Figures 1A and B, respectively. The S1 total dry mass decreased at 30.2, 32.7, 44.8 and 57.5%, respectively, in low light stress during five, 10, 15, and 20 days compared to the control and for S404 total dry mass decreased at 34.7, 60.8, 67.7 and 71.3%, respectively, compared to the control. This indicated that the effect of low light on total dry mass in S1 was significantly lower than that of S404. The leaf area of S1 was noticeably larger in contrast to S404 in response to low light (Figure 1B). During low light treatment for 15 days, S1 leaf area remained unaffected in comparison to control, and however, after 20 days, it declined to only 34.2%. However, the reduction in the leaf area of S404 was continuous compared to control during the whole low light treatment.

**PSI oxygen consumption rate and PSII oxygen evolution rate**

The PSII oxygen evolution rates (Figures 2C and D) was significantly higher than the PSI oxygen consumption rates (Figures 2A and B) under either short- or long-term light-stress condition. However, no change in PSI oxygen consumption rates was detected during the short-term stress period (Figure 2A). Therefore, it could be concluded that the 24 h exposure to low light in our experiment had no substantial effects on the rates of PSI oxygen consumption.

The periodic trend of PSII oxygen evolution was similar in S1 and S404 during short-term stress (Figure 2C). The oxygen evolution rates ascended and reached maximal values, up to about 174.6% (S1) and 106.7% (S404) in 8 h in contrast to their respective rates under low light stress, after which the rates declined to their minimal values by 16 h, and subsequently rose after 24 h. Nevertheless, the whole trend of oxygen evolution rates in S1 and S404 decreased upon prolonged duration of low light-stress. The decline in the levels of S404 was markedly higher compared to S1.

The PSI oxygen evolution rates of S1 and S404 dropped to 29.4 and 67.6%, respectively compared to the control during the fifth day after long-term low light stress (Figure 2D). On the tenth day of low light stress, S1 decreased to 7.9% compared to the control, while S404 increased by 6.1%. With prolonged low light stress, PSII...
oxygen evolution rates in S404 increased significantly compared to S1.

Effect of low light on the transcript levels of *cab* gene

Polymerase chain reaction analysis indicated that the target product had single band with 422 bp (Figure 3). The influence of low light stress on *cab* gene is shown in Figure 4. The expression of *cab* to low light stress was similar both in S1 and S404. In S1 the transcript levels sharply declined during 8 h of low light stress, and then increased gradually to its highest levels by 24 h period. However, S404 still did not recover to reach its respective levels.

Effect of low light on the enzymes activities of carbon metabolism

Under low light stress conditions, reduction in the rubisco carboxylase activities was observed (Figure 5A). With prolonged stress, rubisco carboxylase activities declined to 10.4 to 19.8% in S1 leaves in comparison to the control. However, those of S404 leaves dropped to 30.6 to 47.2% compared to control. This showed that the S1
leaves maintained higher rubisco carboxylase activities than S404 under low light stress. Furthermore the biosynthesis of photosynthetic products were decreased and restricted in the S404 leaves.

Under low light stress, the variation in the FBPase activities were similar in both S1 and S404 leaves (Figure 5B). During the fifth day of stress treatment, the FBPase activities of S1 and S404 declined to 1.65 and 12.2%, respectively, in relation to the control. During the tenth day of stress, FBPase activities of S1 and S404 increased by 23.3 and 11.7%, respectively compared to the control.

The influence of low light stress on NADP-MDH activities of S1 and S404 leaves are shown in Figure 5C. During five, 10, 15, and 20 days under low light conditions, NADP-MDH activities of S1 leaves increased to 58.2, 45.3, 16.0 and 36.5%, respectively, compared to the control.
Northern blot using a cab-specific probe confirming the translation levels of S1 and S404 under short-term low light stress.

DISCUSSION

It is well known that photosynthesis is one of the major factors affecting plant growth. Low light stress decreases the rate of photosynthesis in plants. In our study, the total dry mass and leaf area in the low-light sensitive line S1 decreased compared to the low light tolerant line S404.

Adaptation to the low-light state occurs through loss of excitonic interaction between antennae of PSI and their reaction-centers. The fluorescence yield remains virtually constant during adaptation to the low-light state, suggesting the possibility of cyclic electron flow around PSII in this state. These showed that these antennae complexes participate directly in the adaptation to low light intensities (Canaani and Malkin, 1984). The substantial changes in the stroma lamellae/grana ratios in chloroplasts receiving low light, possibly as a mechanism for re-establishing optimal PSI/PSII ratios (Mazzuca et al., 2009). In this study, the effect of low light on PSII oxygen evolution was higher than the PSI oxygen consumption. PSII oxygen evolution increased markedly during 8 h of low light and subsequently decreased at 16 h, and then, its activity was reactivated in 24 h. PSII oxygen evolution rate at different time points (hours) in low light-tolerant line S1 was higher than that of low light-sensitive line S404. This indicated that chloroplast photochemical activity in S1 leaves was higher when compared to S404 leaves.

In this study, both PSI oxygen consumption rate and PSII oxygen evolution rate were affected during long-term low light stress. By comparison, low light had pronounced effect on PSII than PSI. After five days under low light, both rates decreased in not only S1 but also in the S404 leaves, and after which the rates in S404 declined compared to S1. This indicates that both photosynthetic reducing capacity (Jordan et al., 2001; Scheller et al., 2001) and photosynthetic driving capacity (Oxborough, 2004) in S404 leaves were inferior than that of S1. After 10 days, PSI oxygen consumption rate in leaves increased and the PSII oxygen consumption rate in S1 reduced in comparison to control. Contrasting results were observed in the S404 leaves. These observations implied that the photosynthetic reducing capacity in S1 leaves was reinforced, and more reactive oxygen species (ROS) were eliminated by any possibility. On the contrary, more ROS were generated in S404 leaves. If not promptly eliminated, they would cause damages to the chloroplast membrane.

