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High Level Expression of C_4-Specific NADP-Malic Enzyme in Leaves and Impairment of Photoautotrophic Growth in a C_3 Plant, Rice

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The chloroplastic NADP-malic enzyme (NADP-ME) is a key enzyme of the C_4 photosynthesis pathway in NADP-ME type C_4 plants such as maize. To express the chloroplastic NADP-ME in leaves of a C_3 plant, rice, full-length cDNAs encoding the rice C_3-specific isoform and the maize C_4-specific isoform of the enzyme were expressed under the control of the rice *Cab* promoter. Transformants carrying the rice cDNA showed the NADP-ME activities in the leaves less than several-fold that of non-transformants, while those carrying the maize cDNA showed activities up to 30-fold that of non-transformants or about 60% of the NADP-ME activity of maize leaves. These results indicate that expression of the rice C_3-specific NADP-ME is suppressed at co- and/or post-transcriptional levels by some regulation mechanisms intrinsic to rice, while that of the foreign C_4-specific isoform can escape from such suppression. The accumulation of the maize C_4-specific NADP-ME led to bleaching of leaf color and growth hindrance in rice plants under natural light. These deteriorative effects resulted from enhanced photoinhibition of photosynthesis due to an increase in the level of NADPH inside the chloroplast by the action of the maize enzyme.

Key words: C_4 photosynthesis — Maize — NADP-malic enzyme (EC 1.1.1.40) — Photoinhibition — Rice — Transformation.

Abbreviations: CAM, Crassulacean acid metabolism; DTT, dithiothreitol; NADP-MDH, NADP-malate dehydrogenase; NADP-ME, NADP-malic enzyme; OAA, oxaloacetate; PEPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate, orthophosphate dikinase; PPFD, photosynthetically active photon flux density.

Introduction

Terrestrial plants are classified into C_3, C_4 and Crassulacean acid metabolism (CAM) plants, according to the mechanism of their photosynthetic carbon assimilation. C_3 plants, which include major crops such as rice and wheat, assimilate CO_2 through the C_3 photosynthetic pathway, also known as the Calvin cycle. C_4 and CAM plants possess a unique photosynthetic pathway in addition to the C_3 pathway, and it is considered that they evolved from C_3 plants in response to changes in environmental conditions that caused a decrease in CO_2 availability. CAM plants such as stonecrops and cactus adapt to extreme arid conditions but their photosynthetic capacity is very low (Black 1973). By contrast, C_4 plants such as maize and sugarcane adapt to high light, arid and warm environments, and achieve high photosynthetic capacity, and high water and nitrogen use efficiencies, by means of the C_4 photosynthetic pathway that acts to concentrate CO_2 at the site of the reactions of the C_3 pathway (Hatch 1987). The transfer of C_4 traits to C_3 plants is thus one strategy being adopted for improving the photosynthetic performance of C_3 plants.

The C_4 pathway consists of three key steps: (i) the initial fixation of CO_2 in the cytosol of the mesophyll cells by phospho*enol*pyruvate carboxylase (PEPC) to form a C_4 acid, (ii) decarboxylation of a C_4 acid in the bundle sheath cells to release CO_2, and (iii) regeneration of the primary CO_2 acceptor phospho*enol*pyruvate in the mesophyll cell chloroplasts by pyruvate, orthophosphate dikinase (PPDK) (Hatch 1987). From the decarboxylating enzyme in the bundle sheath cells, C_4 plants are classified into three subtypes. Maize and sugarcane use NADP-malic enzyme (NADP-ME) for the decarboxylation and these are classified as the NADP-ME type (Hatch 1987).

NADP-ME (EC 1.1.1.40) catalyzes the decarboxylation of malate as follows:

$$\text{L-malate} + NADP^+ \leftrightarrow \text{pyruvate} + NADPH + CO_2.$$

It acts in a wide range of metabolic pathways in both plants and animals. In mammalian liver, it is a major enzyme that generates NADPH for lipogenic pathways (Edwards and Andreo 1992). In plants, two different forms, namely, the cytosolic and chloroplastic forms are present, and they play distinct roles. A variety of functions are proposed for the cytosolic form: it acts as a pH stat in combination with PEPC, furnishes NADPH and

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pyruvate for catabolic and anabolic pathways, and converts NADH to NADPH during glycolysis together with PEPC and NAD-malate dehydrogenase (Edwards and Andreo 1992). It is also suggested that the cytosolic form participates in defense reactions and/or stress responses (Schaaf et al. 1995, Casati et al. 1999). In the NADP-ME type CAM plants, this form acts as the decarboxylating enzyme to donate CO_2 to the C_3 pathway (Cushman and Bohnert 1997). The chloroplastic form is well known for its role in C_4 photosynthesis. In the NADP-ME type C_4 plants, it is located in the bundle sheath cells and acts as the decarboxylating enzyme (Hatch 1987).

