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# Modulation *in vivo* by nitrate salts of the activity and properties of phospho*enol*pyruvate carboxylase in leaves of *Alternanthera pungens* ($C_4$ plant) and *A. sessilis* ($C_3$ species)

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## Abstract

Feeding $K^+$ or $Na^+$ nitrate salts *in vivo* enhanced the activity of phospho*enol*pyruvate carboxylase (PEPC) in the leaf extracts of *Alternanthera pungens* ($C_4$ plant) and *A. sessilis* ($C_3$ species). The increase was more pronounced in *A. pungens* than in *A. sessilis*. Chloride salts increased the PEPC activity only marginally. However, the sulfate salts were either not effective or inhibitory. Feeding nitrate modulated the regulatory properties of PEPC in *A. pungens*, resulting in increased $K_I$ (malate) and decreased $K_A$ (glucose-6-P). The sensitivity of PEPC to malate, which gives a measure of phosphorylation status of the enzyme, indicated that feeding leaves with $NO_3^-$ enhanced the phosphorylation status of the enzyme. The reduction in PEPC activity due to cycloheximide treatment suggested that increased synthesis of PEPC protein kinase may be one of the reasons for the enhancement in PEPC activity, after the nitrate feeding. We suggest that nitrate salts could be used as a tool to modulate and analyze the properties of PEPC in $C_3$ and $C_4$ plants.

*Additional key words*: cycloheximide; glucose-6-phosphate; inorganic salts; L-malate; nitrate; phosphorylation; regulation; sulphate.

## Introduction

Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) catalyses the irreversible carboxylation of PEP and plays an important role in $C_3$, $C_4$, and CAM plants (Chollet *et al.* 1996, Miyao and Fukayama 2003). The active form of PEPC is a homotetramer (Andreo *et al.* 1987, Rajagopalan *et al.* 1994). In the presence of NaCl, the $C_4$ enzyme tends to dissociate into dimers but the presence of PEP, $Mg^{2+}$, or glucose-6-phosphate (Glc-6-P) can prevent this dissociation. Jensen *et al.* (1995) reported that the stability of PEPC is influenced by various salts. They noticed cations such as $Na^+$ and $K^+$ to be relatively inert, ions like $NH_4^+$or $Cl^-$ to be destabilizing, and $SO_4^{2-}$ to be stabilizing the enzyme. The destabilization was attributed to the dissociation of the enzyme into its dimeric and monomeric forms.

Since the inorganic ion composition of the cytosol affects a number of processes including the activity of cytosolic enzymes, it is expected that *in situ* feeding of inorganic salts can affect the properties of PEPC. Several plant enzymes are activated by low concentration of neutral salts. The stimulation in enzyme activity (*e.g.* pyruvate kinase or 6-phosphofructokinase) often occurs at 50 to 150 mM concentration of univalent salts, particularly potassium and sodium (Wyn Jones and Pollard 1983).

Reports on the effects of feeding nitrate on PEPC in leaves of $C_3$ and $C_4$ plants are contradictory. *In vivo* feeding of nitrate-starved leaves with nitrate salts enhanced the light activation (LA) of PEPC from the $C_3$ and $C_4$ species, *e.g.* wheat (Van Quy *et al.* 1991, Van Quy and Champigny 1992, Duff and Chollet 1995, Li *et al.* 1996) and maize (Gupta *et al.* 1994). However, Gupta *et al.* (1994) reported that feeding nitrate to nitrate-starved plants had no effect on LA of PEPC in wheat.

Therefore, it is necessary to evaluate the *in vivo* effects of nitrate on the activity and LA of PEPC from plant species grown under normal field conditions. We

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*Abbreviations*: Chl = chlorophyll; CHX = cycloheximide; Glc-6-P = glucose-6-phosphate; Gln = glutamine; GS = glutamine synthetase; LA = light activation; L/D ratio = light/dark activity ratio; MSX = methionine sulfoximine; NADP-ME = NADP-malic enzyme; NR = nitrate reductase; PEP = phospho*enol*pyruvate; PEPC = PEP carboxylase; PEPC-PK = PEPC protein kinase; SPS = sucrose phosphate synthase.
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tried to assess if nitrate or other inorganic ions affect PEPC activity in leaves of only $C_3$ or $C_4$ species, or both. The activation status of PEPC was examined in leaves of $C_3$ and $C_4$ species of *Alternanthera,* after *in vivo* treatment with different inorganic salts.

