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Relationship between calcium and pyruvate kinase

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Abstract

Tobacco plants (*Nicotiana tabacum* L. cv. Sevilla) were grown under controlled conditions. The leaf content of Ca^{2+}, Mg^{2+} and K^+, and the activity of the pyruvate kinase were analyzed. The increased application of Ca^{2+} diminished the content of K^+ and Mg^{2+} in leaves, and decreased the activity of pyruvate kinase. Taking into account these results, we suggest the pyruvate kinase activity as an bioindicator of the contents of the Ca^{2+}, Mg^{2+} and K^+.

Additional key words: bioindicator, magnesium, *Nicotiana tabacum* L., potassium.

Introduction

The enzyme pyruvate kinase (PK) taking part in nitrogen assimilation and glycolysis is essential for the growth and development of plants (*e.g.*, Lin *et al.* 1989, Schuller *et al.* 1990, Vanlerberghe *et al.* 1990). PK catalyses the synthesis of pyruvate and ATP from phosphoenolpyruvic acid and ADP. Studies on the kinetics of this enzyme reveal that its activity is dependent on content of PEP and ADP, and in addition on the presence of the cofactors K^+ and Mg^{2+} (Podestá and Plaxton 1991, 1992). Various studies suggest that PK activity may be a good physiological indicator of K^+, Mg^{2+} and Ca^{2+} contents in plant tissues (Bar-Akiva *et al.* 1976, Pulgar *et al.* 1996, Lavon and Goldschmidt 1999, Ruiz *et al.* 1999a).

The aim of present study was to analyze the effect of different application rates of Ca^{2+}, given the strong competition between this element and the cations K^+ and Mg^{2+}, on the activity of PK in tobacco leaves. In addition, we seek to determine whether the PK activity can be considered as a reliable bioindicator of cation contents in tobacco leaves.

Materials and methods

Plants: Seeds of *Nicotiana tabacum* cv. Sevilla were sown in September 1996. The seedlings were grown in pots with peat in a greenhouse in southern Spain (Granada) for 45 d and then transferred to a cultivation chamber and grown in vermiculite with nutrient solution under controlled conditions (day/night relative humidity of 60/80% and temperature 30/20 °C, and 16-h photoperiod at a photosynthetic photon flux density of 245 μmol m^{-2} s^{-1} measured at pot level with a *190 SB* quantum sensor, *LI-COR Inc.*, Lincoln, USA). From day 45 until day 75 after sowing the plants received a nutrient solution containing: 5 mM KNO_3, 2 mM NaH_2PO_4, 1.25 mM $CaCl_2 . 2 H_2O$, 1.5 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4 . H_2O$, 1 μM $ZnSO_4 . 7 H_2O$, 0.25 μM $CuSO_4 . 5 H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24} . 4 H_2O$ and 5 μM H_3BO_3, pH 5.5 to 6.0, every 3 d. At 75 d after sowing, the plants were supplied with $CaCl_2 . 2 H_2O$ at three concentrations: 1.25 (Ca1), 2.5 (Ca2) and 5 (Ca3) mM. The experimental design was a randomized complete block with three treatments and three plants per treatment.

The plants were sampled beginning at the 14-leaf stage, just before the onset of flowering. From the same plants, leaves were subjected to two samplings: in the first, day 105 after sowing, leaves were picked from nodes 10 and 11; in the second sampling, two weeks later, leaves from nodes 12 and 13 were picked. All the sampled leaves were in the mature state with lengths of more than 10 cm.

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Abbreviations: ADP - adenosinediphosphate; ATP - adenosinetriphosphate; PEP - phosphoenolpyruvic acid; PK - pyruvate kinase.

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Assay of basal PK: The activity of basal PK was determined by the method of Bar-Akiva *et al.* (1976). A total of 0.5 g of fresh leaf samples were ground with a mortar and pestle (at 0 - 4 °C) in 50 mM of Tris-HCl buffer (pH 7.5), 50 % glycerol (v/v) and 10 mM 2-mercapto-ethanol. The homogenate was centrifuged at 3 000 *g* for 5 min at 0 °C and then the supernatant was centrifuged again at 24 000 *g* for 15 min resulting enzymatic extract. To 0.1 cm^3 of the desalted extract, 0.5 cm^3 of 50 mM Tris-HCl buffer (pH 7.4) was added, together with 0.25 mM sodium molybdate, 25 mM PEP, 5 mM ADP, 5 mM $MgCl_2$ and 0.2 ml of H_2O. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by adding 0.5 cm^3 of 2,4-dinitrophenylhydrazine in 2M HCl and 0.5 cm^3 2M NaOH to avoid possible absorbance changes from altering the pH of the reaction mixture. After centrifugation for 5 min at 3 000 *g*, the absorbance at 510 nm was measured against a standard curve of pyruvate. Triplicate assays were performed for each extract, and enzyme activity was expressed as nmol (pyruvate) mg^{-1}(protein) s^{-1}.

