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INHIBITION OF ALFALFA ROOT NODULE PHOSPHOENOLPYRUVATE CARBOXYLASE THROUGH AN ANTISENSE STRATEGY IMPACTS NITROGEN FIXATION AND PLANT GROWTH

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Abstract—Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) plays a paramount role in nodule metabolism and several reports have shown that PEPC provides substantial carbon for N_2-fixation and N assimilation. To study the short- and long-term implications of reduced nodule CO_2 fixation for N_2 fixation in alfalfa, PEPC enzyme expression was reduced through an antisense strategy. The full-length root nodule-enhanced PEPC cDNA in antisense orientation driven by the nodule-enhanced AAT-2 promoter was transformed into alfalfa. Out of 105 transformed plants, 14 showed reduced *in vitro* nodule PEPC activity. Three plants were selected for further evaluation. RNA and protein blots showed reduced PEPC transcript and protein. Nodules of these plants also displayed reduced *in vivo* CO_2 fixation. Total nitrogenase activity as measured by H_2 evolution was reduced, although there was no change in apparent nitrogenase. The nodule electron allocation coefficient of antisense plants was reduced. All antisense plants accumulated less dry matter and nitrogen in a 6-week growing period under controlled conditions. The data confirm a strong interdependence of nodule PEPC and nitrogenase activity.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the irreversible carboxylation of phosphoenolpyruvate to oxaloacetate [1]. PEPC is expressed in alfalfa nodules 10- to 15-fold greater than in other organs [2] and can comprise up to 2% of the soluble protein in the nodules [3]. Alfalfa PEPC is encoded by either a single gene or a small gene family and an alfalfa PEPC cDNA has been isolated using monospecific antibodies to alfalfa PEPC [4]. A cDNA encoding pea nodule PEPC has also been isolated [5]. Most recently, an alfalfa PEPC gene controlling nodule PEPC activity has been characterized in detail [6].

It is well established that CO_2 fixation via PEPC plays a significant role in legume nodules by providing carbon for the synthesis of malate and aspartate [7, 8]. Aspartate is formed from oxaloacetate as a major N-exporting compound, whereas malate is imported into the bacteroid and rapidly respired to provide energy for N_2 reduction [9, 10]. It is estimated that up to 25% of the carbon required for nitrogenase activity and nitrogen assimilation may be supplied by PEPC [11], thereby to some extent alleviating the high carbon burden required for nitrogen fixation [12, 13]. Besides these two well-established roles of PEPC in legume nodule functioning, several others have been proposed, including the maintenance of cell charge as a counterion for cation transport [9] and a role in the

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regulation of the O_2 diffusion barrier [6, 14]. A multiple metabolic role of PEPC in alfalfa nodules is also suggested by the location of PEPC in various cell types [6, 14]. Whole plant studies have shown that elevated CO_2 concentrations around nodules results in enhanced N_2 fixation [15]. Moreover, several studies have demonstrated an interdependence between nodule CO_2 and N_2 fixation [16–19]. There are, however, considerable difficulties connected with measuring nodule *in vivo* CO_2 fixation, especially over longer periods of time. The high concomitant nodule respiration, masks accurate measurement of CO_2 uptake and dilutes any measurements made with $^{14}CO_2$ [7, 8, 19].

The objective of this work was to specifically reduce nodule PEPC activity by decreasing expression of the enzyme through antisense technology and to study the short- and long-term implications for N_2 fixation and N assimilation.

RESULTS

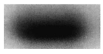
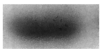
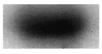
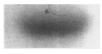
Nodule PEPC activity and protein

Transformation of alfalfa genotype Regen SY yielded 105 independently transformed plants. Transformation was verified through GUS staining and PCR analysis. From these 105 antisense PEPC transformants, 14 showed reduced *in vitro* PEPC enzyme activity. The reductions ranged from 40 to 80%, as compared with an untransformed control. Three plants with a range of reduction in PEPC enzyme activity were selected for more detailed studies (Fig. 1). The three selected transformants along with a transformed control were vegetatively propagated and nodules from these plants were collected and analyzed for PEPC enzyme activity, enzyme protein, and mRNA. The transformed control had undergone the transformation process but showed neither a reduction in PEPC activity nor yielded a correct PCR product but, however, was positive for GUS staining. The nodules of the antisense plants with reduced PEPC activity did not differ in number or appearance from nodules of untransformed control plants. Other than an apparent reduction in shoot growth, the antisense plants look normal.

