Antisense reduction of NADP-malic enzyme in *Flaveria bidentis* reduces flow of CO₂ through the C₄ cycle.

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Abstract

An antisense construct targeting the C4 isoform of NADP-malic enzyme (NADP-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 34-75% reduction in NADP-ME activity relative to wild-type with no visible growth phenotype. We characterised the effect of reducing NADP-ME on photosynthesis by measuring in vitro photosynthetic enzyme activity, gas exchange and real-time carbon isotope discrimination. In α-NADP-ME plants with less than 40% of wild-type NADP-ME activity, CO2 assimilation rates at high intercellular CO2 (Ci) were significantly reduced, whereas the in vitro activity of both PEP carboxylase and Rubisco were increased. Carbon isotope discrimination (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 (α-SSu) 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 (Cₜ). The comparison of α-NADP-ME and α-SSu demonstrates that the coordination of the C₃ and C₄ cycle that exists 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.
Introduction

In the leaves of a range of plants including maize, sorghum, sugarcane and millet, 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 PEP carboxylase (PEPC) in the mesophyll cells into 4-carbon acids which diffuse to an inner ring of bundle sheath cells where they are decarboxylated and the CO₂ refixed by Rubisco. Plants using the C₄ photosynthetic mechanism have been subdivided into 3 primary sub-types, the NADP-ME, NAD-ME and PCK types, according to the decarboxylating enzyme used to generate CO₂ from C₄ acids in the bundle sheath cells (Hatch 1987; Furbank 2011). \textit{Flaveria bidentis} is a typical NADP-ME dicot in which malate and aspartate 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 cell 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 are still unclear however and concrete evidence for the co-ordinated 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 \textit{Flaveria 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 Phosphoenolpyruvate carboxylase 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 potentially alter the balance between the C₃ and C₄ cycles.

In this study we targeted the gene encoding the chloroplastic C₄ isozyme of NADP-Malic enzyme (NADP-ME) in \textit{F. bidentis} (Marshall et al., 1996) with an antisense construct designed to reduce its activity \textit{in vivo}. This isoform is thought to catalyse the decarboxylation of L-malate to pyruvate and CO₂, and NADP to NADPH in bundle sheath chloroplasts during C₄ photosynthesis (Ashton, 1997; Drincovich 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* species – a chloroplastic but potentially non-photosynthetic NADP-ME form, and a cytosolic NADP-ME (Marshall et al., 1996; Drincovich 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, 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 cycle 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 bundle sheath CO₂ concentration (C₅), 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, facilitating estimates of leakiness, C₄ cycle rate and the possible range of C₅ 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 C₃/C₄ 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 characterisation of transformants

Agrobacterium transformation of *F. bidentis* with an antisense construct targeting the chloroplastic NADP-ME yielded twelve independent primary transformants. Of these, 8 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 reduced 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 screened for low NADP-ME activity. Six plants were selected from each line (18 plants in total) encompassing a range of NADP-ME activity’s 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 (Supp. Fig. 1). 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) (Supp. Fig. 3 Legend).

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 three days during which the CO₂ assimilation rate of each plant was measured 3 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. 1B, C) while no change in the PEPC/Rubisco was observed (Supp. Fig. 2A). A slight negative correlation was also observed between leaf nitrogen (N) and NADP-ME activity indicating an increase in N 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 mmol m⁻². This differed from the average total leaf nitrogen for NADP-ME plants (with less than 30 µmol m⁻¹
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 (Supp. Fig. 2B, C).

**CO₂ response of CO₂ assimilation rate**

Fig. 2A and B show the response of the CO₂ assimilation rate to increasing intercellular CO₂ partial pressure (Cᵢ) in 4 wild-type and 8 α-NADP-ME mutant plants. The mutant population included 4 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 WT). The steep initial rise in CO₂ assimilation rate from 10-60 μbar Cᵢ characteristic of C₄ plants did not differ between 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 carbon isotope discrimination**

Carbon isotope composition measured on dried leaf discs (δ¹³C, relative to the standard V-Pee Dee Belemnite) taken from glass house grown wild-type and α-NADP-ME plants showed no significant differences (Fig. 1D). Online measurements of carbon isotope discrimination (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 wild-type and mutants (Fig. 3B, 4B) yet as assimilation rates were lower in the α-NADP-ME plants (Fig. 3A, 4A), C/Cₐ was greater in mutants than in wild-type plants (Fig. 3C, 4C). Online measurements indicated that Δ was significantly lower in α-NADP-ME plants (Fig. 3D, 4D) and leakiness (φ) was also calculated to be less than in wild-type plants (Fig. 3E, 4E).

