Antisense Reduction of NADP-Malic Enzyme in *Flaveria bidentis* Reduces Flow of CO2 through the C4 Cycle\[W]\[OA]

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An antisense construct targeting the C4 isozyme of NADP-malic enzyme (ME), the primary enzyme decarboxylating malate in bundle sheath cells to supply CO2 to Rubisco, was used to transform the dicot *Flaveria bidentis*. Transgenic plants (α-NADP-ME) exhibited a 34% to 75% reduction in NADP-ME activity relative to the wild type with no visible growth phenotype. We characterized the effect of reducing NADP-ME on photosynthesis by measuring in vitro photosynthetic enzyme activity, gas exchange, and real-time carbon isotope discrimination (Δ). In α-NADP-ME plants with less than 40% of wild-type NADP-ME activity, CO2 assimilation rates at high intercellular CO2 were significantly reduced, whereas the in vitro activities of both phosphoenolpyruvate carboxylase and Rubisco were increased. Δ measured concurrently with gas exchange in these plants showed a lower Δ and thus a lower calculated leakiness of CO2 (the ratio of CO2 leak rate from the bundle sheath to the rate of CO2 supply). Comparative measurements on antisense Rubisco small subunit *F. bidentis* plants showed the opposite effect of increased Δ and leakiness. We use these measurements to estimate the C4 cycle rate, bundle sheath leak rate, and bundle sheath CO2 concentration. The comparison of α-NADP-ME and antisense Rubisco small subunit demonstrates that the coordination of the C3 and C4 cycles that exist during environmental perturbations by light and CO2 can be disrupted through transgenic manipulations. Furthermore, our results suggest that the efficiency of the C4 pathway could potentially be improved through a reduction in C4 cycle activity or increased C3 cycle activity.

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In the leaves of a range of plants including maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), and millet (*Pennisetum americanum*), a biochemical pathway known as C4 photosynthesis has evolved to concentrate CO2 at the site of Rubisco such that Rubisco can operate at close to its maximal activity and photorespiration is reduced, enhancing the rate of photosynthesis in air (Hatch, 1987; Sage, 2004). In most C4 plants, CO2 is fixed by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells into four-carbon acids, which diffuse to an inner ring of bundle sheath cells, where they are decarboxylated and the CO2 is refluxed by Rubisco. Plants using the C4 photosynthetic mechanism have been subdivided into three primary subtypes, the NADP-malic enzyme (ME), NAD-ME, and phosphoenolpyruvate carboxykinase types, according to the decarboxylating enzyme used to generate CO2 from C4 acids in the bundle sheath cells (Hatch, 1987). *Flaveria bidentis* is a typical NADP-ME dicot in which malate and Asp contribute equally in the transfer of CO2 to bundle sheath cells (Meister et al., 1996). Presumably, in most C4 plants, the reactions that facilitate the appropriation, transformation, transport, and eventual concentration of CO2 in the bundle sheath chloroplasts (C4 cycle) are balanced with the reactions that incorporate CO2 into usable carbon compounds for energy (C3/Calvin cycle) such that energy is not lost or wasted as environmental conditions fluctuate. This process is important in maintaining the efficiency of the CO2-concentrating mechanism and of C4 photosynthesis overall. The nature of the controlling mechanisms for balance and coordination between the C3 and C4 cycles is still unclear, however, and concrete evidence for the coordinated regulation of primary carboxylation in the mesophyll and decarboxylation of C4 acids in the bundle sheath has not been forthcoming. A key approach to revealing these mechanisms has been the use of antisense RNA in the C4 dicot *F. bidentis* to reduce levels of key photosynthetic enzymes, including Rubisco (Furbank et al., 1996), NADP-malate dehydrogenase and pyruvate phosphate dikinase (Furbank et al., 1997), Rubisco activase (von Caemmerer et al., 2005), carbonic anhydrase (Cousins et al., 2006), and PEPC protein kinase (Furumoto et al., 2007). This has proven to be a valuable method to help gain insight into enzyme function and regulation during C4 photosynthesis and to potentially alter the balance between the C3 and C4 cycles.

In this study, we targeted the gene encoding the chloroplastic C4 isozyme of NADP-ME in *F. bidentis*...
(Marshall et al., 1996) with an antisense construct designed to reduce its activity in vivo. This isoform is thought to catalyze the decarboxylation of l-malate to pyruvate and CO₂ and of NADP to NADPH in bundle sheath chloroplasts during C₄ photosynthesis (Ashton, 1997; Drinovitch et al., 2001), allowing the CO₂ to be fixed into the C₃ cycle by Rubisco and pyruvate to return back to mesophyll cells to be recycled into PEP. These antisense lines were generated for two purposes. First, these plants could be used to confirm the identity of the gene encoding the NADP-ME isozyme involved in C₄ photosynthesis. Several other functioning isoforms of NADP-ME have also been identified within Flaveria spp.: a chloroplastic but potentially nonphotosynthetic NADP-ME form and a cytosolic NADP-ME (Marshall et al., 1996; Drinovitch et al., 1998; Lai et al., 2002). The specific role and regulation of a C₄ NADP-ME isozyme in F. bidentis is of interest in relation to the “transfer” or “generation” of a functioning C₄ cycle to C₃ plants (Sheehy et al., 2007; Furbank et al., 2009). A greater understanding of the balance and interactions between this enzyme and others in the C₄ and C₃ cycles will aid in deciding the expression locations and levels needed for C₃ plants to gain a functional CO₂-concentrating mechanism.