## Materials and methods

**Plants** of *Alternanthera pungens* and *A. sessilis* were grown outdoors under a natural photoperiod of *ca*. 12 h and the average temperatures between 40/27 °C, day/night in summer and 28/13 °C in winter. Third to fifth leaves (counting from the fully developed youngest one) were excised under water between 08:00 and 09:00 (about 2–3 h after sunrise) and were used for experiments. The salts ($KNO_3$, $NaNO_3$, KCl, NaCl, and $K_2SO_4$ at the indicated concentrations) were fed to the excised leaves (after 2 h pre-dark treatment) through petiole for 2 h in darkness. Leaves with petioles kept in distilled water for similar periods served as the control.

**Extraction of PEPC**: 80 mg of leaf was homogenized with 320 $mm^3$ of pre-chilled extraction buffer containing 100 mM Hepes-KOH (pH 7.3), 10 mM $MgCl_2$, 2 mM $K_2HPO_4$, 5 % (v/v) glycerol, 1 mM EDTA, 1 mM chymostatin, 2 mM PMSF, 2 mM benzimidine, 5 mM dithiothreitol, 10 mM sodium fluoride, 0.3 M sorbitol, 1 % (m/v) bovine serum albumin, and 2 % (m/v) insoluble polyvinyl pyrrolidone. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15 000×*g* at 4 °C for 1 min. The supernatant was used for assaying the enzyme.

**Assay and properties of PEPC**: The activity of PEPC was assayed by coupling with NAD-MDH as described by Parvathi *et al*. (2000). The assay medium (1 $cm^3$) contained 50 mM Hepes-KOH, pH 7.8, 10 mM $NaHCO_3$, 4 units NAD-MDH, 0.25 mM NADH, and crude leaf extract equivalent to 1 μg chlorophyll (Chl). The reaction was started by the addition of PEP to make a final concentration of 2.5 mM and change in the absorbance was monitored at 340 nm at 30 °C in a dual beam UV-Vis spectrophotometer (*Shimadzu UV-160A*, Japan). PEPC activity was calculated by using the molar extinction coefficient of NADH at 30 °C. The $K_I$ (malate) of PEPC was measured using 0.01–5.00 mM malate and $K_A$ (Glc-6-P) using 0.5–5.0 mM Glc-6-P.

**Cycloheximide (CHX) feeding**: Leaves were incubated in darkness containing 5 μM CHX for 6 h at 30±1 °C, followed by thorough washing with distilled water.

**Chl and protein estimation**: Chl was estimated by extracting into 80 % (v/v) acetone as per Arnon (1949). Total soluble protein was assayed according to Sedmak and Grossberg (1977).

## Results

When leaves of the $C_4$ species (*A. pungens*) were fed in darkness with potassium or sodium chloride, the extractable PEPC activity was enhanced by KCl, while NaCl had no significant effect. The effect on PEPC was maximal after feeding nitrate salts of K or Na *in vivo* for a period of 90–120 min, in case of *A. pungens* (Fig. 1*A*) as well as *A. sessiles* (Fig. 1*B*). In contrast, the sulfate salts of K or Na did not affect much the activity of PEPC in both *A. pungens* (Fig. 1*C*) and *A. sessiles* (Fig. 1*D*). $K_2SO_4$ decreased marginally the activity of PEPC in leaves of *A. pungens*.

Concentrations above 50 mM of either KCl or NaCl were inhibitory. For instance, feeding leaves with 200 mM KCl reduced the *in vivo* PEPC activity by 53 %. Feeding $KNO_3$ or $NaNO_3$ enhanced the activity of PEPC in the leaves of *A. pungens* by nearly three-fold (Table 1). Feeding with $K_2SO_4$ (in darkness) lowered the *in vivo* PEPC activity by 30 %, comparable to that of the control leaves, while sodium sulfate had no effect (Table 1).