When plotted against C/Cₐ (Fig. 5A), Δ measurements for wild-type and α-NADP-ME plants spread discretely along theoretical lines estimating the relationship between Δ and C/Cₐ using the C₄ model by (Farquhar, 1983) - a φ of 0.25 and 0.184 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 3 transgenic α-SSu *F. bidentis* plants (Supp. Fig. 3) showed increased leakiness compared to wild-type plants (φ ~ 0.34) and also plotted alongside the equivalent C₄ model line (Fig. 5A). Fig. 5B shows the different leakiness calculations in wild-type, α-NADP-ME and α-SSu plants, yet comparatively negligible changes to each as a function of increasing Cᵢ.
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 $\alpha$-NADP-ME (Supp. Fig. 4.). No significant difference was found in $S_b$ ($2.1 \pm 0.1 \, \text{m}^2 \, \text{m}^{-2}$ and $2.0 \pm 0.1 \, \text{m}^2 \, \text{m}^{-2}$ respectively) and a small change in $S_m$ for $\alpha$-NADP-ME plants ($15.3 \pm 0.6 \, \text{m}^2 \, \text{m}^{-2}$ compared to wild-types $17.5 \pm 0.7 \, \text{m}^2 \, \text{m}^{-2}$).

Estimation of leakiness, $C_4$ cycle rate, bundle sheath leak rate and bundle sheath $CO_2$

We used the concurrent measurements of $\phi$ and $CO_2$ assimilation rates to estimate the $C_4$ cycle and bundle sheath leak rate (Fig. 6) as outlined in the Method section. In $\alpha$-NADP-ME plants with the lowest $CO_2$ assimilation rates both the rate of the $C_4$ cycle and the bundle sheath leak rate have been reduced (Fig. 6B, C, E, F), whereas in the $\alpha$-SSu plants the bundle sheath leak rate was similar to wild type accompanied by a reduction in the $C_4$ cycle rate.

The anatomical measurements above showed no differences in $S_b$ and we therefore assumed the same bundle sheath resistance of $333 \, \text{m}^2 \, \text{s}^{-1} \, \text{mol}^{-1} \, \text{bar}^{-1}$ to estimate bundle sheath $CO_2$ partial pressure ($C_s$). Low $\alpha$-NADP-ME plants exhibited a reduced $C_s$ estimation (compared to wild-type) in response to increasing $C_i$ (Fig. 7A) and irradiance (Fig. 7B) whereas $C_s$ in $\alpha$-SSu plants was predicted to be more similar to wild-type. 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**

**Antisense construct targets the photosynthetic C₄ NADP-ME isoform in Flaveria bidentis, reducing CO₂ 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 level (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 NADP-ME isoform encoded by *ChlMe1* is in C₄ photosynthesis, as the major enzyme responsible for decarboxylation of the C₄ acids malate and aspartate, 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 C₄ *Flaveria* species, with immunolocalisation of the enzyme at high concentrations in bundle sheath chloroplasts (Marshall et al., 1996; Marshall et al., 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 C₄ model, the CO₂ saturated portion of an A/Cᵢ curve in C₄ plants at high irradiance is naturally limited by either PEP or RuBP 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 was shown to correlate linearly with CO₂ assimilation rates under saturating illumination (Furbank et al., 1996; von Caemmerer et al., 1997) indicating Rubisco was a major limitation for maximal photosynthetic flux with a control coefficient of 0.5-0.7. In contrast, extractable activities of NADP-Malate dehydrogenase (NADP-MDH) (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 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 co-ordinating relative fluxes through the C\(_3\) and C\(_4\) 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 reduced NADP-ME activity (and reduced photosynthetic performance at high C\(_i\)) is intriguing with respect to C\(_3\) – C\(_4\) 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 in Rubisco and PEPC protein expression. However the increase in PEPC and Rubisco activity was matched by an increase in leaf N in this study (Fig. 1E.) with plants exhibiting the most significant reductions in NADP-ME activity (less than 40% of wild-type, n=4) showing on average 30% more leaf nitrogen than wild-type (n=6). In C\(_4\) plants, Rubisco has been estimated to account for between 5-15% of total leaf nitrogen, PEPC 2-6%, and NADP-ME ~1-1.5% (Sage et al., 1987; Evans and von Caemmerer, 2000; Makino et al., 2003; Ghannoum et al., 2005). From the *in vitro* activity (Fig. 1B.) of Rubisco (assuming Rubisco is 16% N by mass, a MW of 550,000 g mol\(^{-1}\) for the holo-enzyme (Ghannoum et al., 2005)) and the total N per leaf area (Fig. 1E) we estimate that 15.7 ± 0.9% and 14.8 ± 0.8% of the total leaf N present in these plants can be attributed to Rubisco in wild type plants (n=6) and low NADP-ME mutants (less than 40% of wild-type, n=4) respectively. These estimates indicate 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 N 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 C\(_4\) leaves.
Antisense reduction of NADP-ME alters C_3-C_4 cycle coordination