To ascertain whether pyruvate was formed exclusively by PK, we determined the activity of acid phosphatase by the method of Besford (1979).

Assay of PK in the presence of K^+, Ca^{2+}, and Mg^{2+} followed the same method as for basal PK, with the difference of adding to the reaction mixture (0.1 cm^3 of the enzymatic extract + 0.5 cm^3 of 50 mM Tris-HCl buffer at pH 7.4, together with 0.25 mM sodium molybdate, 25 mM PEP, 5 mM ADP, 5 mM $MgCl_2$ + 0.1 cm^3 of H_2O) and either 0.1 cm^3 of K^+ in the form of KCl [50 mM], 0.1 cm^3 of Ca^{2+} in the form of $CaCl_2$ [50 mM], or 0.1 cm^3 of Mg^{2+} in the form of $MgSO_4$ [50 mM]. The processes of incubation, centrifugation and activity measurement were the same as described for basal PK.

The soluble proteins from the supernatants or crude enzyme extracts were determined by Bradford's method (1976), with bovine serum albumin as the standard.

Cation determination: Leaf dry matter was digested with 96 % H_2SO_4 in the presence of hydrogen peroxide. Total potassium (K) was determined by the flame photometer (Lachica *et al.* 1973), and total calcium (Ca) and total magnesium (Mg) was analysed by atomic-absorption spectrophotometry (Hocking and Pate 1977). K^+, Ca^{2+} and Mg^{2+} in their uncomplexed or free forms were analyzed in an aqueous extraction (Romero *et al.* 1997) and determined as described for total contents and expressed in μmol g^{-1} (d.m.).

Statistical analysis: Standard analysis of variance were used to assess the significance of experimental values.

Results and discussion

The various Ca treatments applied induced significantly different leaf Ca concentrations, the highest total and free Ca^{2+} concentration was registered at Ca3 (Table 1). The lowest total Ca content was recorded at Ca1 and the lowest free content at Ca2 (Table 1). The Mg concentration was the complete opposite to that of Ca. The highest total concentrations of Mg were recorded at Ca1 and Ca2, while the lowest was at Ca3 (Table 1). The highest concentration of free Mg^{2+} was at Ca2 and the lowest at Ca3 (Table 1). The strong competition or antagonism between the Ca^{2+} and Mg^{2+} (Marschner *et al.* 1996) is reflected in our experiments. Significant negative correlations between total Ca and total Mg, (r = -0.94 ***), and free Ca^{2+} and free Mg^{2+} (r = -0.91 ***) were found.

The total and free K^+ concentration was higher at Ca2 than at Ca1, however, Ca3 had the lowest values (Table 1). The positive effect of Ca^{2+} in relation to the uptake and foliar concentrations of monovalent cations such as K^+ has been shown (Sung and Lo 1990; Fenn *et al.* 1994).

Table 1. Effect of Ca^{2+} treatments (Ca1, Ca2 and Ca3 represent 1.25, 2.5 and 5.0 mM Ca^{2+}, respectively) on the total and free of K^+, Mg^{2+}, and Ca^{2+} concentrations [μmol g^{-1}(d.m.)] in leaves of tobacco plants. Means ± SE, n = 6; *** - significant differences at $P < 0.001$.

Treatments	Total			Free		
	K	Mg	Ca	K^+	Mg^{2+}	Ca^{2+}
Ca1	772.5 ± 26.4	720.5 ± 23.5	556.5 ± 24.2	265.3 ± 17.0	307.9 ± 17.2	308.9 ± 21.6
Ca2	970.3 ± 27.1	584.7 ± 20.2	863.0 ± 28.6	453.0 ± 20.1	433.3 ± 20.0	244.0 ± 20.1
Ca3	444.6 ± 25.2	288.5 ± 18.7	1241.2 ± 30.8	148.2 ± 15.8	130.9 ± 14.3	669.9 ± 25.4
Significance	***	***	***	***	***	***
$LSD_{0.05}$	54.5	25.3	59.8	35.7	23.3	17.7

The highest basal PK activity was recorded at Ca2, while the lowest was registered at Ca3, with a decrease of 65 % (Table 2). The activity of this enzyme is dependent on the concentrations of PEP and ADP, and on the presence of K^+ and Mg^{2+} (Podestá and Plaxton 1991, 1992). In our experiment, the highest basal PK activity was presented at Ca2 (Table 2). This treatment also gave the highest concentrations of K^+ and Mg^{2+} (Table 1). The regression between basal activity PK and K^+ and Mg^{2+} was significant and positive (total K-basal PK activity, r = 0.81 **; free K^+-basal PK activity, r = 0.92 ***; total Mg-basal PK activity, r = 0.63 ns; free Mg^{2+}-basal PK activity, r = 0.89 **).