It was previously considered that the chloroplastic form of NADP-ME was absent in leaves of C_3 plants. Recent analyses have demonstrated that the gene is expressed in leaves of C_3 plants (Lipka et al. 1994) and that the enzyme protein is present inside the chloroplasts of C_3 plants, albeit at a very low level (Maurino et al. 1997, Drincovich et al. 1998). It has been demonstrated in *Flaveria* species that both C_3 and C_4 species have two genes for the chloroplastic NADP-ME, namely, *Me1* and *Me2* for the isoforms specific to C_4 photosynthesis and housekeeping function, respectively (Marshall et al. 1996). *Me1* is expressed in leaves of *Flaveria* species in which C_4 photosynthesis is operative and its expression parallels the degree of C_4 photosynthesis, while *Me2* is constitutively expressed in both roots and leaves at low levels in C_3 and C_4 species (Marshall et al. 1996). The chloroplastic enzymes derived from *Me1* and *Me2* are thus designated C_4- and C_3-specific isoforms, respectively. Like the cytosolic form of NADP-ME, the chloroplastic form appears to participate in stress responses, since its expression is enhanced by anaerobic stress in rice plants (Fushimi et al. 1994).

We are investigating conditions to express key enzymes of the C_4 pathway (C_4 enzymes) from maize in a C_3 plant, rice, at high levels. Until now, PEPC and PPDK could be accumulated at significant levels in leaves of transgenic rice plants by introduction of the intact maize gene (Ku et al. 1999, Fukayama et al. 1999). Here we report that expression of cDNA encoding the chloroplastic NADP-ME of maize but not rice under the control of the rice *Cab* promoter leads to high level expression of the enzyme protein in the chloroplasts of rice leaves. We also found that accumulation of the maize enzyme impairs photoautotrophic growth of rice plants by stimulating photoinhibition of photosynthesis.

Materials and Methods

cDNA cloning, constructs and transformation of rice

A cDNA library was constructed in λZAPII (Stratagene, CA, U.S.A.) from a poly $(A)^+$ RNA fraction prepared from maize (*Zea mays* L. cv. Golden Cross Bantam) seedlings. To obtain a hybridization probe for screening of the cDNA library, a DNA fragment encoding an N-terminal part of the maize C_4-specific NADP-ME was amplified by PCR on the basis of the nucleotide sequence published previously (Rothermel and Nelson 1989) using maize genomic DNA as a template. Primers used were 5′-GAACTCGACGCCACCATGCTGTC-3′ and 5′-CTTGCGACGGAGGTGGCCCA-3′. The nucleotide sequence of the resultant PCR product was essentially the same as the published sequence with a few nucleotide substitutions which might result from the difference in cultivar used. Using this PCR product, the maize cDNA library was screened. Among positive clones, that of the longest insert of 2.2 kb was sequenced and it was confirmed that it contained the full-length cDNA of the maize C_4-specific NADP-ME. The full-length cDNA encoding the rice chloroplastic NADP-ME of 2.4 kb (Fushimi et al. 1994) was kindly provided by Prof. H. Uchimiya, the University of Tokyo. These cDNAs were each fused to the rice *Cab* promoter (–789 to +59 relative to the transcription initiation site; Sakamoto et al. 1991) and the terminator of the nopaline synthase gene of 260 bp, and cloned into a binary vector pIG121Hm containing a hygromycin resistance gene (a generous gift from Prof. K. Nakamura, Nagoya University). The resultant plasmids were introduced into calli derived from rice (*Oryza sativa* L. cv. Kitaake) using *Agrobacterium*-mediated transformation. Transgenic plants were regenerated from hygromycin-resistant calli and planted in soil.

Plant growth conditions

Rice and maize were planted in soil and vermiculite, respectively, and grown under natural light conditions in a temperature-controlled greenhouse at day and night temperatures of 27 and 22°C, respectively. When indicated, rice plants were germinated on wet filter paper, transplanted onto Murashige-Skoog medium containing 3% (w/v) sucrose and 0.4% (w/v) Gellan Gum (Wako, Kyoto) in one-liter beakers of 10 cm in diameter (one plant per beaker), and grown in a growth chamber at 27°C day/22°C night cycle with a day period for 14 h under illumination with white light at photosynthetically active photon flux density (PPFD) of 20–30 $\mu mol\ m^{-2}\ s^{-1}$.

Extraction of leaf soluble protein

Segments of about 3 cm were harvested from the mid-section of the uppermost fully expanded leaves and immediately frozen in liquid nitrogen until use. Samples were ground using a chilled mortar and pestle in an extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 5% insoluble polyvinylpolypyrrolidone and 10% (w/v) glycerol, with a small amount of sea sand. After total maceration, the homogenate was centrifuged at 15,000×*g* for 10 min and the resultant supernatant was collected as a total leaf soluble protein extract. All these procedures were performed at 0–4°C.