As a whole, both PSI and PSII activities were increased in S1 or S404 based on stress duration. This showed both cucumbers varieties were acclimating to low light stress conditions. In order to investigate the complexities involved in the PSII oxygen evolution during short-term low-light stress, the cab gene transcript levels were determined under low light conditions. The results show that the cab gene expression had a correlation with PSII oxygen evolution rates under low light. The cab transcript levels dropped significantly after 8 h under low light, while PSII oxygen evolution rates declined to minimum after 16 h. Later the levels gradually increased at 16 and 24 h. The effect of low light on cab transcript levels must be attributed to the LHCII. This hysteresis phenomenon displayed the relationship of LHCII and PSII reaction center by directly affecting the PSII activities. Since, cab transcript levels in S1 leaves were significantly higher than those of S404, the ability of capturing and utilizing low light in S1 leaves was stronger than that of S404. As a result, the inclusion of cab transcript level as an evaluation index of low-light resistance should be resolved after further analysis.

Calvin cycle is also known as photosynthetic carbon cycle. Apart from supplying the energy for CO₂
assimilation, light also regulated the activities of photosynthetic enzymes. Rubisco and FBPase are two key light regulatory enzymes in the photosynthetic carbon reduction cycle. Under the normal light intensity and temperature condition, high electron flow rate is propitious to the regeneration of ribulose-1,5-bisphosphate (RuBP), and could improve the activities of NADP-MDH and FBPase, followed by enhanced photosynthetic rate (Harbinson et al., 1990). Rubisco, stromal FBPase and NADP-MDH activities depends on the reducing ability of the electron transport machinery (Holaday et al., 1992).

Low light induces the decrease in the net photosynthetic rate, and increase in the intercellular CO₂ concentration. The principal factor that caused the decline of photosynthesis under the low light is the non-stomatal restriction (Li et al., 2008; Krall and Edward, 1991; Allen and Ort, 2001). In this experiment, Rubisco carboxylation activity in the leaves of two cucumber genotypes decreased under the low light. This showed that low light strengthened the restriction of carboxylation. The regeneration capacity of RuBP could reflect the state of the photosynthetic electron transport and photophosphorylation since the regeneration of RuBP depended on the production of ATP and NADPH which came from photosynthetic electron transport and photophosphorylation (Shen et al., 1998). Under low light conditions, photosynthetic carbon assimilation in different varieties had various restriction factors (Sui, 2006). In S1, the non-stomatal restriction and the decrease in RuBP regeneration rate were affected, while in S404, the decline of rubisco carboxylation activity or rubisco content could be affected. The effect of low light stress on FBPase activity was intricate on the 5th day of low light treatment, the activity of FBPase decreased in the leaves of two cucumber lines, subsequently it increased by the 10th day. This was likely due to an immediate response for maintaining carbon metabolism balance in the plant. Then the FBPase levels gradually dropped again. In general, low light stress induced the decrease of FBPase activity. In the photosynthetic carbon cycle, the influence of low light on activities of rubisco carboxylation and FBPase indicated that low light stress decreases C₃ photosynthetic carbon assimilation. During long-term stress, it is still debatable if the C₃ plants had C₄ photosynthetic pathway. Scheibe et al. (1990) showed that NADP-MDH catalyzed the conversion of oxalic acid (OAA) into malic acid (MA), which exists comprehensively in the nature. Therefore was considered that NADP-MDH in C₃ carbon metabolism had similar role compared to the C₄ metabolism. In recent years, it was reported that angiosperms such as Hydrilla verticilata (Magnin et al., 1997) and Egeria densa (Casati et al., 2000) did not posses Kranz structure, but exhibited C₄ photosynthetic metabolism. Therefore it is possible that individual photosynthetic cells in leaves could process a C₄-microcycle. Similarly, C₄ photosynthetic enzyme system had been found in the leaves of C₃ crops such as soybean (Li et al., 2001), wheat (Hata and Matsuoka, 1987) and rice (Wang et al., 2002). As a result of low

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**Figure 5.** Effect of low light on the enzymes activities of carbon metabolism in cucumber seedlings leaves. (A) rubisco carboxylase activities, (B) FBPase activities, (C) NADP-MDH activities. Values are mean±SE (n=3).
enzyme activity it was presumed that limited C₄ photosynthetic approach may exist in C₃ plants. Chen and Ye (2001) showed that OAA or MA, which was produced by the photosynthetic machinery, could improve the photosynthetic capacity in the leaves of spinach, a C₃ plant. This validates that the leaf cells of C₃ plant could have a turnover of C₄ microcycle. In order to prove the presence of C₄ microcycle and its physiological function, Li et al. (2001) fed low concentrations OAA or MA, which boosted the photosynthetic activity in wild rice.

In this study, the NADP-MDH activities in two cucumber leaves, gradually increased with plant growth and development. However, NADP-MDH activities were enhanced under low light stress in comparison with the control. This indicated that low light stimulated C₄ photosynthetic carbon assimilation microcycle. Although the increase was not significant, the observation could not be neglected under low light condition. Further studies are required to determine the role low light-tolerance to C₄ photosynthetic dominance and NADP-MDH activity to be used as evaluation indices. Low light tolerance of cucumber is a complicated phenomenon which needs further experimental validation to identify new evaluation index.

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