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NADP-Malate Dehydrogenase in the C_4 Plant *Flaveria bidentis*

Cosense Suppression of Activity in Mesophyll and Bundle-Sheath Cells and Consequences for Photosynthesis

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***Flaveria bidentis*, a C_4 dicot, was transformed with sorghum (a monocot) cDNA clones encoding NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82) driven by the cauliflower mosaic virus 35S promoter. Although these constructs were designed for overexpression, many transformants contained between 5 and 50% of normal NADP-MDH activity, presumably by cosense suppression of the native gene. The activities of a range of other photosynthetic enzymes were unaffected. Rates of photosynthesis in plants with less than about 10% of normal activity were reduced at high light and at high $[CO_2]$, but were unaffected at low light or at $[CO_2]$ below about 150 $\mu L\ L^{-1}$. The large decrease in maximum activity of NADP-MDH was accompanied by an increase in the activation state of the enzyme. However, the activation state was unaffected in plants with 50% of normal activity. Metabolic flux control analysis of plants with a range of activities demonstrates that this enzyme is not important in regulating the steady-state flux through C_4 photosynthesis in *F. bidentis*. Cosense suppression of gene expression was similarly effective in both the mesophyll and bundle-sheath cells. Photosynthesis of plants with very low activity of NADP-MDH in the bundle-sheath cells was only slightly inhibited, suggesting that the presence of the enzyme in this compartment is not essential for supporting maximum rates of photosynthesis.**

The photosynthetic efficiency of C_3 plants is often limited by the competition of O_2 with CO_2 at the active site of Rubisco, the primary carboxylating enzyme of photosynthesis (Andrews and Lorimer, 1987). C_4 plants have evolved a complex mechanism involving both structural and biochemical adaptations to overcome this (Hatch, 1987). The primary role of the C_4 mechanism is to increase the ratio of CO_2 to O_2 in the bundle-sheath cell chloroplast, the site of CO_2 fixation by Rubisco. This adaptation increases both the maximum rate of photosynthesis and the efficiency of the process at low light. Other advantages of the C_4 pathway are improved efficiency of water and nitrogen use (Hatch, 1987). However, the advantage conferred to a plant by these adaptations is temperature-dependent, declining as the temperature decreases (Hatch, 1987).

Although the properties of many enzymes of C_4 photosynthesis have been extensively studied in vitro, until recently we have had no way of examining the role of key enzymes in regulating photosynthetic flux in the intact plant. Gene-suppression technology, in particular, antisense, has proven invaluable in identifying the important regulatory enzymes in C_3 photosynthesis under a range of environmental conditions (Furbank and Taylor, 1995). The recent development of an efficient transformation system for the C_4 dicot *Flaveria bidentis* (Chitty et al., 1994) has allowed us to apply these techniques to the study of C_4 photosynthesis. This approach has demonstrated that, as in C_3 plants, Rubisco is an important determinant of the rate of C_4 photosynthesis at air concentrations of CO_2 and at high light intensities (Furbank et al., 1996). However, C_4 photosynthesis is compartmented between two cell types, and the regulatory properties of both the photosynthetic carbon reduction cycle and the C_4 cycle enzymes must be considered. Some of these enzymes have complex regulatory properties (Hatch, 1987), and it is quite likely that one or more of them is also involved in regulating photosynthetic carbon flux. One potential target for regulation is the reaction catalyzed by NADP-MDH (EC 1.1.1.82): oxaloacetate + NADPH + $H^+ \rightleftharpoons$ malate + $NADP^+$.

In maize NADP-MDH is localized exclusively in the mesophyll cell chloroplasts. Activity of the enzyme is regulated by changes in the concentrations of substrates (Ashton and Hatch, 1983a), and also varies in response to changes in light intensity (Johnson and Hatch, 1970) due to changes in activation state. This is due to covalent modification of the enzyme involving the reduction and oxidation

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Abbreviations: CaMV, cauliflower mosaic virus; MDH, malate dehydrogenase; *nos*, nopaline synthase; OAA, oxaloacetate; 2OG, 2-oxoglutarate; PEPCX, PEP carboxylase; PGA, phosphoglycerate; PPDK, pyruvate phosphate dikinase.

of Cys residues by thioredoxin *m* (Kagawa and Hatch, 1977; Jacquot et al., 1984). The oxidized form of the enzyme extracted from darkened leaves is almost completely inactive, with less than 0.001% of the activity of the reduced enzyme (Ashton and Hatch, 1983a). The activation state is regulated by the ratio of NADPH to NADP both in vitro (Ashton and Hatch, 1983b) and in vivo (Rebeille and Hatch, 1986). These properties of the enzyme provide it with a sensitive mechanism by which activity can respond to changes in the redox potential of the chloroplast (Edwards et al., 1985). Although this suggests a potential role for the enzyme in regulating photosynthetic flux, the degree to which this occurs in vivo is not known.

The individual amino acids involved in the covalent regulation of the sorghum enzyme have been identified (Issakidis et al., 1992, 1994), and mutagenesis has produced, among others, an always active, thiol-insensitive enzyme (Issakidis et al., 1994). We have used clones encoding both the wild-type and the mutated sorghum enzymes to manipulate the activity of NADP-MDH in transgenic *F. bidentis*. Analysis of these plants has allowed us to assess the role of this enzyme in regulating the rate of C_4 photosynthesis. *F. bidentis* differs from maize in that it has significant activities of NADP-MDH in the bundle-sheath cell chloroplast (Meister et al., 1996). Therefore, we have also considered the significance of this form of the enzyme in the regulation of C_4 photosynthesis in this plant.

MATERIALS AND METHODS

Biochemicals were obtained from either Boehringer Mannheim or Sigma. Thioredoxin was purified from *Escherichia coli* transformed with the multicopy plasmid pPMR18, provided by Marjorie Russel (The Rockefeller University, New York), which contains the *E. coli* thioredoxin (*TrxA*) gene (Russel and Model, 1986). The thioredoxin was purified to homogeneity by a procedure involving sequential $(NH_4)_2SO_4$ and PEG fractionation followed by DEAE-Sepharose chromatography (A.R. Ashton, unpublished data). Restriction enzymes were from New England Biolabs. Molecular biological techniques were performed as described by Sambrook et al. (1989).

Transformation and Growth of Plants

Wild-type and mutant sorghum NADP-MDH clones differing in kinetic and regulatory properties (Issakidis et al., 1992, 1994) in either pUC19 or M13 mp19 were a gift from Emmanuelle Issakidis and Myroslawa Miginiac-Maslow (Centre National de la Recherche Scientifique, Orsay, France). The clones were excised with *Eco*RI and subcloned in the sense orientation into the vector pJ35SN (Landsmann et al., 1988) between the CaMV 35S promoter and the *nos* terminator. The constructs were digested with *Hin*dIII, and the DNA fragments containing the 35S promoter-sorghum NADP-MDH gene-*nos* terminator were ligated into the binary vector pGA470 (An et al., 1985). This vector contains the neomycin phosphotransferase gene *nptII* driven by the *nos* promoter to confer kanamycin resistance. The NADP-MDH sense construct and the *nptII* gene are transcribed in the same direction from the transferred DNA. This will prevent the generation of antisense NADP-MDH message from the *nos* promoter of the *nptII* gene. The constructs produced in this way were (a) wtSorg (wild-type sorghum cDNA clone), (b) dmNSorg (double N-terminal mutant, C64S/C69S), (c) dmCSorg (double C-terminal mutant, C405A/C417A), and (d) QmSorg (quadruple mutant, C64S/C69S/C405A/C417A). Transformation of *Flaveria bidentis* using *Agrobacterium* strain *AglI* (Lazo et al., 1991) was exactly as described by Chitty et al. (1994). Transformants were selected on kanamycin and then transferred to the greenhouse in pots containing a mixture of 50% (v/v) compost, 50% (v/v) perlite. The plants were initially hardened off under a 50% shade cloth for 2 weeks, and then transferred to full sunlight. The temperature was maintained at 29°C during the day and 21°C at night. Fertilization was with both a slow-release fertilizer (Osmocote, Scotts Australia, Castle Hill, Australia) and daily with a liquid fertilizer, Hoagland no. 2 (Hewitt, 1966). Cuttings were propagated using rooting powder (Rootex-P, Bass Laboratories, Bayswater, Australia). Plants were grown for 2 to 4 months, and enzymatic and physiological analyses were performed on the youngest, fully expanded leaves taken from plants before the onset of flowering.