The second use of these antisense plants was to investigate the degree of coordination between the C₄/C₃ cycles in F. bidentis and the possibility of manipulation to improve photosynthetic efficiency. As mentioned above, the mechanisms of regulation (if any) of the C₃ pathway enzymes such as Rubisco in response to the activity and CO₂ supply rate of the C₄ cycle are unknown. It is similarly unclear how much the reactions of the C₃ cycle affect the rates of the initial CO₂-fixing reactions (carbonic anhydrase and PEPC). Leakiness (ϕ), defined as the ratio of CO₂ leak rate from the bundle sheath to the rate of CO₂ supply, reflects the coordination of the C₄ and C₃ cycles by describing the amount of overcycling of the C₄ cycle that has to occur to support a given rate of net CO₂ assimilation (Furbank et al., 1990; von Caemmerer and Furbank, 1999). As a major C₄ enzyme functioning within the bundle sheath, a reduction in NADP-ME should affect both the C₄ cycle rate and the bundle sheath CO₂ concentration (Cₛ), possibly disrupting the enzymatic balance and coordination in F. bidentis.

Here, we have designed experiments to simultaneously look at in vitro photosynthetic enzyme activity, gas exchange, and real-time carbon isotope discrimination (Δ), facilitating estimates of ϕ, C₄ cycle rate, and the possible range of Cₛ within transgenic α-NADP-ME and antisense Rubisco small subunit (α-SSu) F. bidentis plants (Furbank et al., 1996). These measurements aim to show the impact of our perturbations of the C₄/C₃ balance, highlighting possible communication pathways between the cycles and also other possible targets for future genetic manipulation to improve the rate and/or efficiency of photosynthesis in C₄ plants.

**RESULTS**

**Generation and Characterization of Transformants**

Agrobacterium tumefaciens transformation of F. bidentis with an antisense construct targeting the chloroplastic NADP-ME yielded 12 independent primary transformants. Of these, eight lines were successfully regenerated through selective tissue culture and replanted in soil: 1a4, 1a5, 1a6, 1a7, 1a8, 2a1, 2a2, and 4a1. Primary transformants were screened using the in vitro NADP-ME, Rubisco, and PEPC assays for percentage activity relative to a tissue culture-regenerated control plant and standard wild-type F. bidentis. All mutants showed NADP-ME activity from 34% to 75% of control plants, while Rubisco and PEPC activities were slightly elevated above controls (data not shown). All lines were selfed and grown to seed, and from these, seed was germinated from three of the lines, 1a4, 2a1, and 4a1, and the segregating T1 seedlings again were screened for low NADP-ME activity. Six plants were selected from each line (18 plants in total), encompassing a range of NADP-ME activities for use in experiments. Six plants from a wild-type line with normal NADP-ME activity were grown in parallel for experimental comparison. NADP-ME activity levels in all plants were confirmed by western blotting (Supplemental Fig. S1). Measurements were also made on F. bidentis plants with antisense constructs targeting the Rubisco small subunit (Furbank et al., 1996) grown under identical conditions for comparison. These plants showed wild-type PEPC activity and reduced Rubisco activity (20%–35% of wild-type values; Supplemental Fig. S3).

**Relationship of CO₂ Assimilation Rate, Rubisco and PEPC Activity, and Leaf Nitrogen Content to NADP-ME Activity**

The in vitro activity of NADP-ME, Rubisco, and PEPC was measured in all 24 plants spectrophotometrically (Fig. 1). NADP-ME activity in wild-type plants (n = 6) varied from 57 to 80 μmol m⁻² s⁻¹ (average of 72 ± 3), whereas variation within the mutants (n = 18) was from 25 to 75 μmol m⁻² s⁻¹. Gas exchange of all greenhouse-grown plants was conducted in situ over a period of 3 d, during which the CO₂ assimilation rate of each plant was measured three times. The CO₂ assimilation rate was significantly reduced in mutant plants with less than 30 μmol m⁻² s⁻¹ NADP-ME activity (Fig. 1A), yet above this level, little impact on CO₂ assimilation rate was observed. Activity of PEPC and Rubisco in relation to NADP-ME activity showed a slight negative correlation for both enzymes (Fig. 1, B and C), while no change in the PEPC/Rubisco was observed (Supplemental Fig. S2). A slight negative correlation was also observed between leaf nitrogen and NADP-ME activity, indicating an increase in nitrogen per leaf area in plants with less NADP-ME activity (Fig. 1E). The average total leaf nitrogen for wild-type plants (n = 6) was 112.3 ± 5.3
This differed from the average total leaf nitrogen for NADP-ME plants (with less than 30 μmol m⁻² s⁻¹ NADP-ME activity; n = 4) at 146.6 ± 2.6 mmol m⁻² (P ≤ 0.001). No significant differences were observed in either chlorophyll content or the chlorophyll a/b ratio between mutants and wild-type plants (Supplemental Fig. S2, B and C).