While the mechanisms and extent of the communication between the C_3 and C_4 photosynthetic cycles are not well understood, there are logical reasons (reducing energy loss, maximising energy gain) and experimental evidence for both coordination and balance between the two sets of reactions. Some early evidence for C_3-C_4 cycle interaction includes metabolite measurements made in *Zea mays* (maize) (Leegood and Furbank, 1984, 1984) and *Amaranthus edulis* (Leegood and von Caemmerer, 1988) showing concurrent changes of C_3 and C_4 metabolites levels in response to differing CO_2 concentrations and irradiances. Additional evidence is found in the regulation of the C_4 enzyme PEPC activity in maize by the in vivo concentrations of its substrate PEP and the C_4 acid malate (Doncaster and Leegood, 1987), suggestive of a mechanism by which PEPC activity might be balanced with that of other C_3 and C_4 enzymes.

Here, carbon isotope discrimination (Δ) has been used to estimate leakiness (φ) to gain a measure of coordination and balance between C_3 and C_4 cycles. From the C_4 carbon isotope model it is clear that Δ is strongly influenced by both the ratio of intercellular to ambient CO_2 (C_i/C_a) 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 (δ^{13}C) and the real time carbon isotope discrimination (Δ) in leaves for leakiness 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 reduced NADP-ME (less than 40% of wild type) showed less ^{13}C discrimination and infer reduced leakiness relative to wild type plants over increasing irradiance and C_i (Fig. 3, 4). We used the simplified equations presented in the Methods which make the assumption that C_5 >> C_1 (for discussion see Ubierna et al., 2011). If this were not the case we would have overestimated leakiness slightly in the α-NADP-ME plants relative to the wild type.

Measurements of leakiness in C_4 plants have been shown to be remarkably constant over a range of irradiances, temperatures and intercellular CO_2 concentrations (Henderson et al., 1992). This has been taken as evidence that some level of regulation of the C_3 and C_4 cycles occurs. Our estimations of leakiness (Fig. 3, 4 and Supp. Fig. 3) support these earlier results showing leakiness 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 leakiness 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, Supp. Fig. 3). Reduction in Rubisco resulted in an increased leakiness (φ) relative to 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 C₄ 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 wild type accompanied by a reduction in the C₄ cycle rate despite very similar in vitro PEPC activities (legend to Supp. Fig. 3). 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 bundle sheath leak rate has also decreased (Fig. 6.). To estimate bundle sheath CO₂ partial pressure (Cₛ) and estimate of bundle sheath resistance is needed. Although attempts have been made to estimate bundle sheath resistance by various 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 Zea mays) to estimate bundle sheath CO₂ partial pressures (Cₛ) (Fig. 7). These calculations suggest reduced Cₛ for α-NADP-ME plants whereas α-SSu plants had values similar to wild type. To put these values into context; the apparent Michaelis Menten constant for Rubisco carboxylation in F. bidentis at ambient O₂ partial pressures is approximately 1000 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 it is close to saturation at 4 times the Michaelis Menten constant at high light and above ambient CO₂. Fig. 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 *ChlMe1*) 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 C_i 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 cycle 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 reduction in C4 cycle activity or increased C3 cycle activity.
Methods