Confirmation of Transformation

Activity of neomycin phosphotransferase in leaf discs of *F. bidentis* was measured using the extraction method and dot blot assay described by McDonnell et al. (1987). The presence of the sorghum NADP-MDH gene insert was confirmed by PCR analysis of genomic DNA isolated from leaf material as described by Shure et al. (1983). The oligonucleotide primers used in PCR reactions were NOSSEQ4 (complementary to a region in the *nos* terminator) and TRANSCONF1 (identical to a sequence at the 3′ end of the coding region of the sorghum gene). The sequences of these primers were GGCAACCTTCCGGCGTTCGTG and TAGCGGATGCTATTAAATCC, respectively. These primers amplify a region of about 500 bp from DNA samples containing any of the four sorghum NADP-MDH gene constructs but not from untransformed *F. bidentis* DNA. PCR reactions used about 1 μg of genomic DNA per 20 μL of reaction volume, with an annealing temperature of 50°C. Thirty cycles in a capillary thermal cycler (model FTS1, Corbett Research, Sydney, Australia) were performed.

Enzyme Extraction and Assays

NADP-MDH was extracted from fresh leaf tissue by grinding with 4 to 10 volumes of ice-cold extraction buffer A (25 mM Hepes [K^+], pH 7.5, 10 mM $MgSO_4$, 1 mM Na_2EDTA, 5 mM DTT, 1 mM PMSF, 5% [w/v] insoluble PVP, and 0.05% [v/v] Triton X-100) with a small amount of quartz sand in a 1.5-mL microfuge tube. The enzyme was also extracted from leaf tissue that had been frozen in $N_{2(l)}$ and stored at −80°C by first precooling the microfuge tube containing the sample in $N_{2(l)}$ and then adding extraction buffer A. The tissue was then ground with sand as the extraction buffer thawed. In either case an aliquot was

removed for chlorophyll analysis and the extract then centrifuged for 5 min (14,000*g*, 4°C) to remove insoluble material. The supernatant was retained for measurements of NADP-MDH activity. The enzyme was routinely activated by incubating with approximately 1 μg of *E. coli* thioredoxin in 25 mM Hepes (K^+), pH 7.5, 1 mM Na_2EDTA, 500 mM KCl, 20 mM DTT, and 0.01% (v/v) Triton X-100 for 30 min at 30°C. Alternatively, the enzyme was activated by incubating with 40 mM Tricine (K^+), pH 9, 0.4 mM Na_2EDTA, 120 mM KCl, 100 mM DTT, and 0.0025% (v/v) Triton X-100 for 2 h at 25°C. NADP-MDH activity was measured spectrophotometrically essentially as described by Ashton et al. (1990), except that 150 mM KCl was included in the assay buffer.

Other photosynthetic enzymes were measured in leaf samples extracted in ice-cold buffer A plus 1 mg/mL BSA, 2 mM $MnCl_2$, and 20 μg/mL pyridoxal P. Protease inhibitors, benzamidine (0.5 mM), and ε-amino caproic acid (2.5 mM) were also included in the extraction buffer for the measurement of Rubisco. PPDK was measured in extracts made from illuminated leaves stored at room temperature. Enzymes were assayed as described by Ashton et al. (1990) and Leegood (1990), except for Rubisco, which was measured by following the incorporation of $H^{14}CO_3^-$ into acid-stable products, as described by Furbank et al. (1996). NAD-MDH was assayed in 25 mM Hepes (K^+), pH 7.5, 0.5 mM Na_2EDTA, 0.2 mM NADH, and 0.5 mM OAA.

Light Activation of NADP-MDH in Attached Leaves

We measured the maximum activation state of NADP-MDH by first darkening plants for 1 h to completely oxidize and inactivate the enzyme. While still attached to the plant, a leaf was then placed at 1200 μmol photons m^{-2} s^{-1} for 15 min. Illumination was from a 150-W incandescent lamp with a 2-cm water filter. A small (0.35 cm^2) leaf disc was then removed and ground, together with quartz sand, in 25 volumes of ice-cold extraction buffer B (25 mM Hepes [K^+], pH 6.5, 10 mM $MgSO_4$, 1 mM Na_2EGTA, 5 mM DTT, 1 mM PMSF, 5% [w/v] insoluble PVP, and 0.05% [v/v] Triton X-100). An aliquot was removed for chlorophyll measurement, the sample was centrifuged, and NADP-MDH was assayed as described above. The enzyme was then fully activated with *E. coli* thioredoxin and assayed again.

We also examined the response of activation state to a gradual increase in light intensity by first illuminating an attached leaf at low light (100 μmol photons m^{-2} s^{-1}) for 15 min, removing a small leaf disc, and assaying for NADP-MDH as described above. The incident light on the leaf was then increased to 200 μmol photons m^{-2} s^{-1} for 15 min, and NADP-MDH activity was measured in another leaf disc. The light was gradually increased up to 1600 μmol photons m^{-2} s^{-1} in this manner, with leaf discs being removed and NADP-MDH being assayed at each intensity.

Measurement of NADP-MDH in Bundle-Sheath Cell Strands

Plants were placed in the dark for up to 48 h to deplete starch, and then put in full sunlight for 1 h prior to harvesting. About 5 g of deribbed plant material was blended with 70 mL of ice-cold buffer C (0.3 M sorbitol, 20 mM Hepes [K^+], pH 7.7, 2 mM Na_2EDTA, 2 mM isoascorbate, 2 mM Na_2HPO_4, and 1 mM PMSF) in a mixer (Omnimixer, Sorvall), as described by Meister et al. (1996), to prepare bundle-sheath cell strands for O_2-exchange measurements. A filtrate enriched in mesophyll cell contents was obtained from a portion of the leaf extract after the first blend (10 s at 60% of line voltage) by filtration through an 80-μm nylon net. Samples of the whole leaf extract were taken after the final blending. Bundle-sheath cell strands were resuspended in 10 to 15 mL of buffer D (0.3 M sorbitol, 20 mM Hepes [K^+], pH 7.7, 10 mM KCl, 1 mM Na_2EDTA, and 0.5 mM Na_2HPO_4). DTT (10 mM) and Triton X-100 (0.1%, v/v) were added to all three fractions, which were then extracted in a ground-glass pestle and tube Duall tissue grinder (Kontes Glass Co., Vineland, NJ) and used for measurement of enzymes and chlorophyll. The extent of cross-contamination of cell contents was followed with the use of marker enzymes (NADP-malic enzyme and PEPCX) and by microscopic examination.