Enzyme assays

The NADP-ME activity was assayed by two different protocols. For screening of the primary transgenic rice plants, the activity was measured by protocol 1 as follows. The leaf soluble protein extract of around 1 mg protein ml^{-1} was diluted 20-fold with an assay medium containing 25 mM HEPES-KOH (pH 8.0), 0.1 mM EDTA, 2 mM $MgCl_2$ and 0.5 mM $NADP^+$, and the enzyme reaction was started by adding 1/20 vol. of 100 mM malate (pH 7.0 with NaOH) to give a final concentration of 5 mM. In protocol 2, the extract was desalted by passing through a gel filtration column (NAP-5, Amersham/Pharmacia, U.K.) which had been equilibrated with 50 mM HEPES-KOH (pH 7.4), 0.1 mM EDTA, 5 mM DTT, 10 µM leupeptin and 10% (w/v) glycerol. The resultant eluate was diluted 20-fold with an assay medium containing 25 mM Tricine-KOH (pH 8.3), 0.1 mM EDTA, 5 mM malate and 0.5 mM $NADP^+$. The reaction was started by adding 1/20 vol. of 200 mM $MgCl_2$ (pH 8.3 with NaOH) to give a final concentration of 10 mM (Ashton et al. 1990). In both protocols, the reaction was performed at 30°C and the reduction of $NADP^+$ was monitored by absorbance at 340 nm. The NADP-ME activities of

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maize and non-transgenic rice measured by protocol 1 were 0.250–0.300 and 0.020–0.040 µmol $NADP^+$ reduced (mg protein)$^{-1}$ min^{-1}, respectively, and those measured by protocol 2 were 2.20–2.50 and 0.030–0.050 µmol $NADP^+$ reduced (mg protein)$^{-1}$ min^{-1}, respectively.

The activation level of NADP-malate dehydrogenase (NADP-MDH) was determined by the method of Faske et al. (1997) with slight modifications. A leaf segment of about 1 cm^2 was harvested and immediately frozen in liquid nitrogen. The frozen leaf segment was ground using a chilled mortar and pestle in 0.1 ml of an extraction buffer containing 90 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 5 mM DTT and 10% (w/v) glycerol, which had been degassed by sonication. The homogenate was centrifuged at 15,000×*g* for 2 min and a portion of the resultant supernatant (around 2.5 mg protein ml^{-1}) was diluted 25-fold with an assay medium containing 90 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 70 mM KCl and 1 mM DTT. The reaction was started by adding 1/100 vol. of 200 mM oxaloacetate (OAA) to give a final concentration of 2 mM. The reaction was performed at 30°C and the oxidation of NADPH was monitored by absorbance at 340 nm. The NADP-MDH activity determined in this way was taken as in vivo activity. For determination of maximal potential activity of NADP-MDH, another portion of the supernatant was supplemented with 1/10 vol. of 1.0 M DTT, incubated under nitrogen gas stream at 25°C for 40 min, and then the activity was measured as above.

Analytical procedures

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. SDS-PAGE was carried out by the method of Laemmli (1970) and the gel was stained with Coomassie brilliant blue R-250. For N-terminal amino acid sequencing, polypeptides separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane (Millipore, MA, U.S.A.) and stained with Coomassie brilliant blue R-250. The stained bands were cut out and subjected to sequencing using a gas-phase protein sequencer (477A, Perkin Elmer, NJ, U.S.A.). Immunoblotting was performed as reported previously (Miyao 1994) using an antiserum raised against the recombinant maize C_4-specific NADP-ME produced as follows. The entire coding region of the maize C_4-specific NADP-ME cDNA was inserted into the expression vector pET28b (Novagen, WI, U.S.A.) and expressed in *Escherichia coli* BL21(DE3) cells. The recombinant protein was purified by SDS-PAGE and electroelution, and used to immunize rabbits.

Gas exchange was measured at a leaf temperature of 25°C, 360 µl liter^{-1} CO_2, 21% O_2 and a leaf-to-air vapor pressure difference of 1.2 kPa, using an open gas exchange system (LI-6400, Li-Cor, NE, U.S.A.). Illumination was obtained from light emitting diodes (470 and 665 nm, Li-Cor). The light intensity was increased stepwise and the steady-state rate of CO_2 uptake was measured at each light intensity. Chlorophyll fluorescence was measured at 25°C after dark adaptation of plants for 30min using a modulation fluorometer PAM101–103 (Walz, Germany) equipped with a light source for saturating pulses (KL 1500, Schott, Germany). Minimal fluorescence (F_0) was measured with modulated weak red light (1.6 kHz; 650 nm), variable fluorescence (F_v) with red actinic light (650 nm; 80 µmol m^{-2} s^{-1}), and maximal fluorescence (F_m) was induced with a saturating white light pulse (5,600 µmol m^{-2} s^{-1}; 0.3 s). Chlorophyll content was optically monitored with a chlorophyll meter (SPAD-502, Minolta, Tokyo).