Exogenous feeding of leaves of the $C_3$ species (*A. sessilis*) with 50 mM KCl enhanced the *in vivo* PEPC activity by about 60 %, whereas 50 mM NaCl had no effect on the *in vivo* PEPC activity (Table 1). Concentrations of NaCl above 100 mM decreased the extractable PEPC activity (values not included).

Thus, the salts containing nitrate increased PEPC activity most effectively in the leaves of both $C_3$ and $C_4$ species. Chloride salts enhanced PEPC activity, whereas sulphate salts were either ineffective or suppressed the activity to some extent. Hence, in the further experiments only nitrate or chloride salts were used.

**Effect on LA of PEPC** was evaluated after the *in vivo* feeding of inorganic salts to the leaves. The activation of PEPC was indicated by the ratio (L/D) of the activity of enzyme in extracts from leaves, which were either irradiated (L) or maintained in darkness (D). Nitrate salts of sodium and potassium were the most effective in stimulating the LA of PEPC resulting in high L/D ratio, nearing almost 6. KCl or NaCl enhanced only marginally the extent of LA.

KCl and NaCl had very small effect on the L/D ratio of PEPC in the leaves of *A. sessilis*, a $C_3$ species (Table 1). However, nitrate salts of potassium and sodium enhanced the extent of LA of PEPC nearly 3-fold (Table 1).

Table 1. Effect of exogenously fed inorganic salts (50 mM) on extractable PEPC activity [mmol $kg^{-1}$(Chl) $s^{-1}$] and its L/D ratio from the leaves of *A. pungens*, a $C_4$-dicot and *A. sessilis,* a $C_3$-dicot. Leaves incubated in distilled water were used as the control. ND: Not done.

| Inorganic salt | *A. pungens* | | | *A. sessilis* | | |
|---|---|---|---|---|---|---|
| | PEPC activity | % of control | L/D | PEPC activity | % of control | L/D |
| Control | 173.6±7.8 | 100 | 2.8 | 10.8±1.1 | 100 | 1.5 |
| KCl | 203.9±7.5 | 115 | 3.2 | 17.2±1.9 | 159 | 1.7 |
| NaCl | 187.2±8.1 | 108 | 3.6 | 10.8±1.1 | 100 | 1.6 |
| $KNO_3$ | 409.7±10.8 | 236 | 5.6 | 26.9±1.4 | 249 | 3.8 |
| $NaNO_3$ | 454.4±14.7 | 262 | 5.8 | 25.6±1.9 | 236 | 3.9 |
| $K_2SO_4$ | 118.3±7.8 | 68 | ND | 10.3±0.6 | 95 | ND |
| $Na_2SO_4$ | 171.9±10.6 | 99 | ND | 10.6±0.8 | 97 | ND |