O_2-Exchange Measurements of Isolated Bundle-Sheath Cell Strands

Bundle-sheath cell strands prepared as described above were immediately added to an O_2 electrode (Rank Brothers, Cambridge, UK), and O_2-exchange properties were measured at 25°C as described by Meister et al. (1996). The concentrations of substrates used were as follows: $NaHCO_3$, 10 mM; PGA, 5 mM; Asp, 12.5 mM; 2OG, 12.5 mM; and malate, 12.5 mM.

Chlorophyll Determinations

Chlorophyll was measured in 80% (v/v) acetone, as described by Porra et al. (1989).

Photosynthesis Measurements

Photosynthesis rates of attached leaves were measured at 25°C using a portable IR gas analyzer with a clamp-on leaf chamber (model LCA-2, ADC, Hoddesdon, UK). Illumination was from a 150-W incandescent lamp with a 2-cm water filter.

RESULTS AND DISCUSSION

Reliability of NADP-MDH Extraction and Assay

We confirmed that our extraction and assay methods for NADP-MDH from leaves of *F. bidentis* are reliable as follows. First, in two experiments, the recovery of sorghum NADP-MDH from a mixture of *F. bidentis* and sorghum leaf tissue was 87 and 105%. Second, NADP-MDH activity was completely recovered from leaf samples frozen and stored at −80°C for at least 24 d. In addition, activity was completely recovered even if the samples were thawed and allowed to stand at 25°C for 5 min before extraction. Third, activation of the enzyme by *E. coli* thioredoxin was essentially complete after 5 min at 30°C, with negligible loss of

activity over the next 55 min. We included Triton X-100 in the activation buffer to stabilize the reduced form of the enzyme (Hatch and Agostino, 1992). Fourth, the concentration of each component of the spectrophotometric activity assay was optimized to measure maximum activity. Although the rate could be increased by about 20% by using 100 mM Pi rather than 150 mM KCl in the assay buffer, this concentration of Pi is much higher than what we expected to find in the chloroplast. Together, these results allow us to be confident that our measurements of maximum activity made in a crude extract accurately represent the maximum activity of the enzyme in vivo.

NADP-MDH Activity in Primary Transformants

F. bidentis was transformed with full-length sense constructs containing either the wild-type or mutant forms of the sorghum NADP-MDH cDNA clone (Issakidis et al., 1992, 1994) flanked by the CaMV 35S promoter and the *nos* terminator. The dmNSorg and dmCSorg enzymes are both largely inactive when oxidized. The dmNSorg form is more readily reduced by thioredoxin in vitro than the wild-type enzyme, whereas the reductive activation of the dmCSorg form, unlike that of the wild-type enzyme, is not inhibited by NADP. The QmSorg enzyme cannot form disulfide bonds and is therefore active even under oxidizing conditions. In total, 17 transformation experiments, each with about 150 explants, were performed. Forty-one neomycin phosphotransferase-positive plants from different calli were assayed for maximum catalytic activity of NADP-MDH (Fig. 1). We were unable to detect any sorghum NADP-MDH protein in six transformed plants with the highest NADP-MDH activity by immunoblotting using antibodies specific to the sorghum enzyme (not shown). In 21 of these primary transformants NADP-MDH activity was less than 50% of that in untransformed plants. There was apparently no difference between the four constructs in the frequency of transformants with reduced activity.

The reason we failed to identify transformants expressing sorghum NADP-MDH is uncertain. All four of the clones produce functional protein when expressed in *E. coli* (Issakidis et al., 1992; 1994), and tobacco plants transformed with the wild-type clone correctly target and process the sorghum enzyme (Gallardo et al., 1995). We have also transformed tobacco with wild-type and mutant clones and looked for expression by immunoblotting with antibodies specific for sorghum NADP-MDH. Although we could detect a low-level expression of sorghum NADP-MDH in tobacco transformed with wtSorg or dmCSorg, we could not identify any sorghum protein in plants transformed with dmNsorg or QmSorg (not shown). We have examined the possibility that the lack of expression could be due to cloning artifacts by determining the nucleotide sequence at the junction of the 3′ end of the CaMV 35S promoter and the 5′ end of the NADP-MDH coding sequence in each of these four constructs. In two of them, dmNSorg and QmSorg, there is an upstream, out-of-frame ATG translation initiation codon, whereas no upstream ATG codons were present in the other constructs (not shown). Although the presence of this extra ATG could presumably reduce the efficiency of translation of functional enzyme from dmNSorg and QmSorg (Rogers et al.,

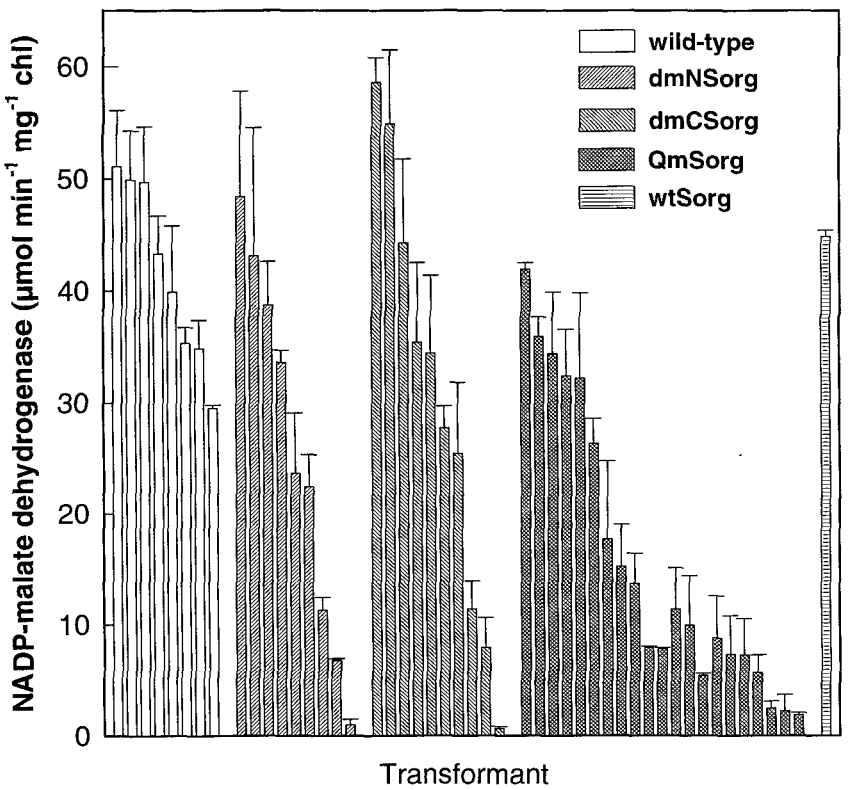

Figure 1. NADP-MDH activities in primary transformants. *F. bidentis* plants transformed with constructs designed to overexpress sorghum NADP-MDH were grown in soil in the greenhouse. The activity of NADP-MDH was measured in leaf discs taken from the youngest, fully expanded leaves of these plants. Activities are mean ± SE ($n \geq 2$) of measurements made on different leaves from an individual plant. chl, Chlorophyll.

1985), it does not explain the apparent lack of expression from all four constructs.

As shown in Figure 1, the majority of *F. bidentis* transformants actually have decreased activity of NADP-MDH, presumably due to cosense suppression of gene expression (Jorgensen, 1991; Flavell, 1994). This phenomenon was first reported for chalcone synthase in petunia (Napoli et al., 1990; van der Krol et al., 1990), and there have since been many other examples reported in the literature (e.g. Vanlerberghe et al., 1994; Brusslan and Tobin, 1995; Paul et al., 1995; Flipse et al., 1996). Three nonmutually exclusive mechanisms have been proposed to explain the occurrence of cosense suppression: (a) alterations in chromatin structure, (b) changes in methylation of promoter sequences, and (c) posttranscriptional mechanism(s) resulting in the degradation of both introduced and endogenous mRNA (Flavell, 1994). We do not have evidence for any one of these particular mechanisms being involved in the suppression of NADP-MDH activity in transformed *F. bidentis*. We suggest that cosense suppression of gene expression is responsible for our inability to detect expression of the sorghum NADP-MDH protein.