Results and Discussion

Expression of NADP-ME in transgenic rice plants

To express the chloroplastic NADP-ME in the mesophyll cells of rice leaves, full-length cDNAs encoding the rice enzyme and the maize C_4-specific isoform were expressed under the control of the rice *Cab* promoter, which directs the mesophyll-specific and light-regulated expression in leaves of C_3 plants (Sakamoto et al. 1991). Since the gene corresponding to the rice cDNA used in this study is expressed constitutively in rice plants (Fushimi et al. 1994), it is likely that the cDNA encodes the C_3-specific isoform of NADP-ME. The level of expression of the introduced gene was screened by assaying the activity of NADP-ME in a leaf soluble protein extract of the primary (T_1) transformants. To simplify the assay procedure, the extract was directly subjected to the assay by protocol 1 (see Materials and Methods). With this protocol, however, the assay could be interfered by the reaction of NADP-malate dehydrogenase (Ashton et al. 1990) and the activity could be underestimated when the malate concentration of the extract was high.

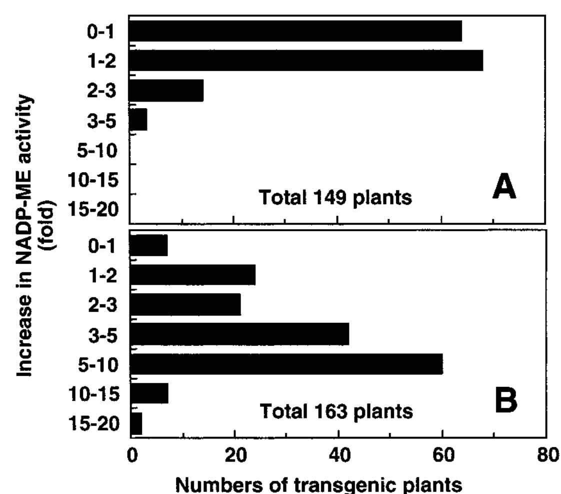

Fig. 1 The activities of NADP-ME of leaves of the primary (T_1) transgenic rice plants. (A) Transformants introduced with the rice C_3-specific NADP-ME cDNA fused to the rice *Cab* promoter. (B) Transformants introduced with the maize C_4-specific NADP-ME cDNA fused to the rice *Cab* promoter. The leaf soluble protein extracts were directly subjected to the assay of NADP-ME activity by protocol 1 (see Materials and Methods). The enzyme activities, determined on the protein basis, were expressed as fold increases relative to that of non-transgenic rice. The maize activity was around 10-fold over that of non-transgenic rice.

Transformants carrying the rice cDNA showed the NADP-ME activities less than several-fold that of non-transformants (Fig. 1A). By contrast, transformants carrying the maize cDNA showed a wide range of activities (Fig. 1B). A majority showed the activities ranged from two- to ten-fold, while nine out of 163 plants showed higher activities up to 18-fold that of non-transformants. SDS-PAGE revealed that the NADP-ME activities of these transformants were correlated well with the level of a polypeptide of about 60 kDa (Fig. 2A).

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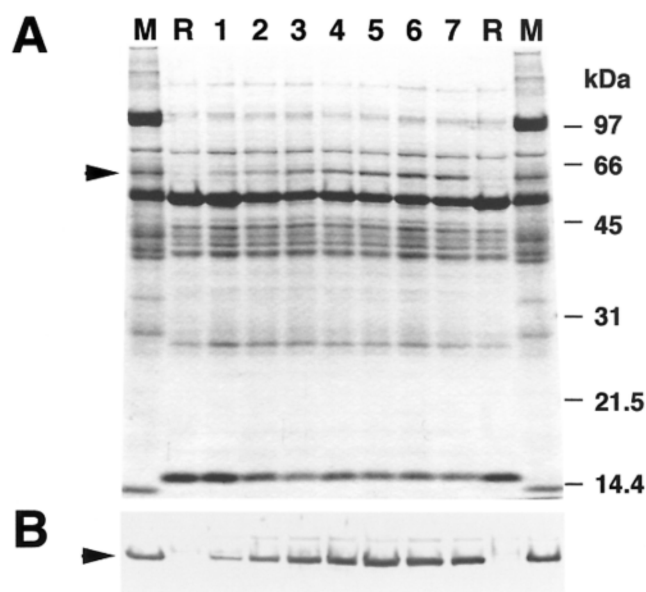

Fig. 2 Accumulation of the NADP-ME protein in leaves of the primary (T_1) transgenic rice plants introduced with the maize C_4-specific NADP-ME cDNA fused to the rice *Cab* promoter. The leaf soluble protein extracts were analyzed by SDS-PAGE (3 µg protein per lane). (A) Polypeptide profiles after Coomassie staining. (B) Immunoblot profiles with the antibody specific to the maize C_4-specific NADP-ME. M, maize; R, non-transgenic rice; 1–7, transgenic rice plants with increasing activities of NADP-ME. The activities of transformants relative to that of non-transgenic rice, assayed by protocol 1, were 3.8 to 17.6 from lane 1 through lane 7. Numbers on the right side of panel A represent the positions of molecular size markers. Arrowheads indicate the position of the band of the NADP-ME protein.