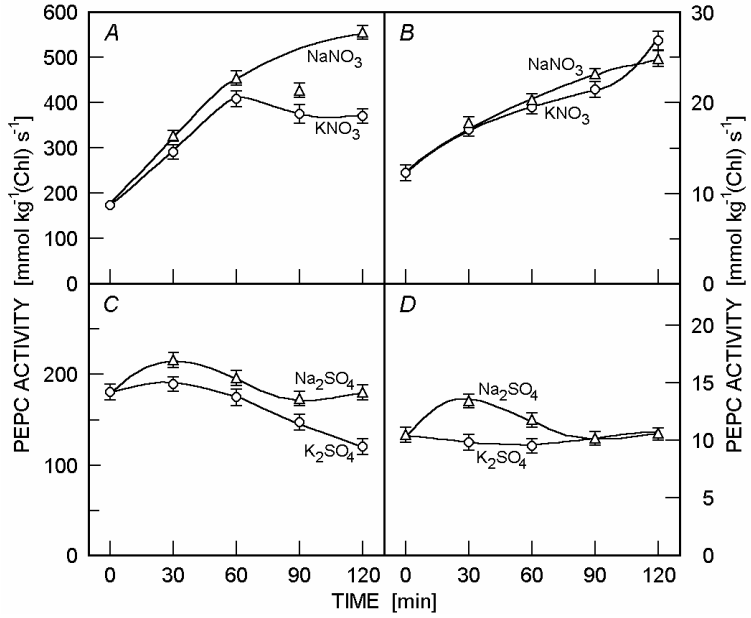


Fig. 1. Changes in PEPC activity with time in leaves of *A. pungens* ($C_4$ species) (*A, C*) or *A. sessilis* ($C_3$ species) (*B, D*) fed *in vivo* with 50 mM nitrate (*A, B*) or sulphate (*C, D*) salts of potassium or sodium. PEPC activity in the extracts was assayed at pH 7.3 and 1.5 mM PEP.

Table 2. Effect of exogenously fed inorganic salts on $K_I$ (L-malate) and $K_A$ (Glc-6-P) [mM] of PEPC extracted from leaves of *A. pungens* ($C_4$ species). Leaves incubated in distilled water were used as control.

| Inorganic salt | $K_I$ (L-malate) | | $K_A$ (Glc-6-P) | |
|---|---|---|---|---|
| | Dark | Light | Dark | Light |
| Control | 0.25 | 1.19 | 2.56 | 1.91 |
| KCl | 0.27 | 1.19 | 2.49 | 1.87 |
| NaCl | 0.27 | 1.13 | 2.57 | 1.94 |
| $KNO_3$ | 0.41 | 2.93 | 2.21 | 1.34 |
| $NaNO_3$ | 0.40 | 2.91 | 2.23 | 1.31 |

**Sensitivity of PEPC to L-malate or Glc-6-P**: Nitrate salts were the most effective and increased $K_I$ (malate) of PEPC in the leaves of *A. pungens* from 0.25 mM (control) to 0.41 mM (Table 3). However, feeding of chloride salts had no significant effect on the $K_I$ (malate) of PEPC (Table 2). Irradiation itself increased the $K_I$ (malate) of PEPC in leaves of *A. pungens*.

Table 3. Effect of 5 µM CHX feeding (6 h) on PEPC activity [µmol $kg^{-1}$(Chl) $s^{-1}$] from leaves of *A. pungens* ($C_4$ species) fed with 50 mM each of $KNO_3$ and $NaNO_3$.

| Inorganic salt | Dark (D) | Light (L) | L/D |
|---|---|---|---|
| Control | 188 (100) | 484 (100) | 2.6 |
| +CHX | 173 (92) | 263 (54) | 1.5 |
| $KNO_3$ | 410 (100) | 2289 (100) | 5.6 |
| +CHX | 299 (73) | 1602 (70) | 5.4 |
| $NaNO_3$ | 454 (100) | 2630 (100) | 5.8 |
| +CHX | 316 (69) | 1736 (66) | 5.5 |

Unlike the marked changes in L-malate sensitivity of PEPC, there were only marginal effects on $K_A$ for

Glc-6-P (Table 2). Feeding leaves with $KNO_3$ or $NaNO_3$ decreased the $K_A$ (Glc-6-P), irrespective of incubation in darkness or under irradiation. Chloride salts did not change $K_A$ (Glc-6-P) of PEPC.

**Effect of CHX on LA of PEPC**: CHX (a eukaryotic protein synthesis inhibitor) can be used to examine if there is any *de novo* synthesis of PEPC- or PEPC-PK protein (Rajagopalan *et al*. 1994). 5 μM CHX fed for 6 h had no major effect on PEPC activity from dark-control samples, inhibiting the activity of light form of PEPC by almost 50 %. As a result, the light/dark PEPC activity ratio decreased from 2.5 (control) to 1.5 (CHX-treated). Feeding of 5 μM CHX caused a marked (nearly 30 %) reduction in the activity of PEPC in the $KNO_3$ and $NaNO_3$ fed leaves, even when the leaves were maintained in darkness (Table 3). However, the LA of PEPC in $KNO_3$ or $NaNO_3$ fed leaves remained high and well above 5-fold.