Initial Characterization of Reduced-Activity T_1 Plants

T_0 transgenic plants were allowed to self-fertilize and produce seed. The biochemical and physiological analyses described in the following sections were performed on T_1 plants germinated from this seed. We identified some transgenic plants in which the activity of NADP-MDH varied significantly between different portions of an individual leaf. This was seen in plants transformed either with the sorghum gene constructs or with homologous antisense constructs (S.J. Trevanion, R.T. Furbank, A.R. Ashton, unpublished data). Since uneven expression of NADP-MDH would significantly complicate analysis of these plants, we analyzed plants in which we were unable to detect this phenotype. A further important consideration when analyzing transgenic plants is to confirm that the reduced expression of the target gene has not affected expression of other photosynthetic enzymes. For example, undesirable alterations in gene expression could occur from the insertion of the transgene into either the regulatory or the coding sections of an endogenous gene. In addition, reducing the activity of the target enzyme could in itself alter the expression of other genes (e.g. Hudson et al., 1992) by as yet undefined mechanisms. We have addressed these possibilities by initially characterizing T_1 plants from three independent lines: 12–7–20, 22–1–6, and 22–8–1. PCR analysis of genomic DNA from each of these individuals confirmed that they are transformed with the sorghum NADP-MDH gene. No comparable DNA fragment was detected in untransformed plants. We measured the activities of a range of enzymes involved in both the C_4 cycle and photosynthetic carbon reduction cycle enzymes (Table I). In each of the transgenic lines the activity of NADP-MDH was less than 25% of that in untransformed plants. There were slight variations in the activities of some other enzymes between the different lines, but we did not observe any consistent trend in these differences. Although the measured activities of Rubisco were low in both untransformed and reduced-activity plants, this was presumably due to problems with extraction and/or activation of the enzyme. Of course, we cannot exclude the possibility that the activity of another, unassayed enzyme may also be affected, but we can be reasonably confident that we are working with plants that have a specific change in the amount of NADP-MDH.

The decrease in NADP-MDH activity was sometimes accompanied by a decrease in chlorophyll content. There was no change in the chlorophyll *a* to chlorophyll *b* ratio in any of the reduced activity plants. The plant with reduced chlorophyll (12–7–20) was characterized by a decrease in growth rate, whereas plants with normal levels of chlorophyll (22–1–6 and 22–8–1) had apparently normal growth rates (not shown). An association between reduced enzyme activity, chlorophyll content, and growth rate has previously been observed in tobacco (Hudson et al., 1992) and *F.*

Table I. *Enzyme activities in untransformed and transformed F. bidentis*

Activities of C_3 and C_4 enzymes were measured in the youngest, fully expanded leaves of both untransformed and individual T_1 plants transformed with the plasmid pBQmsorg (12–7–10) or pBdmNsorg (22–1–6 and 22–8–1). Activities are mean ± SE ($n \geq 3$).

Enzyme	Activity			
	Wild type	12-7-20	22-1-6	22-8-1
	μmol min^{-1} mg^{-1} chlorophyll			
PPDK	2.9 ± 0.3	2.9 ± 0.04	3.4 ± 0.2	2.6 ± 0.5
PEPCX	21 ± 3	21 ± 3	20 ± 2	36 ± 9
NAD-MDH	61 ± 3	63 ± 5	74 ± 6	97 ± 1
NADP-ME	7.5 ± 0.4	9.2 ± 0.3	8.6 ± 0.5	10 ± 1
Asp aminotransferase	10 ± 1	14 ± 1	7.5 ± 0.3	11 ± 0.2
Ala aminotransferase	20 ± 1	25 ± 2	16 ± 0.7	18 ± 0.6
NADP-MDH	25 ± 1	2.1 ± 0.5	4.2 ± 0.7	4.5 ± 0.8
Rubisco	1.8 ± 0.04	3.4 ± 0.4	2.3 ± 0.1	2.1 ± 0.1
Phosphoglycerate kinase	20 ± 2	24 ± 2	24 ± 2	28 ± 5
Phosphoribulokinase	27 ± 1	29 ± 4	24 ± 0.7	56 ± 3
Chlorophyll (mg m^{-2})	535 ± 29	323 ± 10	685 ± 39	460 ± 22
Chlorophyll *a*:Chlorophyll *b*	4.6 ± 0.2	4.6 ± 0.2	4.7 ± 0.1	4.7 ± 0.1

bidentis (Furbank et al., 1996) with decreased levels of Rubisco, in tobacco with reduced Rubisco activase (Mate et al., 1993), and in *F. bidentis* with decreased PPDK (R.T. Furbank, unpublished data).

Response of Photosynthesis to Changes in Light Intensity

We examined the response of photosynthesis to changes in light intensity at saturating concentrations of CO_2 (>400 μL L^{-1}) for untransformed *F. bidentis* and a reduced-activity plant from each of the lines (Fig. 2). At the maximum light intensity available using our system (1250 μmol photons m^{-2} s^{-1}), photosynthesis of the untransformed plant was about 5.5 μmol min^{-1} mg^{-1} chlorophyll. The maximum rate of photosynthesis of each of the reduced-activity plants was less than this, although the actual rate varied considerably between the different plants. This decrease in photosynthesis reflects a limitation imposed by the severe decrease in NADP-MDH activity in these plants (see below). There were also differences between the untransformed and the reduced-activity plants in the light intensity required to saturate photosynthesis. The untransformed plant showed a typical response for a C_4 plant, with a doubling of the rate of photosynthesis when the light was increased from 500 to 1250 μmol photons m^{-2} s^{-1}. However, photosynthesis of two of the reduced-activity plants (12–7–20 and 22–8–1) was saturated at about 500 μmol photons m^{-2} s^{-1}, and the other plant (22–1–6) showed only a slight increase in rate at higher light intensities. NADP-MDH activities in these lines were 12, 18, and 17% of normal, respectively. The similar behavior of three different transgenic lines further confirms that we are dealing with transgenic plants that have a specific change in the activity of NADP-MDH.

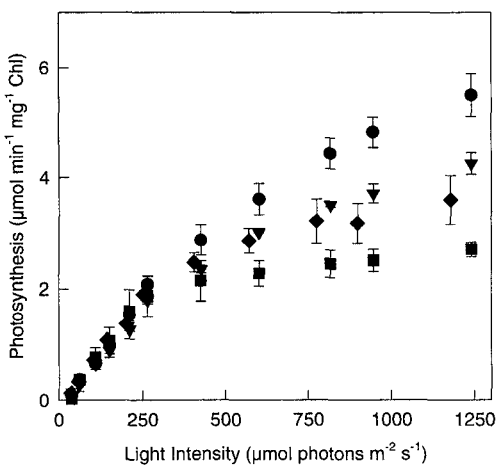

Figure 2. Light-response curve of untransformed and reduced-activity plants. Photosynthesis of attached leaves from untransformed and reduced-activity plants were measured at different light intensities using IR gas analysis. The $[CO_2]$ was between 400 and 500 μL L^{-1}. Results are mean ± SE of measurements made on different leaves from an individual plant. ●, Untransformed (n = 4), NADP-MDH = 30 ± 3 μmol min^{-1} mg^{-1} chlorophyll; ■, 12–7–20 (n = 3), NADP-MDH = 3.9 ± 0.5 μmol min^{-1} mg^{-1} chlorophyll; ▼, 22–1–6 (n = 2), NADP-MDH = 7.7 ± 3.4 μmol min^{-1} mg^{-1} chlorophyll; ◆, 22–8–1 (n = 2), NADP-MDH = 4.0 ± 1.1 μmol min^{-1} mg^{-1} chlorophyll (Chl).