In the transformant of lane 7 with 18-fold activity, the level of the 60-kDa polypeptide accounted for several percent of total leaf soluble protein. As judged from the apparent molecular mass and the cross-reactivity with the antibody specific to the maize NADP-ME (Fig. 2B), the 60-kDa polypeptide corresponds to the maize NADP-ME protein. In transformants carrying the rice cDNA, by contrast, the increase of a polypeptide band in the corresponding position of the gel was barely detected after Coomassie staining, though a faint band could be detected by immunoblotting (data not shown).

As described above, the activity of NADP-ME assayed by protocol 1 could not be accurate. The activities assayed by two different protocols were compared using leaves of a progeny of a transformant carrying the maize cDNA. A transgenic line used was ME210, of which NADP-ME activity of the primary (T_1) transformant (lane 1 of Fig. 2) assayed by protocol 1 was 3.8-fold. The segregation ratio of the level of the NADP-ME protein, determined by SDS-PAGE of young seedlings of T_2 generation, indicated that this line has the transgene inserted in a single locus of the genome. The NADP-ME activity of the heterozygous progeny was 3.8-fold that of non-transformants or 33% of the maize activity when assayed by protocol 1, while it was 6.4-fold that of non-transformants or 12% of the maize activity when assayed by protocol 2. Thus, the NADP-ME activities of the primary transformants assayed by protocol 1 were underestimated in terms of a fold increase over that of non-transformants, while they were overestimated in terms of a percent of the maize activity. The accurate activity of NADP-ME of the primary transformant with the highest activity (lane7 of Fig. 2) would be around 30-fold that of non-transformants or 60% of the maize activity, since its level of the NADP-ME protein was about five times as high as that of ME210 in lane 2 (Fig. 2).

The N-terminal amino acid sequence of the maize NADP-ME protein in the leaves of the transformants was AVAMVS. This completely matched with residues from Ala60 to Ser65 of the sequence deduced from the nucleotide sequence of cDNA of the maize C_4-specific NADP-ME (Rothermel and Nelson 1989). The N-terminal region of 59 amino acid residues of the deduced sequence shares features characteristic to the transit peptide for targeting into chloroplasts (Rothermel and Nelson 1989). Thus it is likely that the maize enzyme was located inside the chloroplasts of transgenic rice leaves.

The above observations clearly show that expression of the maize but not rice cDNA under the control of the rice *Cab* promoter is effective in accumulating NADP-ME inside the chloroplasts of rice leaves. This finding, however, was a surprise to us, since the rice and maize cDNAs are highly homologous with each other. Their nucleotide sequences show a similarity of 80% and their deduced amino acid sequences including the transit peptide portion are 80% identical (Fushimi et al. 1994). In addition, they were driven by the same promoter in transgenic rice, and therefore, their transcriptional activities would be the same. It is likely that expression of the rice enzyme was suppressed at co- and/or post-transcriptional levels by some mechanisms intrinsic to rice, and that expression of the maize enzyme could escape from such suppression.

High level expression of NADP-ME in transgenic rice by introduction of the maize cDNA contrasts with previous observations with other C_4 enzymes that expression of cDNAs under the control of strong promoters, such as *Cab*, *rbcS* and cauliflower mosaic virus 35S promoters, did not significantly increase the enzyme activity and the protein level of PEPC (Hudspeth et al. 1992, Kogami et al. 1994, Gehlen et al. 1996) and of PPDK (Ishimaru et al. 1997, Ishimaru et al. 1998, Sheriff et al. 1998, Fukayama et al. 1999). The reason for such a difference is obscure at present. It might reflect the difference in the evolutionary scenario of the C_4-specific gene between the C_4 enzymes located in the mesophyll cells in C_4 plants such as PEPC and PPDK and those located in the bundle sheath cells such as NADP-ME (see Ku et al. 1996).