## Discussion

Our results indicate that nitrate salts of $K^+$ and $Na^+$ are effective in enhancing *in vivo* the activity of PEPC (Table 1) from both the $C_3$ and $C_4$ species of *Alternanthera*. Phosphorylation of PEPC leads to an increase in activity and a decrease in sensitivity of $C_4$ enzyme to malate (Jiao and Chollet 1991). The sensitivity of PEPC to malate, which was used to assess the phosphorylation status of the enzyme, indicates that there is possibly an enhanced phosphorylation of the enzyme on short-term $NO_3^-$ feeding (Tables 2 and 3). Salts of $SO_4^{2-}$ have not much effect on the L-malate inhibition of PEPC both from darkened and irradiated leaves.

The earlier reports on the effects of nitrate *in vivo* on PEPC are conflicting. Van Quy *et al*. (1991) reported that the supply of nitrate to nitrate-starved (or low nitrate grown) leaves had enhanced significantly the extent of LA of PEPC in leaves of wheat, a $C_3$ species. Duff and Chollet (1995) also reported enhanced PEPC-PK activity as a result of nitrate feeding to nitrate-starved wheat leaves. However, Gupta *et al.* (1994) observed that feeding nitrate had no effect on the already low level of LA of PEPC in leaves of $C_3$ species (wheat), but significantly increased the extent of photoactivation of PEPC from maize, a $C_4$ species. All the above experiments were performed after creating an artificial nitrate deficiency in the plants (Van Quy *et al*. 1991, Manh *et al*. 1993, Gupta *et al*. 1994).

The much greater stimulation of PEPC by $NaNO_3$ than by $KNO_3$ is intriguing, but can be interpreted as due to the beneficial effect of sodium as a micronutrient for $C_4$ species (Brownell 1979). Chloride salts enhanced the PEPC activity and its LA in *A. pungens* ($C_4$ species) only marginally. Our results also suggest that the effects observed on feeding of leaves with various salts are mainly due to the anions rather than the cations.

Sugiharto *et al*. (1990, 1992) observed a selective accumulation of PEPC in N-starved leaves of maize when supplied with nitrate or glutamine. The rise in the content of PEPC-mRNA is more pronounced in maize plants supplemented with $NH_4^+$ or glutamine than in those supplemented with nitrate (Sugiharto and Sugiyama 1992). Pre-treatment of excised leaves with CHX in the dark for 6 h caused only marginal reduction in the PEPC activity in the subsequent irradiation *plus* nitrate treatments (Table 3). The decrease in activity was about 35 % compared to the 46 % reduction in the control. Thus, the present study indicates that protein synthesis (obviously of PEPC-PK) may be needed, but the sustained LA of PEPC in the $C_3$ or the $C_4$ species is not entirely dependent on protein synthesis machinery. We speculate that a possible activation of existing PEPC-PK also plays a significant role in enhancing the PEPC activity.

In spite of similarity in LA of $C_3$ and $C_4$ leaf PEPC upon nitrate feeding, the details of their respective signal transduction pathways are likely to differ. For example, LA of $C_3$ PEPC-PK is inhibited strikingly by inhibitors of glutamine synthetase, such as methionine sulfoximine or phosphinothricine, whereas these compounds have no detectable effect on the LA of maize ($C_4$) PEPC-PK (Li *et al*. 1996). These results indicate that a disruption of leaf N metabolism does not have the same impact on the regulatory phosphorylation of PEPC in irradiated leaves of $C_3$ and $C_4$ species. Gln specifically antagonizes the inhibitory effect of MSX on LA of PEPC-PK in tobacco leaves, suggesting that this product of catalysis by GS may be a critical component in the $C_3$ light-signal transduction mechanism.

We suggest that nitrate salts can be used as a tool to modulate PEPC activity in dark/light conditions and further work may possibly be useful monitoring pH changes and precise phosphorylation status of the enzyme.

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