**Plasmid construction, transformation and mutant regeneration**

Total RNA was isolated and purified from *Flaveria bidentis* (L.) Kuntze (von Caemmerer et al., 1997) leaves using the TRIzol reagent (Invitrogen, Carlsbad, USA). cDNA was synthesized using the Superscript III First-Strand Synthesis kit (Invitrogen) from 1 μg total RNA using the reverse specific primer NADPME-R2 (5′–ATGGGTGGATCCGTACTTTTCAAG–3’). NADPME-R2 and NADPME-F2 (5′–TTGGAGCTCTTGGTGGTGGTGTTG–3’), primers designed based on the chloroplastic C₄ isofrom of the NADP-malic enzyme (NADP-ME) open reading frame ME1 from *Flaveria trinervia* and *Flaveria bidentis* (Genbank accession numbers: X57142) and AY863144). These primers were used to amplify, via PCR, an 845 bp fragment from the F. bidentis cDNA library and introduce BamHI and SacI restriction enzyme sites at the ends of the section. This PCR fragment was ligated into the pGEMT-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/SacI digested pBI121 binary vector under the control of a 3S cauliflower mosaic virus promoter and named pANME2. pANME2 was subsequently transformed into *Agrobacterium tumefaciens* strain AgL1 and maintained in selective culture. *F. bidentis* was transformed with pANME2 using the *Agrobacterium* method (Chitty et al., 1994) in two separate transformation experiments involving 100-400 explants each. Transformants were grown in 3 rounds of selective tissue culture then transferred to soil after roots had grown.

**Plant growth**

Wild-type and antisense NADP-ME *F. bidentis* plants were grown to seed in growth cabinets under a 12/12 day-night cycle at 28/25°C, 70% humidity at 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 litre pots in a garden soil mix with fertilizer (Osmocote, Scotts Australia Pty Ltd., NSW, Australia) and watered daily. Experimental measurements were conducted on fully expanded leaves from plants ~ 30-40 days 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 phosphoenolpyruvate carboxylase (PEPC)
were measured as previously described (Cousins et al., 2007; Pengelly et al., 2010). 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; Drincovich 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 homogenisers with 500 μL of extraction buffer (50 mM Tricine-KOH pH 7.8, 1 mM EDTA, 0.1% Triton-X, 10 mM dithiothreitol and 1% polyvinylpolypyrrolidone) and 10 μL of protease inhibitor cocktail (Sigma). Homogenate was briefly centrifuged and the supernatant used for NADP-ME assays. For each assay, 20 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 initiated by the addition of 10 μL of 200 mM MgCl₂. Activity of NADP-ME was calculated by monitoring the increase of NADPH absorbance at 340 nm with a diode array spectrophotometer (Hewlett Packard) after initiation of the reaction. Chlorophyll was extracted from frozen leaf disks in a Tissuelyser II ball mill (Retsch, Haan, Germany) with 80% acetone. Chlorophyll *a* and *b* content were spectrophotometrically quantified according to (Porra et al., 1989).

**Gas exchange and carbon isotope discrimination**

Gas exchange measurements on T1 plants were done *in situ* on the youngest fully expanded leaves using a LI-6400 (Li-Cor, Lincoln, NE, USA). The CO₂ assimilation rate was first measured at ambient CO₂ using a leaf temperature of 25° C at a blue-red irradiance of 1500 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 carbon isotope discrimination 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 tuneable diode laser (TDL, model TGA100, Campbell Scientific, Inc., Logan, Utah, USA) 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 tested leaves, snap frozen in liquid nitrogen and stored at -80 °C for subsequent measurements of enzyme activities, chlorophyll, nitrogen content and dry matter carbon isotope discrimination.

Calculation of leakiness (ϕ) from online carbon isotope discrimination 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
\[
\Delta = \frac{1}{1-t} a' + \frac{1+t}{1-t} \left( a_t - a' \right) \frac{A}{g_m c_a} + \frac{1+t}{1-t} \left( b'_4 + (b'_3 - s) \phi - a' \right) \frac{(c_l \frac{A}{g_m})}{c_a}, \tag{1}
\]

where \( t = \frac{(1+a')E}{2\theta_{LC}} \). \( E \) denotes the transpiration rate and \( g^{t}_{m} \) the total conductance to \( CO_2 \) 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 and

\[ a' = a_h\frac{(c_a-c_{tie}) + a(c_{tie}-c_{l})}{(c_a-c_{l})} \]  