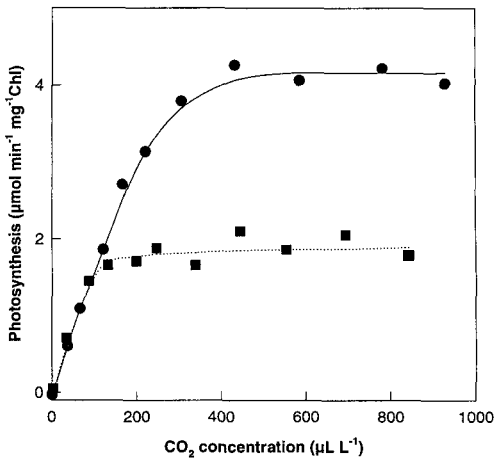

Figure 3. CO_2-response curve of untransformed and reduced-activity plants. Photosynthesis of attached leaves from an untransformed and a reduced-activity plant (12–7–20) were measured at different CO_2 concentrations using IR gas analysis. Results shown are from a single experiment. Light intensity was 1150 μmol photons m^{-2} s^{-1}. ●, Untransformed, NADP-MDH = 20 μmol min^{-1} mg^{-1} chlorophyll; ■, 12–7–20, NADP-MDH = 3.2 μmol min^{-1} mg^{-1} chlorophyll (Chl).

Unlike the situation at high light, the response of photosynthesis to changes in light intensity below 250 μmol photons m^{-2} s^{-1} was identical in the untransformed plant and in the three reduced-activity plants. This strongly implies that the quantum yield of photosynthesis was unaffected in these plants and supports the idea that photosynthesis in C_4 plants is limited by the regeneration of either PEP or ribulose bisphosphate (Collatz et al., 1992).

Response of Photosynthesis to Changes in $[CO_2]$

The typical response of photosynthesis to changes in $[CO_2]$ in an untransformed and a reduced-activity plant (12–7–20) are shown in Figure 3. These experiments were done at a light intensity of 1150 μmol photons m^{-2} s^{-1}. Compared with the untransformed plant, 12–7–20 had a much lower rate of photosynthesis at saturating $[CO_2]$. The $[CO_2]$ required to saturate photosynthesis was also decreased, from about 400 μL L^{-1} in the untransformed plant to about 150 μL L^{-1} in 12–7–20. There was no difference in the response of photosynthetic rate of the two plants to changes in $[CO_2]$ between 0 and 150 μL L^{-1}. This is not surprising given that the response of photosynthesis to $[CO_2]$ in C_4 plants is thought to be largely determined by the activity and kinetic properties of PEPCX (Edwards and Walker, 1983).

Activation State of NADP-MDH

The activation state of maize NADP-MDH is dependent on the redox state of thioredoxin *m*, and on the NADPH/NADP ratio (Ashton and Hatch, 1983b). Slight changes in either of these parameters may have large consequences for

Table II. *Activation state of NADP-MDH in untransformed and reduced-activity plants*

The activation state of NADP-MDH was measured in leaves of untransformed and reduced-activity plants illuminated at 1200 μmol photons m^{-2} s^{-1} for 15 min. Results are mean ± SE (n = 3).

Plant	Activity		Activation State
	μmol min^{-1} mg^{-1} chlorophyll	% of untransformed	%
Untransformed	32 ± 10	100	52 ± 7
12–7–7	16 ± 3	51	44 ± 7
12–7–20	3.3 ± 0.2	10	107 ± 6

the activation state of the enzyme. Assuming that the *F. bidentis* enzyme behaves similarly to the maize enzyme, two observations suggest that the activation state of NADP-MDH might differ between the untransformed and the reduced-activity plants. First, since the maximum rate of photosynthesis is reduced in transgenic plants, this will affect the rate of chloroplast electron transport and the consumption of reducing power by the reactions of the photosynthetic reduction carbon cycle. This could potentially lead to changes in both the NADPH/NADP ratio and the redox state of thioredoxin *m*. Second, since NADPH is a substrate and NADP is a product of the NADP-MDH reaction, changes in the activity of the enzyme could itself alter the NADPH/NADP ratio.

This possibility of changes in activation state is of particular significance for measurements of the metabolic flux control coefficient of NADP-MDH (see below). Therefore, we measured the activation state of NADP-MDH in different transgenic lines at a range of light intensities. The activation state is expressed as the activity of the unactivated enzyme as a percentage of that of the thioredoxin-activated enzyme. We first confirmed that the extraction method used did not alter the activation state of either the inactivated or activated enzyme by extracting NADP-MDH from leaves either predarkened for 1 h, or predarkened and then illuminated at 800 μmol photons m^{-2} s^{-1} for 15 min, respectively, and following the activity of the extracted enzyme over time (not shown). Next, we measured the maximum activation state of the enzyme by illuminating attached leaves at 1200 μmol photons m^{-2} s^{-1} for 15 min (Table II). This period was sufficient for the activation state to reach steady state (not shown). The enzymes from either an untransformed plant or a transgenic plant with 50% of normal activity (12–7–7) were only activated to about 50% by this treatment. However, the enzyme from 12–7–20 (10% of normal activity) was completely activated under these conditions.

We also examined the response of the activation state of the enzyme to step-wise changes in light intensity in an untransformed plant and in 12–7–20. This was done by first predarkening the plant and then illuminating an attached leaf at sequentially higher light intensities. The activity in darkened extracts was 0.19 ± 0.04 μmol min^{-1} mg^{-1} chlorophyll from untransformed leaves (n = 5), and 0.12 ± 0.05 μmol min^{-1} mg^{-1} chlorophyll from 12–7–20 (n = 3). These equate to activation states of 0.46 ± 0.04% and 5.0 ± 2.3%, respectively, demonstrating the effectiveness of the covalent modification in inactivating the *F. bidentis* enzyme. The results of changes in light intensity on activation state in one experiment are shown in Figure 4. For the enzyme extracted from the untransformed plant, the highest activation state of NADP-MDH achieved (only 50% of maximum) was reached at a light intensity of about 800 μmol photons m^{-2} s^{-1}. However, 100% activation of the enzyme from 12–7–20 was observed at a much lower light intensity, about 200 μmol photons m^{-2} s^{-1}. The slight decrease in the activation state of enzyme extracted from untransformed leaves illuminated at 1600 μmol photons m^{-2} s^{-1} was observed on each of the three occasions this experiment was performed. The significance of this is not clear. Although we did not measure photosynthesis at such high light intensities, Furbank et al. (1996) did not see any decline in the rate of CO_2 fixation in untransformed plants when the light intensity was increased from 1100 to 1800 μmol photons m^{-2} s^{-1}.