**CO₂ Response of CO₂ Assimilation Rate**

Figure 2 shows the response of the CO₂ assimilation rate to increasing intercellular CO₂ partial pressure (Ci) in four wild-type and eight α-NADP-ME mutant plants. The mutant population included four plants that exhibited the most significant reduction in the initial NADP-ME activity screening relative to the wild-type (activities between 34% and 40% of wild-type values). The steep initial rise in CO₂ assimilation rate from 10 to 60 μbar Ci characteristic of C₄ plants did not differ between the wild type and mutants (Fig. 2B), yet assimilation rate at high Ci was significantly reduced in the α-NADP-ME plants, with NADP-ME activity below 40% of the wild-type level (Fig. 2A).

**Concurrent Gas Exchange and Δ**

Carbon isotope composition measured on dried leaf discs (δ¹³C, relative to the standard V-Pee Dee Belemnite) taken from glasshouse-grown wild-type and α-NADP-ME plants showed no significant differences (Fig. 1D). Online measurements of Δ measured concurrently with gas exchange were performed on three individual wild-type and three α-NADP-ME plants with low (under 30 μmol CO₂ m⁻² s⁻¹) NADP-ME activity over a range of both increasing Ci (Fig. 3) and irradiance (Fig. 4). Stomatal conductance remained similar between the wild type and mutants (Figs. 3B and 4B), yet as assimilation rates were lower in the α-NADP-ME plants (Figs. 3A and 4A), the ratio of intercellular to ambient CO₂ (Ci/Ca) was greater in mutants than in wild-type plants (Figs. 3C and 4C). Online measurements indicated that Δ was significantly lower in α-NADP-ME plants (Figs. 3D and 4D), and φ was also calculated to be less than in wild-type plants (Figs. 3E and 4E).

When plotted against Ci/Ca (Fig. 5A), Δ measurements for wild-type and α-NADP-ME plants spread discretely along theoretical lines estimating the relationship between Δ and Ci/Ca using the C₄ model by Farquhar (1983): φ values of 0.25 and 0.184 were used, respectively, and assuming saturating amounts of carbonic anhydrase such that the reversible conversion of CO₂ and HCO₃⁻ is at isotopic equilibrium (Cousins et al., 2006). Identical measurements made for comparison on three transgenic α-SSu F. bidentis plants (Supplemental Fig. S3) showed increased φ compared with wild-type plants (φ of approximately 0.34) and also plotted alongside the equivalent C₄ model line.
(2.1 ± 0.1 and 2.0 ± 0.1 m² m⁻², respectively), and a small change was found in $S_m$ for α-NADP-ME plants (15.3 ± 0.6 m² m⁻² compared with the wild type at 17.5 ± 0.7 m² m⁻²).

**Figure 2.** Gas exchange of wild-type ($n = 4$) and α-NADP-ME ($n = 8$) *F. bidentis* plants. CO₂ assimilation rates are given over a complete (A) and low (B) range of $C_i$. Lines depicted are four wild-type plants (open circles), four α-NADP-ME plants with NADP-ME activity from 55% to 95% of the wild type (closed triangles), and four α-NADP-ME plants with NADP-ME activity below 40% of the wild type (closed circles). Error bars show three technical replicates of individual plants. Measurements were made in the glasshouse at 1,500 μmol quanta m⁻² s⁻¹ and a leaf temperature of 25°C.

(Fig. 5A). Figure 5B shows the different $\phi$ calculations in wild-type, α-NADP-ME, and α-SSu plants yet comparatively negligible changes to each as a function of increasing $C_i$.

**Anatomical Measurement of $S_m$ and $S_b$**

The surface area of mesophyll cells exposed to intercellular airspace ($S_m$) and the bundle sheath cell surface area ($S_b$) were measured in embedded leaf sections of wild-type and α-NADP-ME plants (Supplemental Fig. S4). No significant difference was found in $S_b$, but a small change was found in $S_m$ for α-NADP-ME plants (15.3 ± 0.6 m² m⁻² compared with the wild type at 17.5 ± 0.7 m² m⁻²).