As shown in Fig. 1, antisense PEPC plants were reduced in both *in vitro* PEPC activity and *in vivo* CO_2 fixation. The reduction for *in vitro* enzyme activity was greater than that for *in vivo* CO_2 fixation. Reduced PEPC enzyme activity was accompanied by a reduction in enzyme protein as measured by immunoblots. Two of the three antisense PEPC plants had reduced PEPC mRNA. Parallel enzyme activity tests for nodule NADH-glutamate synthase (GOGAT; EC 1.4.1.13), aspartate aminotransferase (AAT; EC 2.6.1.1), malate dehydrogenase (MDH; EC 1.1.1.37), and glutamate dehydrogenase (GDH); EC 1.4.1.2) showed that *in vitro* activities of these enzymes in antisense plants were comparable to those in the control plants (data not shown).

Plant growth and nitrogen fixation

Plants in the growth and N_2 fixation experiment received only N-free nutrient solution and the N-content of the growth substrate was negligible. Thus, the amount of N accumulated reflects that acquired through symbiotic N_2 fixation. All antisense plants accumulated less total dry matter than did the control. Dry matter was reduced in both shoots and roots (Table 1). The greatest reduction occurred in antisense

	Control	Antisense 1	Antisense 2	Antisense 3	LSD [1] (0.05%)
PEPC mRNA					
(cpm) [2]	13,984	10,508	13,021	7,695	
PEPC Protein					
in vitro PEPC Activity [3]	12.1 [a]	4.3 [c]	3.3 [c]	6.5 [b]	1.8
in vivo PEPC Activity [4]	10.0 [a]	7.8 [b]	6.8 [b]	6.3 [b]	1.6

[1] Data are given as mean of 4 replications. LSD is to compare within a row
[2] Counts per minute in PEPC mRNA
[3] nKAT / mg protein in nodule extract
[4] nmol CO_2 / min and g nodule fresh weight.

Fig. 1. Antisense transformants are reduced in phosphoenolpyruvate carboxylase (PEPC) mRNA, protein and enzyme activity (Values in each column followed by a different letter are significantly different at P 0.05)

Table 1. Dry matter accumulation, nitrogen concentration, and total nitrogen of antisense PEPC and control plants grown in a growth chamber

	Dm shoot (g)	% N shoots	Dm root (g)	% N roots	Dm shoot + root (g)	Total N in shoots + roots (mg)
Control	1.37[a]	3.28[ab]	0.80[a]	2.71[ab]	2.17[a]	66.30[a]
Antisense 1	0.57[b]	3.50[a]	0.42[c]	2.60[ab]	1.00[b]	30.10[b]
Antisense 2	0.75[c]	3.09[bc]	0.53[b]	2.41[b]	1.28[c]	36.10[c]
Antisense 3	0.44[d]	2.89[c]	0.33[c]	2.06[c]	0.77[d]	19.30[d]
LSD, (0.05%)	0.14	0.25	0.10	0.33	0.22	4.40

The plants received no N-fertilization, data are given per plant as means of six replications.
DIM = dry matter.
Values in each column followed by a different letter are significantly different at P 0.05.

plant 3. There was a concomitant decrease in % N for antisense plants 2 and 3 as compared to controls. However, antisense plant 1 showed no change in % N. Total nitrogen assimilated into shoots and roots was reduced 40 to 70% as compared to the control. Thus, expression of antisense PEPC in alfalfa root nodules impacted both growth and N accumulation.

Root/nodule hydrogen and CO_2 evolution

H_2 and CO_2 evolution from the root space was measured on 6-week-old plants grown in quartz sand in a growth chamber. Antisense 3 plants showed very poor growth performance under these conditions, whereas the other transgenic lines grew at rates comparable to that seen in the growth experiment. At the beginning of the assays, the antisense 3 plants were too small to allow reasonable measurements and therefore were not included.

Table 2 shows that while apparent nitrogenase activity (ANA; H_2 evolution in N_2:O_2, 79/21, v/v) was not different between control and antisense plants, there were significant reductions in total nitrogenase activity (TNA; H_2 evolution in Ar:O_2, 79/21, v/v) of antisense plants. The reduced TNA as compared to ANA resulted in a significantly reduced electron allocation coefficient (EAC) for antisense plants. CO_2 evolution was also reduced in the antisense plants and regression analysis revealed an interdependence of CO_2 evolution ($P < 0.0247$) and TNA ($P < 0.026$) with total N accumulation of these plants in the growth experiment.