(2)

where \( C_h \) is the \( CO_2 \) partial pressure at the leaf surface, \( a_h \) (2.9‰) is the fractionation occurring through diffusion in the boundary layer and \( a \) (4.4‰) is the fractionation due to diffusion in air (Evans et al., 1986). The fractionation factor associated with the dissolution of \( CO_2 \) and diffusion through water is given by \( a_i \) (1.8‰). Here we assume that \( s = a_i \).

\[ b'_3 = b_3 - e \left( \frac{R_d}{A + R_d} - \frac{0.5R_d}{A + 0.5R_d} \right) \]  

(3)

and

\[ b'_4 = b_4 - e \left( \frac{0.5R_d}{A + 0.5R_d} \right) \]  

(4)

where \( b_3 \) is the fractionation by Rubisco (30‰), \( b_4 \) is the combined fractionation of the conversion of \( CO_2 \) to \( HCO_3^- \) and PEP carboxylation (-5.74‰ at 25°C). The fractionation factor \( e \) associated with respiration was calculated from the difference between \( \delta^{13}C \) in the \( CO_2 \) cylinder (-2.5 to -4.5‰) used during experiments and that in the atmosphere under growth conditions (-8‰) (Tazoe et al., 2009). \( A \) and \( R_d \) denote the \( CO_2 \) assimilation rate and day respiration respectively. \( R_d \) was assumed to equal dark respiration. Equation (1) can be rearranged to calculate leakiness \( \phi \) and

\[ \phi = \frac{\frac{1-t}{1+t} \frac{a'}{a} - \frac{A}{g_m c_a} (b'_4 - a') \frac{c_l}{c_a}}{(b'_3 - s) \frac{(c_l - \frac{A}{g_m})}{c_a}} \]  

(5)

In equation (1) the assumption is made that \( C_s > C_m \). If not the case this may slightly overestimate leakiness. More complex equations are given by (Ubierna et al., 2011) and (Farquhar and Cernusak, 2012). We assumed a mesophyll conductance \( g_m = 1 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1} \) for these calculations.

Calculations of \( C_4 \) cycle rates, bundle sheath leak rate and bundle sheath \( CO_2 \) partial pressure.
We used the values of leakiness calculated from carbon isotope discrimination measurements together with the measurements of CO$_2$ assimilation rate to calculate the C$_4$ cycle rate ($V_p$) and the bundle sheath leak rate (L) from the equation:

$$V_p = \frac{A + R_d}{1 - \phi} \quad (6)$$

in which $A$ denotes the CO$_2$ assimilation rate, $R_d$ the rate of mitochondrial day respiration and $\phi$ leakiness (von Caemmerer and Furbank, 1999). Bundle sheath leak rate (L) was calculated from

$$L = \phi \cdot V_p. \quad (7)$$

We used L to estimate the concentration of CO$_2$ within the bundle sheath ($C_s$) from:

$$C_s = C_m + \frac{L}{g_s} \quad (8)$$

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

$$C_m = C_i - \frac{A}{g_m} \quad (9),$$

with $g_m = 1$ mol m$^{-2}$ s$^{-1}$ bar$^{-1}$.

**Anatomical measurements**

Leaf sections measuring ~ 2 mm by 5 mm from mature plants were fixed and embedded in London Resin White (LR White, Electron Microscopy Sciences, Fort Washington, PA, USA) acrylic resin. Leaf cross sections were cut, visualised and the mesophyll surface area exposed to intercellular airspace/leaf area ($S_m$) and the bundle sheath surface area/leaf area ($S_b$) measured as described previously (Pengelly et al., 2010). Measurements were averaged from data from 20 sections from 4 different wild-type and $\alpha$-NADP-ME plants.
References


Fig. 1.
CO₂ assimilation rate (A), PEPC activity (B), Rubisco activity (C), dry matter carbon isotope composition (D) and Nitrogen (E) as a function of NADP-ME activity in wild-type (○) and α-NADP-ME (●) F. bidentis plants. Error bars show 3 technical replicates of individual plants. Linear correlations were fitted to B, C, D and E yielding R² values of 0.17, 0.33, <0.01 and 0.46 respectively.

Fig. 2.
Gas exchange of wild-type (n=4) and α-NADP-ME (n=8) Flaveria bidentis plants. CO₂ assimilation rates are given over a complete (A) and low (B) range of intercellular CO₂ (Cᵢ) partial pressures. Lines depicted are 4 wild-plants (○), four α-NADP-ME plants with NADP-ME activity from 55-95% of wild type (☑) and four α-NADP-ME plants with NADP-ME activity below 40% of wild type (●). Error bars show 3 technical replicates of individual plants. Measurements were made in the glasshouse at 1500 µmol quanta m⁻² s⁻¹ and a leaf temperature of 25° C.