**Figure 3.** Concurrent measurement of CO₂ assimilation rate (A), stomatal conductance (B), $C_i/C_a$ (C), $\Delta$ (D), and $\phi$ (E) as a function of $C_i$. Lines and error bars represent averages and SE of measurements on three individual wild-type (open circles) and α-NADP-ME (closed circles) *F. bidentis* plants, respectively. Measurements were made at 1,500 μmol quanta m⁻² s⁻¹ and a leaf temperature of 25°C.
Estimation of $f$, C₄ Cycle Rate, Bundle Sheath Leak Rate, and Bundle Sheath CO₂

We used the concurrent measurements of $f$ and CO₂ assimilation rates to estimate the C₄ cycle and bundle sheath leak rate (Fig. 6), as outlined in “Materials and Methods.” In α-NADP-ME plants with the lowest CO₂ assimilation rates, both the rate of the C₄ cycle and the bundle sheath leak rate have been reduced (Fig. 6, B, C, E, and F), whereas in the α-SSu plants, the bundle sheath leak rate was similar to the wild-type rate accompanied by a reduction in the C₄ cycle rate.

The anatomical measurements above showed no differences in $S_{io}$; therefore, we assumed the same bundle sheath resistance of 333 m² s⁻¹ mol⁻¹ bar⁻¹ to estimate $C_s$. Low α-NADP-ME plants exhibited a reduced $C_s$ estimation (compared with the wild-type) in response to increasing $C_i$ (Fig. 7A) and irradiance (Fig. 7B), whereas $C_s$ in α-SSu plants was predicted to be more similar to the wild-type value. Estimation of $C_s$ is linearly dependent on the assumed bundle sheath resistances, and this...
highlights the uncertainty in the absolute values of $C_s$ but relative differences remain the same (Fig. 7C).

DISCUSSION

An Antisense Construct Targets the Photosynthetic $C_4$ NADP-ME Isoform in *F. bidentis*, Reducing CO$_2$ Assimilation Rate

An antisense construct targeted to the central coding region of *ChlMe1* reduced total NADP-ME leaf activity in transformed *F. bidentis* plants considerably. In *F. bidentis* leaves, *ChlMe1* has been shown to be the dominant expressing isoform at both the mRNA and protein levels (Marshall et al., 1996; Drincovich et al., 1998), so it is likely that the reduction seen in the in vitro NADP-ME activity from whole leaf extracts is mostly due to the reduction in *ChlMe1* transcripts rather than other isoforms. Despite this, enough sequence similarity exists between *ChlMe1* and *ChlMe2* that *ChlMe2* transcripts may also have been reduced in the antisense plants. The probable function of the

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**Figure 6.** CO$_2$ assimilation rate (A and D), $C_4$ cycle rate (B and E), and bundle sheath leak rate (C and F) as a function of $C_i$ (A–C) and irradiance (D–F) in wild-type (open circles, open squares), $\alpha$-NADP-ME (closed circles), and $\alpha$-Ssu (closed squares) *F. bidentis* plants. The $C_4$ cycle and bundle sheath leak rate were calculated from Equations 6 and 7.

**Figure 7.** Estimated $C_s$ as a function of $C_i$ (A), irradiance (B), and bundle sheath resistance (C). Symbols represent estimated bundle sheath CO$_2$ levels at the bundle sheath resistance assumed in this study for $\phi$ measurements (approximately 333 m$^2$ s$^{-1}$ bar$^{-1}$ mol$^{-1}$).
NADP-ME isoenzyme encoded by *ChlMe1* is in *C₄* photosynthesis, as the major enzyme responsible for decarboxylation of the *C₄* acids malate and Asp, releasing CO₂ in close proximity to Rubisco for CO₂ assimilation in the Calvin cycle. This role is based on studies linking the light-regulated, leaf-specific expression of *ChlMe1* in *F. bidentis* species with immunolocalization of the enzyme at high concentrations in bundle sheath chloroplasts (Marshall et al., 1996, 1997; Drincovich et al., 1998). The data shown here support this role, linking the specific decline of NADP-ME activity (less than 30 μmol CO₂ m⁻² s⁻¹) in *F. bidentis* leaves to a measurable decrease in photosynthetic CO₂ assimilation rate at saturating Cᵢ.