Effects of high level expression of NADP-ME on rice plants

The accumulation of the maize C_4-specific NADP-ME inside the chloroplasts showed serious deteriorative effects on

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the growth of rice plants. The primary transformants regenerated from calli were first grown photoheterotrophically in the presence of sucrose under weak light conditions (PPFD of the day period of 20–30 µmol m^{-2} s^{-1}). During this stage, they grew normally and their leaf color was bright green. After they were transplanted in soil and grown photoautotrophically under natural light conditions, however, leaf color was gradually bleached and growth was suppressed. The extents of bleaching and stunt were more pronounced with increasing activities of NADP-ME in leaves. In transformants with the activities of some-fold, their leaf color faded slightly just after transfer to photoautotrophic growth, but they continued to grow almost normally and set seeds, though the final plant height was about 70–80% of that of non-transformants. In transformants with the activities above ten-fold, their leaf color turned faded greenish yellow, the final plant height was less than half of that of non-transformants, and they were sterile. The rate of CO_2 assimilation also decreased with increasing activity of NADP-ME (data not shown). Together with these symptoms, the level of total leaf soluble protein on the leaf area basis (not shown) and the level of Rubisco per total leaf soluble protein were also decreased (Fig. 2A). Preliminary experiments indicated that malate content was much higher in bleached leaves of the transformants than in normal leaves of non-transformants, a typical feature of plants under stress conditions (Lance and Rustin 1984).

These phenomena were also observed in their progenies of T_2 generation. When progenies of ME210 were grown photoheterotrophically under weak light conditions, they grew normally and the polypeptide profiles of total leaf soluble protein were almost the same except the band of the maize NADP-ME protein (data not shown). After transfer to photoautotrophic growth, their leaf color faded and the growth was stunted (Fig. 3), and the level of Rubisco in total leaf soluble protein was also reduced as observed in the primary transformants (data not shown). These symptoms were more pronounced in the homozygous transformants than in the heterozygous transformants.

In transformants carrying the rice cDNA, by contrast, such deteriorative effects of the introduced gene were not observed even if the NADP-ME activity was five-fold that of non-transformants. This difference would result from differences in kinetic characteristics between the C_3- and C_4-specific isoforms. At pH around 7.5, V_m of the C_3-specific isoform of wheat is less than 10% of that of the C_4-specific isoform of maize, and the K_m values for malate and $NADP^+$ of the wheat C_3-specific isoform are five to ten times as high as those of the maize C_4-specific isoform (Casati et al. 1997). It is thus likely that the in vivo activity of the maize enzyme was much higher than that of the rice enzyme when their protein levels were the same.

Effects of accumulation of the maize C_4-specific NADP-ME on photosynthesis were investigated using rice plants grown photoheterotrophically under weak light conditions. After growth under these conditions for one month, plant heights and chlorophyll contents of leaves of the transformants were almost comparable to or slightly lower than those of non-transformants. The F_v/F_m value and the effective quantum yield of photosynthetic electron transport, monitored by chlorophyll fluorescence according to Genty et al. (1989), were lower by 5–10% in the transformants than in non-transformants (data not

Fig. 3 Effects of accumulation of the maize C_4-specific NADP-ME in leaves on growth of rice plants. Rice plants were grown photoheterotrophically in the presence of sucrose in a growth chamber with PPFD during the day period of 20–30 µmol m^{-2} s^{-1} for 16 d, then planted in soil and further grown in a greenhouse under shaded conditions for 1 month. Under these conditions, PPFD at noon on sunny days was around 200 µmol m^{-2} s^{-1}. (a) Non-transgenic rice; (b) and (c) transgenic rice plants introduced with the maize C_4-specific NADP-ME cDNA fused to the rice *Cab* promoter. Plants of (b) and (c) were heterozygous and homozygous progenies of T_2 generation, respectively, of the same primary transformant (ME210) of which activity of NADP-ME assayed by protocol 1 was 3.8-fold. The NADP-ME activities of these progenies after photoautotrophic growth were 6.4- and 23-fold over that of non-transformants or 12 and 45% of the maize activity, when assayed by protocol 2.

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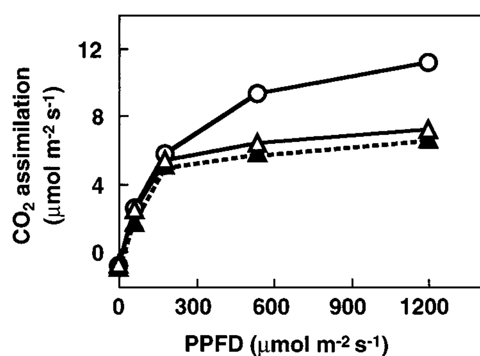

Fig. 4 Light-intensity dependence of CO_2 assimilation rate of rice plants. Rice plants were grown photoheterotrophically under weak light conditions as in the legend for Fig. 3 for 1 month. The fully-expanded fifth leaves were used for gas-exchange measurements at 25°C, 360 μl liter^{-1} CO_2 and 21% O_2. Open circles, non-transgenic rice; open and closed triangles, heterozygous and homozygous progenies of T_2 generation, respectively, of ME210.