Fig. 3.
Concurrent measurement of CO₂ assimilation rate (A), stomatal conductance (B), ratio of intercellular to ambient CO₂, Cᵢ/Cₐ (C), carbon isotope discrimination, Δ (D) and leakiness, φ (E) as a function of intercellular CO₂, Cᵢ. Lines and error bars represent averages and standard errors of measurements on 3 individual wild-type (○) and α-NADP-ME (●) F. bidentis plants respectively. Measurements were made at 1500 µmol quanta m⁻² s⁻¹ and a leaf temperature of 25° C.

Fig. 4.
Concurrent measurement of CO₂ assimilation rate (A), stomatal conductance (B), ratio of intercellular to ambient CO₂, Cᵢ/Cₐ (C), carbon isotope discrimination, Δ (D) and leakiness, φ (E) as a function of irradiance. Lines and error bars represent averages and standard errors of measurements on 3 individual wild-type (○) and α-NADP-ME (●) F. bidentis plants respectively. Measurements were made at a CO₂ concentration of 380 µmol mol⁻¹ and a leaf temperature of 25° C.
Fig. 5.
A: Carbon isotope discrimination (Δ) of wild-type (○, □), α-NADP-ME (●) and α-SSu (■) *F. bidentis* plants as a function of the ratio of intercellular to ambient CO2 (Ci/Ca). Lines represent the theoretical relationship between Δ and Ci/Ca during C4 photosynthesis (equation 1) at infinite gm with a leakiness (φ) of 0.184, 0.25 and 0.34. (Δ=4.64+(-5.9-4.64+29.2*φ)*(Ci/Ca)). Measurements were made as described in Fig. 3.
B: Leakiness (φ) of wild-type (○, □), α-NADP-ME (●) and α-SSu (■) as a function of intercellular CO2 (Ci). Measurements made as described in Fig. 3.

Fig. 6.
CO2 assimilation rate (A, D), C4 cycle rate (B, E) and Bundle sheath leak rate (C, F) as a function of intercellular CO2 (Ci) (A, B, C) and irradiance (D, E, F) in wild-type (○, □), α-NADP-ME (●) and α-SSu (■) *F. bidentis* plants. The C4 cycle and bundle sheath leak rate were calculated from equations (6) and (7) in the Method section.

Fig. 7.
Estimated bundle sheath CO2 (Cs) as a function of intercellular CO2 (Ci) (A), irradiance (B), and bundle sheath resistance. Symbols represent estimated bundle sheath CO2 levels at the bundle sheath resistance assumed in this study for leakiness measurements (~333 m² s bar mol⁻¹).

Supp. Fig. 1.
A: Western blot of NADP-ME content in wild-type and α-NADP-ME *F. bidentis* lines. Protein samples were extracted from frozen leaf disks and separated using SDS-PAGE. NADP-ME protein was detected using a maize-specific antibody (courtesy of B. Furbank, CSIRO Plant Industry, Canberra, Australia). Labels represent individual WT and NADP-ME plant lines.
B: NADP-ME *in vitro* activity as a function of NADP-ME content as measured by western blotting of wild-type (○) and α-NADP-ME (●) *F. bidentis* plants. A linear correlation was fitted (dotted line) giving an R² value of 0.71.

Supp. Fig. 2.
Ratio of PEPC: Rubisco activity (A), Chlorophyll a/b ratio (B) and Chlorophyll a+b content (C) as a function of NADP-ME activity in *F. bidentis* wild-type (○) and α-NADP-ME (●) plants. Error bars show 3 technical replicates of individual plants.
Supp. Fig. 3.
Concurrent measurement of CO₂ assimilation rate (A, F), stomatal conductance (B, G), ratio of intercellular to ambient CO₂, Cᵢ/Cₐ (C, H), carbon isotope discrimination, Δ (D, I) and leakiness, φ (E, J) as a function of intercellular CO₂, Cᵢ (A-E) and irradiance (F-J) in 3 individual wild-type (○) and α-SSu (■) *F. bidentis* plants respectively. Measurements were made at a leaf temperature of 25°C, a CO₂ concentration of 380 µmol mol⁻¹ and irradiance of 1500 µmol quanta m⁻² s⁻¹. Lines and error bars represent averages and standard errors. The mean *in vitro* PEPC and Rubisco activities were 150±15 & 44±2 and 146±12 & 14±2 in wild type and α-SSu plants respectively.