This effect on CO₂ assimilation rate is similar to that observed in *F. bidentis* plants with reduced Rubisco small subunit levels (Furbank et al., 1996; von Caemmerer et al., 1997). As predicted in the C₂ model, the CO₂-saturated portion of an A/Cᵢ curve (response of CO₂ assimilation rate to intercellular CO₂) in *C₄* plants at high irradiance is naturally limited by either PEP or ribulose 1,5-bisphosphate regeneration or by maximum Rubisco activity (Berry and Farquhar, 1978; von Caemmerer and Furbank, 1999). As Rubisco activity in vitro was not reduced in α-NADP-ME plants (Fig. 1C), the reduction in CO₂ assimilation rate at high CO₂ in the α-NADP-ME plants is most likely due to reduced C₄ cycle regeneration rate rather than a Rubisco limitation. The similarity of maximum CO₂ assimilation rates in wild-type and antisense plants exhibiting greater than 30 μmol CO₂ m⁻² s⁻¹ NADP-ME activity suggests that in wild-type leaves the *C₄* NADP-ME is either present in excess or is regulated to limit its activation, as observed with other C₄ enzymes. In α-SSu lines, both Rubisco content and maximal activity were shown to correlate linearly with CO₂ assimilation rates under saturating illumination (Furbank et al., 1996; von Caemmerer et al., 1997), indicating that Rubisco was a major limitation for maximal photosynthetic flux, with a control coefficient of 0.5 to 0.7. In contrast, extractable activities of NADP-malate dehydrogenase (Furbank et al., 1997) have been shown to be 10 times that required to support CO₂ assimilation rates (i.e. an effective flux control coefficient of zero). Similarly, it seems that in *F. bidentis*, NADP-ME can be reduced to approximately one-half the wild-type content (based on in vitro activity assays) without affecting photosynthetic rates or growth, again indicating a control coefficient of effectively zero. It seems unlikely that in *F. bidentis*, regulation of NADP-ME is limiting photosynthetic flux in any major way or playing a direct regulatory role in coordinating relative fluxes through the C₃ and C₄ cycles in wild-type plants.

**Increase in Rubisco and PEPC Activity and Leaf Nitrogen in Low-NADP-ME Antisense Plants**

The increased activity of PEPC and Rubisco measured in plants with NADPH-ME activity (and reduced photosynthetic performance at high Cᵢ) is intriguing with respect to C₃-C₄ cycle coordination. Strong positive correlations are commonly observed between both PEPC and Rubisco activity and maximum photosynthetic rates (Sage et al., 1987; von Caemmerer et al., 1997), yet here we observed the opposite in antisense NADP-ME plants. One possibility for these observations is that the decline in the amount of NADP-ME has altered the amount of nitrogen available for enzyme production, causing general increases inRubisco and PEPC protein expression. However, the increase in PEPC and Rubisco activity was matched by an increase in leaf nitrogen in this study (Fig. 1E), with plants exhibiting the most significant reductions in NADP-ME activity (less than 40% of wild-type values; *n* = 4) showing on average 30% more leaf nitrogen than the wild type (*n* = 6). In *C₄* plants, Rubisco has been estimated to account for between 5% and 15% of total leaf nitrogen, PEPC for between 2% and 6%, and NADP-ME for approximately 1% to 1.5% (Sage et al., 1987; Evans and von Caemmerer, 2000; Makino et al., 2003; Ghanoum et al., 2005). From the in vitro activity (Fig. 1B) of Rubisco (assuming that Rubisco is 16% nitrogen by mass and has a molecular weight of 550,000 g mol⁻¹; Ghanoum et al., 2005) and the total nitrogen per leaf area (Fig. 1E), we estimate that 15.7% ± 0.9% and 14.8% ± 0.8% of the total leaf nitrogen present in these plants can be attributed to Rubisco in wild-type plants (*n* = 6) and low-NADP-ME mutants (less than 40% of the wild type; *n* = 4), respectively. These estimates indicate that the increase in total leaf nitrogen has been mirrored by a proportional increase in Rubisco. The substantially lower proportion of nitrogen estimated for NADP-ME (Evans and von Caemmerer, 2000) would indicate that its reduction alone in antisense plants would not account directly for the increase in leaf nitrogen observed. It is possible, though, that its reduction may have an indirect effect, possibly altering common transcription factors, metabolites, or other regulators of activity/expression for PEPC and Rubisco. A more detailed study of photosynthetic metabolite levels would be required to examine changes in photosynthetic flux, but even this approach would be hampered by the complexities of the anatomical separation of photosynthetic processes within *C₄* leaves.

**Antisense Reduction of NADP-ME Alters C₃-C₄ Cycle Coordination**

While the mechanisms and extent of the communication between the C₃ and C₄ photosynthetic cycles are not well understood, there are logical reasons (reducing energy loss, maximizing energy gain) and experimental evidence for both coordination and balance between the two sets of reactions. Some early evidence for C₃-C₄ cycle interaction includes metabolite measurements made in maize (Leegood and Furbank, 1984a, 1984b) and *Amaranthus edulis* (Leegood and von Caemmerer, 1988) showing concurrent changes of C₃
and C₄ metabolite levels in response to differing CO₂ concentrations and irradiances. Additional evidence is found in the regulation of the C₄ enzyme PEPC activity in maize by the in vivo concentrations of its substrate PEP and the C₄ acid malate (Doncaster and Leegood, 1987), suggestive of a mechanism by which PEPC activity might be balanced with that of other C₃ and C₄ enzymes.