Compared with measurements of photosynthetic rates (Fig. 2), these results suggest that regulation of the activation state of NADP-MDH plays a role in maintaining rates of photosynthesis in the reduced activity plants. Figure 5, a and b, shows the relationship between actual activity of the enzyme (i.e. activation state × maximum activity) and photosynthesis in untransformed and reduced-activity plants, respectively. As expected, the activity of NADP-MDH in both plants is in excess of the rate of photosyn-

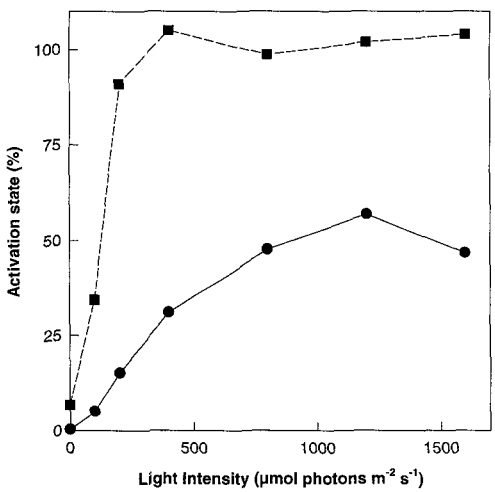

Figure 4. Activation state of NADP-MDH in untransformed and reduced-activity (12–7–20) plants. Plants were placed in the dark for 1 h to completely inactivate NADP-MDH. Individual leaves, still attached to the plant, were then illuminated at 100 μmol photons m^{-2} s^{-1} for 15 min, and the activation state of NADP-MDH was measured in a small disc. The light intensity was then sequentially increased to 1600 μmol photons m^{-2} s^{-1}, and the activation state of NADP-MDH was measured after 15 min of illumination of the leaf at each light intensity. All measurements were made on a single leaf from each plant. The results shown are from a single experiment. ●, Untransformed, NADP-MDH = 52 ± 2 μmol min^{-1} mg^{-1} chlorophyll; ■, 12–7–20, NADP-MDH = 2.9 ± 0.2 μmol min^{-1} mg^{-1} chlorophyll.

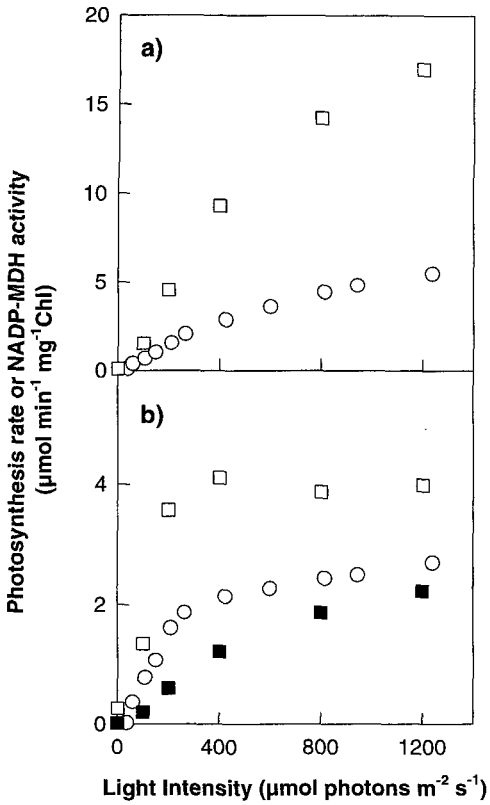

Figure 5. Relationship between activity of NADP-MDH and rate of photosynthesis in untransformed and reduced-activity (12–7–20) plants. Activity of NADP-MDH was calculated by multiplying the maximum catalytic activity of the enzyme in leaves used for measurements of photosynthesis rates (Fig. 2) by the activation state of the enzyme in leaves from identical plants (Fig. 4). ○, Photosynthesis rates; □, NADP-MDH activities; ■, activity of NADP-MDH in the reduced-activity plant, assuming that the activation state is equal to that in the untransformed plant. a, Untransformed; b, 12–7–20. Chl, Chlorophyll.

thesis. However, Figure 5b also shows that in the absence of the increase in activation state of the enzyme in the reduced-activity plants, there would be insufficient activity of NADP-MDH to maintain the observed rates of photosynthesis.

Changes in the activation state of the enzyme are most readily explained by differences in the NADPH/NADP ratio and/or the redox state of thioredoxin *m* between the different lines. Indeed, it is hard to imagine the redox state of the thioredoxin pool not changing when the redox state of the NADPH/NADP pool changes and vice versa because of the multiple connections between the pools. Both are reversibly reduced by Fd, and NADPH can reduce some chloroplast thiols via glutathione reductase, thus to some extent influencing the rate of oxidation of the thioredoxin pool. Of the connections between the thioredoxin pool and the NADPH/NADP pool, NADP-MDH may itself be most important, since altering the redox state of thioredoxin will alter NADP-MDH activity and thus the rate of consumption of NADPH. Chlorophyll fluorescence measurements can be used to monitor the redox state of some chloroplast components. Furbank (1988) found in experiments with isolated maize mesophyll chloroplasts that the redox state of the primary electron-accepting plastoquinone of PSII was considerably more oxidized when the chloroplasts were reducing OAA than in the absence of an electron acceptor. However, measurements of chlorophyll fluorescence quenching of untransformed and reduced-activity plants did not reveal any significant difference in either the effective quantum yield of PSII (a measure of the redox state of the primary electron-accepting plastoquinone of PSII) (Genty et al., 1989) or nonphotochemical quenching of chlorophyll fluorescence (not shown). This suggests that untransformed and reduced-activity plants do not differ greatly in the redox state of the chloroplast electron transport chain, the photoprotective function of PSII, or thylakoid pH, or, alternatively, that any changes of redox state in the reduced-activity plants are not as extreme as the differences occurring in isolated chloroplasts in the presence and absence of an electron acceptor such as OAA.

Because the activation state of NADP-MDH depends on the redox states of both the thioredoxin pool and the NADP(H) pool (Ashton and Hatch, 1983b; Rebeille and Hatch, 1986), and the interaction between these two regulators seems to be synergistic, it may be that small changes in both are sufficient to achieve the changes in NADP-MDH activation state seen in the transformed plants. Although it would be interesting to measure the redox state of the NADP(H) pool in the transformed and untransformed plants, such an experiment would be technically demanding, since we would need to distinguish the chloroplastic from the cytoplasmic pools, the mesophyll from the bundle-sheath pools, and, most difficult of all, the bound from the free pool.

NADP-MDH Activities in Isolated Bundle-Sheath Cell Strands

Unlike C_4 monocot grasses, *F. bidentis* contains significant NADP-MDH activity in the bundle-sheath cell chloroplast (Meister et al., 1996). The effectiveness of cosense suppression of NADP-MDH in each of these compartments will depend on the sequence similarity between the transgene and the endogenous gene. Although we do not have any direct evidence for NADP-MDH gene copy number in *F. bidentis*, there is only one gene for NADP-MDH in the range of C_3, C_3-C_4, and C_4 species of the genus *Flaveria* (McGonigle and Nelson, 1995). There are two genes for NADP-MDH in sorghum (Luchetta et al., 1991), but only one of these is light-regulated and expressed to a high level. It is therefore highly probable that there is only one gene encoding NADP-MDH in *F. bidentis*, which is expressed in both the bundle-sheath cells and the mesophyll cells. In this situation cosense suppression should be equally effective in both compartments. However, if the mechanism of cosense suppression in these plants involves posttranscriptional degradation of mRNA, then its effectiveness will also depend on the abundance of the transgene and the endogenous gene transcripts (Lindbo et al., 1993). If these differ between the bundle sheath and the

mesophyll, then the degree to which gene expression is suppressed could vary between the two compartments. This would obviously have important consequences for our interpretation of the analysis of these plants. Although the relative levels of expression of the CaMV 35S promoter appear to be similar in both compartments (Chitty et al., 1994), they have not been accurately quantified. It is therefore possible that the levels of sorghum transcript could vary between the two cell types. In addition, since the amounts of NADP-MDH protein in the two compartments are very different, it is probable that the levels of endogenous mRNA also differ widely between the cell types (McGonigle and Nelson, 1995).