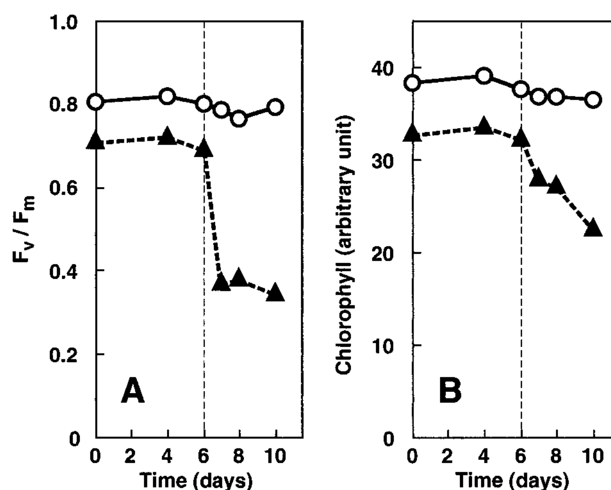

Fig. 5 Effects of illumination with strong light on F_v/F_m and chlorophyll content of rice leaves. Rice plants were grown photoheterotrophically under weak light conditions as in the legend for Fig. 3 for 1 month, until the fifth leaves had been fully expanded. Plants were then planted in soil (day 0), further grown under weak light conditions for 6 d, and then PPFD during the day period was increased to 500 μmol m^{-2} s^{-1} on day 7. Chlorophyll fluorescence and chlorophyll content of the mid-portion of the fifth leaf were measured at 17:00. Averages of two and five measurements were presented for F_v/F_m and chlorophyll content, respectively. Standard deviations of results were less than 0.04 and 1.0 for F_v/F_m and chlorophyll content, respectively. Open circles, non-transgenic rice; closed triangles, homozygous progeny of T_2 generation of ME210.

shown). This was also the case when plants were transplanted in soil but kept under weak light conditions. Thus, it is likely that photosystems and photosynthetic electron transport of the transformants were not significantly affected when grown under weak light conditions.

A significant difference was observed in the light-intensity dependence of the rate of CO_2 assimilation (Fig. 4). At PPFD up to 200 μmol m^{-2} s^{-1}, the rate steeply increased with increasing PPFD in almost the same way in all plants examined. At PPFD above 200 μmol m^{-2} s^{-1}, however, the rate of non-transformant further increased with increasing PPFD while those of the transformants increased only slightly. These observations indicate that light energy becomes more excessive for the transformants than non-transformant under illumination with strong light, and suggest that the transformants are more susceptible to photoinhibition of photosynthesis.

This hypothesis was examined by monitoring the chlorophyll fluorescence parameter F_v/F_m and the chlorophyll content of leaves during growth under strong light conditions. The F_v/F_m value is a sensitive indicator of photoinhibition of photosynthesis (Björkman and Demmig 1987) and the decrease in chlorophyll content reflects photooxidative damage following the photoinhibition (Powles 1984). Rice plants which had been grown photoheterotrophically under weak light conditions were planted in soil, and PPFD during the day period was increased to 500 μmol m^{-2} s^{-1}. As shown in Fig. 5A, the F_v/F_m value of the transformant drastically declined just after transfer to strong light conditions and then continued to decrease gradually, while that of non-transformant decreased only slightly for 2 d and then recovered afterward. The chlorophyll content also showed similar changes (Fig. 5B). It decreased gradually after transfer to strong light conditions in the transformant but remained unchanged in non-transformant. Thus, it is evident that accumulation of the maize NADP-ME inside the chloroplasts renders photosynthesis more susceptible to photoinhibition and the following photooxidative stress. The photooxidative stress causes irreversible damage to the photosynthetic machinery (Powles 1984). The decrease in the levels of Rubisco and total leaf soluble protein, observed in the transformants grown under natural light conditions, likely resulted from such photooxidative damage.

In general, the photoinhibition occurs when light energy absorbed by the light-harvesting complexes exceeds consumption of reducing power generated by photosystem I (PSI) through the Calvin cycle reactions, photorespiration and the reduction of oxygen at the acceptor side of PSI (Osmond and Grace 1995, Asada 1999). Since NADP-ME catalyzes the reduction of NADP$^+$ to NADPH, it is likely that the enzyme increases the level of NADPH in the chloroplast stroma to stimulate the photoinhibition. This hypothesis was examined by measuring the activation level of NADP-MDH, an indirect measure of the NADPH/(NADP$^+$ + NADPH) ratio in the stroma (Scheibe 1987, Fridlyand et al. 1998). The activation level was measured under growth light conditions (PPFD of 30 μmol m^{-2} s^{-1}) and after illumination at PPFD of 100 μmol m^{-2} s^{-1} for 10 min. As shown in Table 1, the activation level was increased with increased light intensity in both the trans-