Here, Δ has been used to estimate φ to gain a measure of coordination and balance between C₃ and C₄ cycles. From the C₄ carbon isotope model, it is clear that Δ is strongly influenced by both Cᵢ/C₄ and φ (Farquhar, 1983), mostly due to the compartmentalization of Rubisco in the bundle sheath, reducing its opportunity to discriminate. We measured both the stored dry matter (Δ₁³C) and the real-time Δ in leaves for φ estimations. Dry matter measurement of discrimination did not show a difference between wild-type and mutant populations. As discussed previously (Pengelly et al., 2011), this likely reflects the integrated nature of this measurement, which includes post-photosynthetic fractionation rather than just photosynthetic discrimination.

Using real-time Δ measurements, we clearly show that plants with NADP-ME (less than 40% of the wild type) showed less ¹³C discrimination and infer reduced φ relative to wild-type plants over increasing irradiance and Cᵢ (Figs. 3 and 4). We used the simplified equations presented in “Materials and Methods,” which make the assumption that Cₛ > > Cᵢ (for discussion, see Ubierna et al., 2011). If this were not the case, we would have overestimated φ slightly in the α-NADP-ME plants relative to the wild type.

Measurements of φ in C₄ plants have been shown to be remarkably constant over a range of irradiances, temperatures, and intercellular CO₂ concentrations (Henderson et al., 1992). This has been taken as evidence that some level of regulation of the C₃ and C₄ cycles occurs. Our estimations of φ (Figs. 3 and 4; Supplemental Fig. S3) support these earlier results showing that φ within the plant is generally maintained at a relatively constant level. The exception to this, as observed previously (Pengelly et al., 2010), is the apparent increased φ at low irradiance, although this has recently been attributed to an overestimation due to the incorrect assumption that a large difference in CO₂ concentration is maintained between the mesophyll and bundle sheath at low irradiances (Ubierna et al., 2011).

Our measurements of Δ in α-NADP-ME plants contrasts with Δ measurements (repeated in this study) made on antisense F. bidentis plants with reduced levels of the Rubisco small subunit (α-SSu; Furbank et al., 1996; von Caemmerer et al., 1997; Fig. 5A; Supplemental Fig. S3). Reduction in Rubisco resulted in an increased φ relative to the wild type. We have used φ inferred from Δ measurements from both α-NADP-ME and α-SSu plants together with the concurrent measurements of CO₂ assimilation rates to calculate estimates of the rate of the C₄ cycle and the bundle sheath leak rate (Fig. 6). In the α-NADP-ME plants with the lowest CO₂ assimilation rates, both the rate of the C₃ cycle and the bundle sheath leak rate have been reduced, whereas in the α-SSu plants, although φ was increased, the bundle sheath leak rate was similar to the wild-type value, accompanied by a reduction in the C₄ cycle rate despite very similar in vitro PEPC activities (see legend to Supplemental Fig. S3). This suggests some feedback from the C₃ to the C₄ cycle. The comparison of the performance of α-NADP-ME and α-SSu plants examined here suggests that the coordination of the C₃ and C₄ cycles that is apparent during environmental perturbations by light and CO₂ can be disrupted through transgenic manipulations.

In effect, the reduction of the decarboxylating action of NADP-ME within the bundle sheath chloroplasts of the antisense plants created here has reduced the C₄ cycle rate, and the bundle sheath leak rate has also decreased (Fig. 6). To estimate Cₛ, an estimate of bundle sheath resistance is needed. Although attempts have been made to estimate bundle sheath resistance by varies techniques, great uncertainty exists about the actual magnitude of this resistance. We assumed a value of 333 m² s⁻¹ mol⁻¹ bar⁻¹ (which is within the range of estimates made by Yin et al. [2011] for maize) to estimate Cₛ (Fig. 7). These calculations suggest reduced Cₛ for α-NADP-ME plants, whereas α-SSu plants had values similar to the wild type. To put these values into context, the apparent Michaelis-Menten constant for Rubisco carboxylation in F. bidentis at ambient oxygen partial pressures is approximately 1,000 μbar and greater if oxygen is elevated in the bundle sheath (Whitney et al., 2011), which suggests that Cₛ is not saturating for Rubisco in α-NADP-ME plants. For wild-type and α-SSu plants, Cₛ is close to saturation at four times the Michaelis-Menten constant at high light and above ambient CO₂. Figure 7C illustrates how the estimates of Cₛ depend on the bundle sheath resistance used in the calculations.

**CONCLUSION**

From our observations in this study, we confirm the function of NADP-ME (encoded by ChlMel) in the reduction of malate for CO₂ supply to Rubisco as part of the C₄ cycle in F. bidentis. We have shown that under standard light and temperature conditions in F. bidentis, NADP-ME activity exceeds what is required for maximum photosynthetic flux at high Cᵢ and that this enzyme is unlikely to be rate limiting in the C₄ photosynthetic pathway. The comparison of the photosynthetic performance of α-NADP-ME and α-SSu plants demonstrates that the coordination of the C₃ and C₄ cycles apparent during environmental perturbations by light and CO₂ can be disrupted through transgenic manipulation. Furthermore, our results suggest that the efficiency of the C₄ pathway could potentially be improved through a reduction in C₄ cycle activity or increased C₃ cycle activity.
Materials and Methods