DISCUSSION

Using an alfalfa nodule-enhanced PEPC cDNA driven by the promoter of nodule-enhanced *AAT*-2 we have shown that it is possible to specifically reduce the expression and activity of PEPC in alfalfa root nodules. Plants containing the antisense PEPC construct showed not only reduced PEPC activity but also reduced growth, % N, total N accumulation, and total nitrogenase activity. Moreover, the fact that the activities of other enzymes involved in N metabolism were not reduced, confirms the effectiveness of the antisense strategy in specifically affecting a single target in the N_2 fixation and N metabolism metabolic scheme. It is logical to conclude that root nodule PEPC plays an integral role in nodule functioning and N_2 fixation, as was predicted from $^{14}CO_2$ labeling experiments [7, 8, 11, 19] and studies with ineffective nodules [8, 19, 20]. Impaired nodule function has also been reported when AAT-2 and uricase are down-regulated via antisense in lupine [21] and bean [22], respectively.

Based upon the reduced EAC found in nodules

Table 2. Apparent nitrogenase (ANA), total nitrogenase (TNA), electron allocation coefficient (EAC), and carbon dioxide (CO_2) evolution for nodulated root systems of antisense PEPC and control plants grown in a growth chamber.

	ANA μmol $H_2 \cdot p^{-1} \cdot h^{-1}$	TNA μmol $H_2 \cdot p^{-1} \cdot h^{-1}$	EAC	CO_2 evolution mmol $CO_2 \cdot p^{-1} \cdot h^{-1}$
Control	6.2[a]	15.2[a]	0.59[a]	0.46[a]
Antisense 1	5.5[a]	11.9[b]	0.53[b]	0.34[b]
Antisense 2	5.7[a]	12.8[b]	0.55[b]	0.38[b]
LSD, (0.05%)	0.9	1.3	0.04	0.08

Plants were grown in quartz sand without nitrogen fertilization, data are given per plant as mean of 4 replications.
Values in each column followed by a different letter are significantly different at P 0.05.

of antisense plants in H_2 evolution experiments, they appear to divert more energy to H^+ reduction than to N_2 reduction as compared to control plants. The antisense PEPC plants in essence have reduced efficiency for N_2 fixation. This result may be due to the diversion of nitrogenase toward H^+ generation when N assimilation is restricted [23, 24]. The fact that ANA is not altered while TNA is reduced in PEPC antisense plants supports this interpretation. In addition, the data suggest that reduced PEPC activity mainly impairs N assimilation in nodules rather than malate supply to bacteroids. This interpretation supports the N feed-back theory for control of nodule nitrogenase activity [23, 24].

It is interesting to note that the reduction in PEPC enzyme activity in the antisense plants was directly related to reduced PEPC enzyme protein but not to PEPC transcript in antisense 1 and 2 plants. The presence of abundant PEPC transcripts in antisense 1 and 2 plants indicates that regulation of PEPC expression in these plants may be occurring at the translational level. Temple *et al*. [25] have noted that expression of antisense glutamine synthetase (GS) in alfalfa leaves resulted in reduced GS activity and enzyme protein but not GS transcripts. They suggested that duplexes formed between GS mRNA and antisense GS transcript molecules disturbed translational efficiency.

The high interdependence of PEPC, nodule CO_2 evolution, and TNA is consistent with whole plant selection studies showing that there was a very high correlation between *in vitro* PEPC activity and selection for increased nitrogenase activity [12, 26]. Furthermore, Jessen *et al*. [26] showed that whole plant selection for reduced nodule *in vitro* PEPC activity reduced plant growth and N assimilation. Their data in conjunction with the antisense expression data presented here indicate that PEPC may be a useful target for genetic improvement to enhance N_2 fixation in alfalfa.

EXPERIMENTAL

Chimeric gene construction and alfalfa transformation

A 1108 bp fragment of AAT2 5′ upstream region starting at position −49 nt relative to the translation start site was generated by PCR and subcloned initially into pBluescript KS- and then transferred to pMon999 at the *Pst*I and *Sac*I restriction sites, respectively. A full-length alfalfa PEPC cDNA (3.3 kb) was inserted into pMon999 in the antisense orientation between the AAT-2 promoter and the *nos* poly(A) addition signal at the *Sac*I and *Kpn*I sites. The DNA fragment containing the AAT-2 promoter, PEPC cDNA antisense orientation, and the *nos* poly(A) addition site was removed by *Not*I digestion and ligated into the corresponding site in the plant transformation vector PARC100, thereby producing the PARC(AAT2:a PEPC) construct.

The plasmid PARC(AAT2:aPEPC) was introduced into *Agrobacterium tumefaciens* strain LBA4404 using the triparental mating method [27]. Transgenic alfalfa was obtained via *Agrobacterium*-mediated transformation of alfalfa cv Regen SY leaf explants [28]. GUS staining [29] and PCR were performed to confirm the existence of introduced genes in transgenic alfalfa.