Therefore, we have examined the extent of cosense suppression in each cell type by measuring NADP-MDH activity in bundle-sheath cell strands and mesophyll cell-enriched fractions prepared from plants differing in total activity of NADP-MDH. We initially confirmed that we can routinely activate NADP-MDH from different compartments using *E. coli* thioredoxin by activating the enzyme from the whole leaf or from mesophyll-enriched and purified bundle-sheath cell strands prepared from untransformed and reduced activity (12–7–20) plants. In each case the maximum activity of NADP-MDH was the same whether activation of the enzyme was with *E. coli* thioredoxin or with high pH (not shown). The distribution of enzymes between mesophyll and bundle-sheath cells was then determined by measuring the maximum activities of NADP-MDH and marker enzymes in each of these fractions (Table III). Untransformed plants had about 2.4 μmol min^{-1} mg^{-1} chlorophyll NADP-MDH in the bundle-sheath cells. This is considerably less than the 11 μmol min^{-1} mg^{-1} chlorophyll reported by Meister et al. (1996), but is nonetheless a significant activity. In reduced-activity plants, the activity of the bundle-sheath cell NADP-MDH was much reduced, about 0.12 μmol min^{-1} mg^{-1} chlorophyll. When expressed as a percentage, 12-7-20 had 9% of normal activity in mesophyll cell-enriched fractions, and 5% in bundle-sheath cell strands, demonstrating that cosense suppression is similarly effective in reducing activity of NADP-MDH in both the bundle-sheath and the mesophyll compartments.

Table III. *Enzyme activities in bundle-sheath and mesophyll cell fractions from untransformed and transformed F. bidentis*

Activities of NADP-MDH and the marker enzymes PEPCX and NADP-malic enzyme were measured in whole-leaf extracts, and in mesophyll-enriched and purified bundle-sheath cell strands prepared from both untransformed and reduced-activity plants. Activities are mean ± SE (n = 3).

Fraction	Activity		
	PEPCX	NADP-malic enzyme	NADP-MDH
	μmol min^{-1} mg^{-1} chlorophyll		
Untransformed			
Whole leaf	9.0 ± 3.4	8.7 ± 2.3	21 ± 5
Mesophyll-enriched	24 ± 7	6.5 ± 0.7	49 ± 2
Bundle sheath	0.8 ± 0.1	23 ± 4	2.4 ± 1.1
Reduced			
Whole leaf	16 ± 5	11 ± 1	3.6 ± 1.9
Mesophyll-enriched	30 ± 6	7.3 ± 0.4	4.2 ± 1.5
Bundle sheath	0.9 ± 0.1	29 ± 9	0.12 ± 0.06

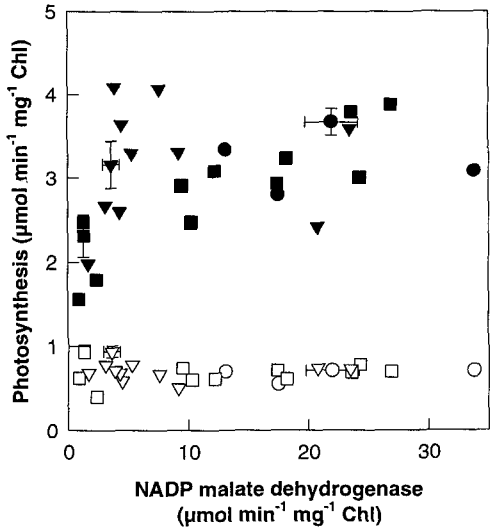

Figure 6. Control analysis of NADP-MDH. The rate of photosynthesis and NADP-MDH activity were measured in leaves of untransformed plants and T_1 progeny from two reduced-activity lines. Closed symbols, Measured at high light (1200 μmol photons m^{-2} s^{-1}); open symbols, measured at low light (150 μmol photons m^{-2} s^{-1}). The symbols represent single measurements except where error bars are shown (mean ± SE, $n \geq 3$). ●, Untransformed; ■, line 12–7–20; ▼, line 22–1–6. Chl, Chlorophyll.

Metabolic Flux Control Analysis of NADP-MDH

Although the maximum rate of photosynthesis is inhibited in reduced-activity plants, the decrease was difficult to quantitate due to the variability of the rate between different leaves that was seen for both the untransformed and the reduced-activity plants (Fig. 2). We therefore examined the relationship between maximum catalytic activity of NADP-MDH activity and photosynthesis rate by measuring these parameters in a large number of T_1 plants with a wide range of activities. We used untransformed and reduced-activity plants from two lines for this analysis (Fig. 6). These data show that at high light, the rate of photosynthesis was affected only in plants with a severe (less than about 10% of normal) decrease in activity; plants with greater than about 10% of normal activity had apparently normal rates of photosynthesis. When measurements were made at low light, even plants with the most severe reduction in activity had normal rates of photosynthesis.

The control over the rate of flux through a metabolic pathway that is exerted by a particular enzyme can be quantified by metabolic flux control analysis (Kacser and Burns, 1973). The metabolic flux control coefficient for an enzyme (C_j) can be calculated from a graph such as that shown in Figure 6, as the slope of the line where activity is reduced from 100%. We conclude from our data that even under high light, when there is the greatest flux of fixed carbon through NADP-MDH, C_j for NADP-MDH is low, i.e. the enzyme exerts little control over the steady-state

rate of photosynthesis. It is only in transformants with a large decrease in activity that NADP-MDH becomes limiting and the maximum rate of photosynthesis is reduced. Since the activation state of the enzyme does not change until there is a severe decrease in total activity (Table II), we can be confident that our measurements of activity in untransformed and T_1 plants with less than a 50% decrease in activity are comparable.

As shown in Table III, cosense suppression of expression appears to be equally effective at reducing activity of NADP-MDH in the bundle-sheath cell and in the mesophyll cell chloroplast of 12–7–20 (10% of normal activity). It is quite probable that T_1 plants with just a slight decrease in total activity of NADP-MDH are reduced in both bundle-sheath and mesophyll cell NADP-MDH. If this is indeed the case, we can conclude that C_i for both bundle-sheath and mesophyll enzyme will be low.

Metabolic Activities of Isolated Bundle-Sheath Cell Strands

In maize, fixed carbon is transported from the mesophyll cells to the bundle-sheath cells largely as malate (Hatch, 1971). However, from measurements of the metabolic properties of bundle-sheath cell strands isolated from *F. bidentis*, and from $^{14}CO_2$-labeling experiments, Meister et al. (1996) suggested that in this plant approximately 35 to 40% of fixed carbon is transported to the bundle-sheath cells as Asp rather than malate. The Asp is transaminated to OAA by Asp aminotransferase, which is then reduced to malate by bundle-sheath-localized NADP-MDH. As described above, the transgenic plants with low total activity of NADP-MDH have extremely low activities of this enzyme in the bundle-sheath cells, and might therefore be expected to differ from untransformed plants in the pathway of carbon transport between the mesophyll and bundle-sheath cells. We have examined the light-dependent metabolic activities of isolated bundle-sheath cell strands prepared from untransformed and reduced-activity plants to investigate this possibility. The results from a typical experiment are shown in Table IV. Strands isolated from both an untransformed plant and 12-7-20 showed substantial rates of light-dependent O_2 evolution when supplied with Asp, 2OG, PGA, and $NaHCO_3$. However, strands from the two plants differed in the extent to which they could metabolize Asp and 2OG alone; whereas those from untransformed plants were able to support significant rates of O_2 evolution (about 38% of maximum) under these conditions, strands from 12–7–20 could support rates of only about 13% of maximum (experiments 1 and 3). Similarly, whereas the addition of Asp and 2OG in the presence of PGA and $NaHCO_3$ stimulated the rate of O_2 evolution of untransformed strands, it had no effect on metabolism of strands from 12–7–20 (experiments 2 and 4). The rate of Asp plus 2OG-dependent O_2 evolution in the 12–7–20 strands (0.09–0.12 μmol min^{-1} mg^{-1} chlorophyll) is similar to the maximum activity of NADP-MDH in the bundle-sheath cell strands (0.12 μmol min^{-1} mg^{-1} chlorophyll). In untransformed plants the rate of Asp plus 2OG-

Table IV. *O_2 exchange of isolated bundle-sheath cell strands from untransformed and reduced-activity plants*

Bundle-sheath cell strands prepared from untransformed or reduced-activity (12–7–20) plants were incubated in an O_2 electrode at 25°C. The rate of O_2 release into the medium in the presence of the appropriate substrates was measured.