Plasmid Construction, Transformation, and Mutant Regeneration

Total RNA was isolated and purified from Flaveria bidentis (von Caemmerer et al., 1997) leaves using the TRizol reagent (Invitrogen). Complementary DNA was synthesized using the SuperScript III First-Strand Synthesis kit (Invitrogen) from 1 µg of total RNA using the reverse specific primer NADPME-R2 (5'-ATGGTGATCTCTTCAACG-3'). NADPME-R2 and NADPME-F2 (5'-TTGGCCTTGGTGGTGGTGTGG-3') primers were designed based on the chloroplastic C4 isoform of the NADP-ME open reading frame ME1 from Flaveria trinervia and F. bidentis (GenBank accession nos. X57142 and AY863144). These primers were used to amplify, via PCR, an 845-bp fragment from the F. bidentis complementary DNA library and introduce BamHI and SacI restriction enzyme sites at the ends of the PCR fragment. The PCR fragment was ligated into the pGEM T-Easy vector (Invitrogen), sequenced to confirm identity, and designated ME1. The fragment was then digested out and inserted in the antisense orientation into the BamHI/Sacl-digested pB121 binary vector under control of a cauliflower mosaic virus promoter and named pANME2. pANME2 was subsequently transformed into Agrobacterium tumefaciens strain AgL1 and maintained in selective culture.

Plant Growth

Wild-type and antisense NADP-ME F. bidentis plants were grown to seed in growth cabinets under a 12/12-h day/night cycle at 28°C/25°C, 70% humidity, and an irradiance of 400 µmol quanta m⁻² s⁻¹. Subsequently, seed was germinated and plants grown for experiments during the summer months in a glasshouse under natural light conditions (28°C day and 18°C night temperatures). Plants were grown in 30-L pots in a garden soil mix with fertilizer (Osmocote; Scotts Australia) and watered daily. Experimental measurements were conducted on fully expanded leaves from plants approximately 30 to 40 d after germination. F. bidentis plants containing an antisense construct to the small subunit of Rubisco (Furbank et al., 1996) were grown under identical conditions for the purpose of comparative measurements.

Measurements of NADP-ME, Rubisco, and PEPC Activity in Vitro

The activity of photosynthetic enzymes was measured in vitro on leaf extracts from frozen leaf discs sampled directly after gas exchange. Rubisco and PEPC were measured as described previously (Cousins et al., 2007; Pengelly et al., 2010). The activity of NADP-ME was measured indirectly, as described previously by Hatch and Mau (1977) with minor changes outlined below, by following the formation of NADPH as malate is decarboxylated to pyruvate. As the chloroplastic type of NADP-ME is the dominant isoform in F. bidentis (Marshall et al., 1996; Drinovich et al., 1998), measurements on a whole leaf extract should predominantly represent the activity of this isoform. A 0.7-cm² frozen leaf disc was processed in ice-cold glass homogenizers with 500 µL of assay buffer (50 mM Tricine-KOH, pH 7.8, 1 mM EDTA, 0.1% Triton-X, 10 mM dithiothreitol, and 1% polyvinylpyrrolidone) and 10 µL of protease inhibitor cocktail (Sigma). Homogenate was briefly centrifuged, and the supernatant was used for NADP-ME assays. For each assay, 50 µL of leaf extract was added to 980 µL of assay buffer (50 mM Tricine-KOH, pH 8.3, 5 mM malate, 0.1 mM EDTA, and 0.5 mM NADP), and the reaction was initiated by the addition of 10 µL of 200 mM MgCl₂. Activity of NADP-ME was calculated by monitoring the increase of NADPH using a diode array spectrophotometer (Hewlett-Packard) after initiation of the reaction. Chlorophyll was extracted from frozen leaf discs in a TissueLyzer II ball mill (Retsch) with 80% acetone. Chlorophyll a and b contents were spectrophotometrically quantified according to Porra et al. (1989).

Gas Exchange and Δ

Gas-exchange measurements on T1 plants were done in situ on the youngest fully expanded leaves using a LI-6400 (Li-Cor). The CO₂ assimilation rate was first measured at ambient CO₂ using a leaf temperature of 25°C at a blue-red irradiance of 1,500 µmol quanta m⁻² s⁻¹. Following this, CO₂ assimilation rate was measured over a range of CO₂ partial pressures from 50 to 600 µbar. Online Δ measurements concurrent with gas exchange were later made on a subset of plants in a constant-temperature growth cabinet using two LI-6400 systems coupled to a tunable diode laser (model TGA100; Campbell Scientific) as described by Tazoe et al. (2009). Measurements were made over a range of CO₂ partial pressures and irradiances. Following gas exchange, 0.5-cm² discs were removed from frozen leaves, snap frozen in liquid nitrogen, and stored at −80°C for subsequent measurements of enzyme activities, chlorophyll, nitrogen content, and dry matter Δ.