Plant growth

Transformed and control plants were grown in sand pots in a glasshouse, fertilized daily with N-free Hoagland's solution, and inoculated with *Rhizobium meliloti* strain 102F51. This strain does not have an uptake hydrogenase system. Glasshouse conditions were as described previously [30]. Nodules were collected from primary transformants grown to initial flowering. Collected nodules were placed on ice and immediately assayed for PEPC enzyme activity. Cuttings were made from three of the antisense plants with substantially lowered PEPC activity along with control plants. Cuttings were treated with rooting hormone mix, planted in Containers with vermiculite and maintained in a controlled environment chamber. After rooting, cuttings were inoculated with *R. meliloti* strain 102F51. Twelve uniform cuttings from each treatment were transplanted into sand pots, inoculated again, and fertilized with N-free Hoagland solution. At flowering six plants were used for *in vitro* and *in vivo* enzyme activity immunoblot analysis, and PEPC transcript levels. The remaining six plants were grown for an additional six weeks and harvested into shoots and roots. Dry matter and nitrogen [31] were determined for each sample. A further four cuttings were made of each antisense and control plant and these were grown in specially designed tubes as described by Blumenthal *et al*. [23]. After six weeks of growth these plants were used to measure root/nodule H_2 and CO_2 evolution. PEPC transcripts were analyzed in nodules of plants used for gas analysis.

Preparation of cell free extracts. Fresh nodules (100 mg) were ground in extraction buffer (100 mM Mes-NaOH [pH 6.8], 100 mM sucrose, 2% v/v 2-mercaptoethanol, 15% v/v ethylene glycol, 2 mm PMSF, 0.2 mM antipain) and centrifuged 15 min at 14,000 *g* to obtain the soluble protein fraction [3, 20].

In vitro enzyme assays PEPC, GOGAT, GDH, AAT, and MDH were assayed spectrophotometrically by monitoring the disappearance of NADH at 340 nm in direct or coupled assays as described by Egli *et al*. [20]. Protein content of extracts was measured by the Lowry assay [32].

SDS-page and western blotting. Soluble proteins in cell-free extracts were electrophoresed in 10% SDS-polyacrylamide gels (Phast Gel, Pharmacia Biotech AB Uppsala Sweden) and transferred to nitrocellulose. Protein load per lane was 2 μg. Rabbit polyclonal antibodies to alfalfa nodule PEPC were used to detect the corresponding antigens on protein immunoblots [19, 33].

In vivo PEPC activity assay of excised nodules. The *in vivo* CO_2 fixation assay for excised nodules was slightly modified from Maxwell *et al*. [19]. Fresh alfalfa nodules (100 mg) were placed on a moist filter paper at the bottom of a sealed 10-ml reaction flask. The assay was initiated by injection of 4 M lactic acid into center wells containing 2.96×10^5 Bq of aqueous $NaH^{14}CO_3$ (1.73×10^9 Bq mmol^{-1}) suspended above the nodules. After incubation for 20 min at 23°C, the reaction was terminated by injection of 1.5 ml of hot 50% (v/v) ethanol, followed by extraction in a 45°C water bath for 20 min and centrifugation at 15,000 *g* for 15 min. An aliquot of the supernatant was treated with HCL and the acid-stable radioactivity was determined by liquid scintillation counting. Carbon dioxide fixation was calculated from the associated radioactivity of nodule tissue and the specific activity of $^{14}CO_2$ which surrounded the nodules during labeling.

RNA extraction and northern blotting. Total RNA was isolated from freshly collected 100 mg nodule samples according to the procedures detailed by Chomczynski and Sacchi [34]. The separation, blotting, and detection of PEPC mRNA followed the procedures described by Pathirana *et al*. [4].

Nitrogenase activity (H_2 evolution, TNA, ANA) and CO_2 evolution was measured in a slightly adapted system described by Minchin *et al*. [35] using a nitrogenase-activity analysis system (Morgan scientific, Havermill, MA) connected with a CO_2 infrared gas analyzer (Lichor). The procedure for the measurement of TNA and ANA is outlined in detail by Blumenthal *et al*. [23]. The flow rate in the assay gas stream was 1,000 ml min^{-1}. ANA, i.e., H_2 evolution in N_2:O_2 (79/21 v/v), of the whole root system was determined first along with the CO_2 evolution from the root space. After stable values for ANA and CO_2 evolution were obtained (about 30 min after the start of the assay), TNA, i.e., the peak H_2 evolution in Ar:O_2 (79:21 v/v), was measured. The EAC, i.e., the proportion of total electron flow through nitrogenase used for N_2 reduction, was calculated using the formula: $EAC = 1 - ANA/TNA$.

Statistics. All data were subjected to analysis of variance and linear regression analysis using SAS (SAS Institute, 1987). All experiments were repeated twice.

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