Substrate	Untransformed		12-7-20	
	μmol min^{-1} mg^{-1} chlorophyll	%[a]	μmol min^{-1} mg^{-1} chlorophyll	%[a]
Experiment 1				
+ Light	0.0	0	0.0	0
+ Asp + 2OG	0.28	36	0.09	11
+ PGA	0.71	89	0.63	80
+ $NaHCO_3$	0.79	100	0.79	100
Experiment 2				
+ Light	0.0	0	0.0	0
+ $NaHCO_3$	0.0	0	0.18	17
+ PGA	0.79	92	1.04	100
+ Asp + 2OG	0.86	100	1.04	100
Experiment 3				
+ Light	0.0	0	0.0	0
+ Asp + 2OG	0.30	39	0.12	15
+ $NaHCO_3$	0.36	48	0.16	20
+ PGA	0.75	100	0.79	100
+ Malate	0.62	82	0.64	81
Experiment 4				
+ Light	0.0	0	0.0	0
+ PGA	0.57	72	0.62	68
+ $NaHCO_3$	0.71	90	0.92	100
+ Asp + 2OG	0.79	100	0.92	100

[a] Percentage of maximum rate during each experiment.

dependent O_2 evolution (0.3 μmol min^{-1} mg^{-1} chlorophyll) was only 12.5% of the maximum NADP-MDH activity.

These measurements confirm that bundle-sheath cell strands isolated from untransformed *F. bidentis* can sustain significant rates of O_2 evolution with Asp and 2OG as substrates. In addition, activities of NADP-MDH measured in bundle-sheath cells are sufficient to account for about 50% of the maximum rate of photosynthesis of untransformed plants, supporting the scheme proposed by Meister et al. (1996). However, two lines of evidence suggest that the pathway of C_4 acid metabolism is altered in the reduced-activity plants. First, as described above, bundle-sheath cell strands isolated from reduced-activity plants can sustain only a low rate of O_2 evolution with Asp and 2OG as substrates. Second, the activity of NADP-MDH in bundle-sheath cell strands prepared from reduced-activity plants is extremely low (Table III). Typical activities in these strands of 0.12 μmol min^{-1} mg^{-1} chlorophyll would be sufficient to catalyze only 6% of the maximum rate of photosynthesis of the reduced-activity plants (2 μmol min^{-1} mg^{-1} chlorophyll). These results suggest that bundle-sheath cell-localized NADP-MDH cannot be essential for sustaining high rates of photosynthesis in *F. bidentis*. It is likely that *F. bidentis* is able to adapt to a decrease in the amount of bundle-sheath cell NADP-MDH by transferring more carbon to this compartment in the form of malate, although further experiments are required to confirm this.

The fact that *F. bidentis* can function reasonably efficiently with very little NADP-MDH in the bundle-sheath cells implies that the presence of the enzyme in this compartment may represent an intermediate step in the evolution of C_4 photosynthesis. There was no increase in the chlorophyll *a* to chlorophyll *b* ratio in the bundle-sheath cell strands of reduced-activity plants (not shown). This indicates that, despite the probable shift in the pathway of C_4 acid metabolism, the plant does not acclimate by reducing activity of PSII in the bundle-sheath cells.

CONCLUDING COMMENTS

NADP-MDH, either in the mesophyll or in bundle-sheath cells, has a low metabolic flux control coefficient for photosynthesis in the C_4 plant *F. bidentis*. Previous work in this laboratory has shown that Rubisco partially limits the steady-state rate of photosynthesis at moderate to high light intensities in *F. bidentis* (Furbank et al., 1996) with an estimated C_i of 0.4 at 2000 μmol photons m^{-2} s^{-1} (from figure 9 of Furbank et al., 1996). Another highly regulated enzyme, PPDK, contributes to the limitation on photosynthesis in *F. bidentis* at high light intensities (C_i approximately 0.3–0.4) but not at low light intensities (R.T. Furbank, J.A. Chitty, C.L.D. Jenkins, S.J. Trevanion, S. von Caemmerer, and A.R. Ashton, unpublished data). Mutants of *Amaranthus edulis*, a NAD-malic enzyme-type C_4 plant deficient in PEPCX, have been produced by Dever et al. (1995). F_1 heterozygotes with about 50% of normal activity appear to grow normally in air, but do have a slightly reduced rate of photosynthesis at very high light intensities (2000 μmol photons m^{-2} s^{-1}). However, this decrease is only slight, and the plants appear to behave normally at lower light. This demonstrates that PEPCX also has very little control over the steady-state rate of photosynthesis in C_4 plants. Together these observations suggest that significant control of the rate of photosynthesis in C_4 plants at high light intensities may be shared by Rubisco in the C_3 cycle and by PPDK in the C_4 cycle. It is also clear that all of the limitations on C_4 photosynthesis at high light intensity have yet to be identified.

Since NADP-MDH is a highly regulated enzyme but does not limit photosynthesis in *F. bidentis*, one could ask what is the purpose of the complex regulation of the enzyme. One role of the covalent regulation may be to switch the enzyme off in the dark to limit the potential for transferring reducing equivalents into and out of the chloroplast. Another, and perhaps more important, role of the covalent regulation may be to maintain the NADP pool largely reduced during steady-state photosynthesis. This would allow the other NADPH-requiring reactions of the chloroplast, such as amino acid, lipid, and secondary product biosynthesis, to compete for NADPH in the face of the vastly greater catalytic capacity of the enzymes of photosynthetic CO_2 assimilation (A.R. Ashton and S.J. Trevanion, unpublished data; R.T. Furbank, J.A. Chitty, C.L.D. Jenkins, S.J. Trevanion, S. von Caemmerer, and A.R. Ashton, unpublished data). Answers to specific questions regarding the role of the regulatory properties of NADP-MDH might have been provided by analysis of plants overexpressing the mutant forms of the enzyme. However, in light of the high frequency of cosense suppression between the sorghum and *F. bidentis* enzymes, this approach of genetic transformation of the nuclear genome is unlikely to be successful.

A review of the literature shows that the sequence identity between an introduced and endogenous gene that is required for cosense suppression is not generally documented, or even known. Although it has generally been assumed that sequences have to be nearly identical to observe this phenomenon, the coding region for NADP-MDH from *F. bidentis*, a dicot, has only 71% overall sequence identity with the coding sequence of the clone from sorghum, a monocot (S.J. Trevanion and A.R. Ashton, unpublished data). This observation extends the range of combinations of clones that should be considered in attempts to produce plants with reduced levels of enzymes. However, it also stresses the need for choosing widely divergent heterologous clones when attempting to overexpress enzymes in plant tissues.

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