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Table 1 The activation level of NADP-MDH under illumination

Plant	PPFD (μmol m^{-2} s^{-1})	NADP-MDH activity [a]		Activation (%)
		In vivo	Maximal	
Non-transgenic rice	30	0.030	0.121	24.8
	100	0.035	0.127	27.6
		0.052	0.165	31.5
		0.045	0.096	46.9
ME210 (homozygous)	30	0.037	0.094	39.4
	100	0.069	0.119	58.0
		0.111	0.128	86.7
		0.130	0.148	87.8

Rice plants were grown photoheterotrophically under weak light conditions as in the legend for Fig. 3 for 3 weeks. The fully expanded fourth leaves were harvested under growth light conditions (PPFD of 30 μmol m^{-2} s^{-1}) or after illumination with white light at PPFD of 100 μmol m^{-2} s^{-1} at 25°C for 10 min, immediately frozen in liquid nitrogen under illumination, and subjected to the assay of NADP-MDH activity. The activation level represents the ratio of in vivo activity to maximal potential activity.

[a] μmol NADPH oxidized (mg protein)$^{-1}$ min^{-1}.

formants and non-transformants, an indication that increases in the level of NADPH were detectable under our experimental conditions. Although deviations were large, it was evident that the activation level was higher in the transformants than in non-transformants under both illumination conditions. The difference was small or negligible under growth light conditions while it was marked after illumination with a stronger light (Table 1). These observations clearly indicated that the level of NADPH in the stroma was higher in the transformants than in non-transformants under illumination, and that the difference in the level of NADPH was larger at higher light intensities.

According to Fridlyand et al. (1998), the rate of export of reducing equivalents from the chloroplast by the malate-OAA shuttle can be increased with increasing level of NADPH to reach a maximum level of around 80 μmol (mg Chl)$^{-1}$ h^{-1}, which corresponds to about 40% of the maximum rate of CO_2 assimilation, in spinach grown under normal light conditions. From the CO_2 assimilation rate of rice plants in this study (6 μmol m^{-2} s^{-1}; see Fig. 4) and the content of soluble protein per leaf area of 6 g m^{-2} (a standard value for rice leaf blade), the maximum export rate of reducing equivalents calculated as 40% of the CO_2 assimilation rate is 0.024 μmol (mg protein)$^{-1}$ min^{-1}. This value corresponds to only about 2% of the maximum activity of NADP-ME in the homozygous transformant of ME210 (1.1 μmol (mg protein)$^{-1}$ min^{-1}). Thus, it is reasonable that the maize NADP-ME acts to increase the level of NADPH. It is noted that the increase in the level of NADPH can occur when malate imported from the cytosol is used as the substrate of the enzyme. If malate is supplied by the reaction of NADP-MDH from OAA, which is imported into the chloroplast by the OAA transporter, the level of NADPH remains constant, since NADP-MDH consumes one NADPH molecule for generation of one malate molecule.

Another action of the maize NADP-ME would be depletion of malate in the stroma through its decarboxylation reaction. Even if the in vivo activity of NADP-ME were to be 2% of the maximum, malate present in the stroma, at around 3 mM under illumination (Heldt et al. 1990), can be consumed within 1 min in the homozygous transformant of ME210. Depletion of malate likely leads to suppression of photorespiration, since 2-oxoglutarate has to be imported into the chloroplast in exchange with malate by the 2-oxoglutarate transporter for photorespiration (Flügge and Heldt 1991). Suppression of photorespiration then retards regeneration of riburose-1,5-bisphosphate for the Calvin cycle (Leegood et al. 1995), again leading to stimulation of photoinhibition. It is also likely that the decarboxylation of malate acts to waste once assimilated carbon. Such an effect would retard the growth of rice plants even under weak light conditions.

The activity of the C_4-specific NADP-ME is increased under illumination through increases in pH and concentration of Mg^{2+} in the stroma (Edwards and Andreo 1992). Therefore, it is reasonable that deteriorative effects of the maize enzyme were more pronounced at higher light intensities.

The above considerations suggest that carbon metabolism inside the chloroplast can greatly be perturbed by introduction of a foreign enzyme. The C_4-specific NADP-ME has a higher V_m value, lower K_m values for substrates, and higher optimum pH, as compared with the C_3-specific isoform (Edwards and Andreo 1992, Casati et al. 1997). Such characteristics are suitable for strict regulation of the enzyme activity in the bundle sheath cell chloroplasts of C_4 plants (Edwards and Andreo 1992), but these allow the enzyme to express its activity in leaves of C_3 plants up to the level that causes serious deteriorative effects on growth. This, however, has an important implication that the carbon metabolism in the mesophyll cells of C_3

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plants has significant flexibility, especially in terms of import of metabolites into the chloroplasts.

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