Calculation of Δ from online Δ measurements were similar to the previous description by Pengelly et al. (2010) except that the tertiary formulation suggested by Farquhar and Cernusak (2012) was used, such that

\[ \Delta = \frac{1 - t}{1 + t} \left( a' \right) \frac{A}{(\delta a' + \delta) A + \delta} \left( \frac{C_i - A}{C_s} \right) \]

where \( t = \frac{1 + x e^f}{2 x} \), \( E \) denotes the transpiration rate and \( \delta \), the total conducance to CO₂ diffusion including boundary layer and stomatal conductance (von Caemmerer and Farquhar, 1981). The symbol \( a' \) denotes the combined fractionation factor through the leaf boundary layer and through stomata

\[ a' = \frac{a_b (C_s - C_b) + b (C_b - C_i)}{(C_s - C_i)} \]

and

\[ b = b_t - \frac{0.5 R_d}{(A + 0.5 R_d)} \]

where \( b_t \) is the fractionation by Rubisco (30%) and \( b_b \) is the combined fractionation of the conversion of CO₂ to HCO₃⁻ and PEP carboxylation (~5.4% at 25°C). The fractionation factor \( a \) associated with respiration was calculated from the difference between 6°C in the CO₂ cylinder (~2.5 to ~5.5%) used during experiments and that in the atmosphere under growth conditions (~8% Tazoe et al., 2009). \( A \) and \( R_d \) denote the CO₂ assimilation rate and day respiration, respectively; \( R_d \) was assumed to equal dark respiration. Equation 1 can be rearranged to calculate \( \phi \):

\[ \phi = \frac{1 - t}{1 + t} \left( a' \right) \frac{A}{(\delta a' + \delta) A + \delta} \left( \frac{b_t - b_i}{(b_t - b_i)} \right) \left( \frac{C_i}{C_s} \right) \frac{b_t}{(b_t - b_i)} \]

In Equation 1, the assumption is made that \( C_s > C_i + \delta \). If that is not the case, this may slightly overestimate \( \phi \). More complex equations are given by Ubierna et al. (2011) and Farquhar and Cernusak (2012). We assumed a mesophyll conductance \( g_m = 1 \) mol m⁻² s⁻¹ bar⁻¹ for these calculations.

Calculations of C₄ Cycle Rates, Bundle Sheath Leak Rate, and Bundle Sheath CO₂ Partial Pressure

We used the values of \( \phi \) calculated from Δ measurements together with the measurements of CO₂ assimilation rate to calculate the C₄ cycle rate \( (V_p) \) and the bundles sheath leak rate from the equation:

\[ V_p = \frac{(A + R_d)}{(1 - \phi)} \]

where \( A \) denotes the CO₂ assimilation rate and \( R_d \) the rate of mitochondrial day respiration (von Caemmerer and Farquhar, 1998). Bundle sheath leak rate \( (L) \) was calculated from

\[ L = \phi V_p \]

We used \( L \) to estimate the \( C_i \) from:
\[ C_s = C_m + \frac{L/\delta_s}{\delta_s} \]

assuming a bundle sheath conductance \((g_s)\) of 0.003 mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\) for the wild type and mutants, as there was no difference in the \(S_b\) (von Caemmerer and Furbank, 1999). Mesophyll \(\text{CO}_2\) concentration \((C_m)\) was calculated from \(A\) and \(C_s\) as

\[ C_m = C_s - A/\delta_s \]

with \(g_m = 1\) mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\).

**Anatomical Measurements**

Leaf sections measuring approximately 2 mm \(\times\) 5 mm from mature plants were fixed and embedded in London Resin White (Electron Microscopy Sciences) acrylic resin. Leaf cross-sections were cut and visualized, and the \(S_m\) and \(S_b\) were measured as described previously (Pengelly et al., 2010). Measurements were averaged from data from 20 sections from four different wild-type and \(\alpha\)-NADP-ME plants.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Western blot of NADP-ME content in wild-type and \(\alpha\)-NADP-ME *F. bidentis* lines.

**Supplemental Figure S2.** Ratio of PEPC to Rubisco activity, chlorophyll \(a/b\) ratio, and chlorophyll \(a/b\) content as a function of NADP-ME activity in *F. bidentis* wild-type and \(\alpha\)-NADP-ME plants.

**Supplemental Figure S3.** Concurrent measurement of \(\text{CO}_2\) assimilation rate, stomatal conductance, \(C_i/C_s\), \(\Delta\), and \(\delta\) as a function of intercellular \(\text{CO}_2\) and irradiance in three individual wild-type and \(\alpha\)-SSu *F. bidentis* plants.

**Supplemental Figure S4.** Representative images of the leaf cross-sections in \(\alpha\)-NADP-ME and wild-type plants used to measure \(S_m\) and \(S_b\).

Received July 5, 2012; accepted July 24, 2012; published July 30, 2012.

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