Supp. Fig. 4.
Representative images of the leaf cross sections in α-NADP-ME (A,B,C) and wild-type (D,E,F) plants used to measure Sᵣ and Sᵣ.
Fig. 1.

CO₂ assimilation rate (A), PEPC activity (B), Rubisco activity (C), dry matter carbon isotope composition (D) and Nitrogen (E) as a function of NADP-ME activity in wild-type (○) and α-NADP-ME (●) *F. bidentis* plants. Error bars show 3 technical replicates of individual plants. Linear correlations were fitted to B, C, D and E yielding R² values of 0.17, 0.33, <0.01 and 0.46 respectively.
Fig. 2.
Gas exchange of wild-type (n=4) and α-NADP-ME (n=8) *Flaveria bidentis* plants. CO$_2$ assimilation rates are given over a complete (A) and low (B) range of intercellular CO$_2$ (C$_i$) partial pressures. Lines depicted are 4 wild-plants (O), four α-NADP-ME plants with NADP-ME activity from 55-95% of wild type (▲) and four α-NADP-ME plants with NADP-ME activity below 40% of wild type (●). Error bars show 3 technical replicates of individual plants. Measurements were made in the glasshouse at 1500 µmol quanta m$^{-2}$ s$^{-1}$ and a leaf temperature of 25°C.
Fig. 3.
Concurrent measurement of CO$_2$ assimilation rate (A), stomatal conductance (B), ratio of intercellular to ambient CO$_2$, C$_i$/C$_a$ (C), carbon isotope discrimination, $\Delta$ (D) and leakiness, $\phi$ (E) as a function of intercellular CO$_2$, C$_i$. Lines and error bars represent averages and standard errors of measurements on 3 individual wild-type (○) and α-NADP-ME (●) *F. bidentis* plants respectively. Measurements were made at 1500 µmol quanta m$^{-2}$ s$^{-1}$ and a leaf temperature of 25° C.
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Concurrent measurement of CO₂ assimilation rate (A), stomatal conductance (B), ratio of intercellular to ambient CO₂, Cᵢ/Cₐ (C), carbon isotope discrimination, Δ (D) and leakiness, φ (E) as a function of irradiance. Lines and error bars represent averages and standard errors of measurements on 3 individual wild-type (○) and α-NADP-ME (●) F. bidentis plants respectively. Measurements were made at a CO₂ concentration of 380 µmol mol⁻¹ and a leaf temperature of 25°C.
Fig. 5.

A: Carbon isotope discrimination (Δ) of wild-type (○, □), α-NADP-ME (●) and α-SSu (■) F. bidentis plants as a function of the ratio of intercellular to ambient CO₂ (Cᵢ/Cₐ). Lines represent the theoretical relationship between Δ and Cᵢ/Cₐ during C₄ photosynthesis (equation 1) at infinite gₘ with a leakiness (ϕ) of 0.184, 0.25 and 0.34. (Δ=4.64+(-5.9-4.64+29.2*ϕ)*(Cᵢ/Cₐ)). Measurements were made as described in Fig. 3.

B: Leakiness (ϕ) of wild-type (○, □), α-NADP-ME (●) and α-SSu (■) as a function of intercellular CO₂ (Cᵢ). Measurements made as described in Fig. 3.
Fig. 6.
CO₂ assimilation rate (A, D), C₄ cycle rate (B, E) and Bundle sheath leak rate (C, F) as a function of intercellular CO₂ (Cₜ) (A, B, C) and irradiance (D, E, F) in wild-type (○, □), α-NADP-ME (●) and α-SSu (■) F. bidentis plants. The C₄ cycle and bundle sheath leak rate were calculated from equations (6) and (7) in the Method section.
Fig. 7.
Estimated bundle sheath CO$_2$ ($C_s$) as a function of intercellular CO$_2$ ($C_i$) (A), irradiance (B), and bundle sheath resistance. Symbols represent estimated bundle sheath CO$_2$ levels at the bundle sheath resistance assumed in this study for leakiness measurements (~333 m$^2$ s bar mol$^{-1}$).