In relation to the lowest basal PK activity at Ca3 (Fig. 1), the greatest leaf Ca^{2+} concentration in this treatment reduced the leaf concentration both of K^+ and Mg^{2+} (Marschner *et al.* 1996), this perhaps being the cause of the reduction of the basal PK activity. On the other hand, as occurs with the Mg^{2+} at high concentrations, in the treatment Ca3, Ca^{2+} could bond to the substrates (PEP and ADP) of the reaction which catalyses the PK, thereby impeding or reducing the affinity between these and PK, and therefore the activity of this enzyme (Podestá and Plaxton 1992). Finally, bearing in mind the close relationship between the nitrogenous metabolism and glycolysis (Baysdorfer and Bassham 1984, Turpin *et al.* 1990, Geigenberger and Stitt 1991, Van Quy *et al.* 1991), we suppose, as other researchers (Kafkafi *et al.* 1992, Bharti *et al.* 1996, Ruiz *et al.* 1999b), a decrease of the nitrogenous metabolism at Ca3 which could influence the decline in PK activity in this treatment.

On the other hand, various studies showed PK activity to be an indicator of the K^+, Mg^{2+}, and Ca^{2+} content in the organ under study (Bar-Akiva *et al.* 1976, Pulgar *et al.* 1996, Lavon and Goldschmidt 1999, Ruiz *et al.* 1999a), based on the differences between basal PK activity and the PK activities resulting from the addition of the different cations to the enzymatic reaction medium.

The highest PK activity in the presence of K^+ was at Ca1 and Ca2, while the lowest at Ca3. However, in this treatment PK in the presence of K^+ showed a greatest increase of 40 % with respect to the basal PK activity (Table 2). On the contrary, the least increase was recorded at Ca2, this being only 2 %. The same trend was found for the PK activity in the presence of Mg^{2+} (Table 2). It is worth noting that in our experiment the increases in the PK activities, in the presence of K^+ and Mg^{2+}, were inversely proportional to the leaf concentrations of the free forms of both cations.

As indicated above, the treatment Ca3 brought about the greatest increase in PK activity in the presence of K^+ and Mg^{2+} (Table 2). This is due to the fact that in this treatment the levels of free K^+ and Mg^{2+} were lowest (Table 1) due possibly to the antagonism with the Ca^{2+}. Therefore, an increase in the concentration of these cations in the reaction medium implies a rise in the level of PK activity with respect to the basal activity. This rise in PK activity also is reflected in the Ca1 treatment (Table 2), where the levels of free K^+ and Mg^{2+} were also low (Table 1). Finally, the increase of PK activity in the presence of K^+ and Mg^{2+} in the Ca1 and Ca3 treatments indicates the possible need of these cations. The contrary trend was noted in the Ca2 treatment, where the increased PK activity in the presence of K^+ and Mg^{2+} with respect to the basal PK activity was lowest (Table 2), indicating that in this treatment the concentrations of K^+ and Mg^{2+} were apparently adequate.

Table 2. Effect of Ca^{2+} treatments (Ca1, Ca2 and Ca3 represent 1.25, 2.5 and 5.0 mM Ca^{2+}, respectively) on basal PK activity and on the PK activity in the presence of 50 mM K^+ (PK-K^+), 50 mM Mg^{2+} (PK-Mg^{2+}) and 50 mM Ca^{2+} (PK-Ca^{2+}) in leaves of tobacco plants. Values in brackets indicate increases (+%) or decreases (-%) in PK activity in the presence of ions versus basal PK activity. Means ± SE, n = 6; *** - significant differences at $P < 0.001$.

Treatments	PK activity [nmol(pyruvate) mg^{-1}(protein) s^{-1}]			
	basal PK	PK-K^+	PK-Mg^{2+}	PK-Ca^{2+}
Ca1	52.3 ± 5.83	64.5 ± 6.01 (23)	59.8 ± 6.83 (14)	31.5 ± 2.51 (-40)
Ca2	91.8 ± 7.01	93.1 ± 7.33 (1)	95.5 ± 8.01 (4)	70.7 ± 4.51 (-32)
Ca3	32.2 ± 3.83	45.3 ± 5.83 (41)	48.8 ± 5.16 (52)	7.83 ± 1.52 (-75)
Significance	***	***	***	***
$LSD_{0.05}$	5.66	3.51	3.01	3.83

The PK activity in the presence of Ca^{2+} (Table 2) was highest at Ca2 and lowest at Ca3. It was striking that in all of the treatments the PK activity stimulated by Ca^{2+} was lower than was the basal PK activity (Table 2), caused possibly either by an increase in the formation of the Ca^{2+}-PEP and Ca^{2+}-ADP in the reaction medium, or by a direct action of Ca^{2+} on the PK activity, explaining the results obtained. This trend of PK activity in the presence of Ca^{2+} with respect to the basal PK activity indicates that the Ca^{2+} contents are adequate at Ca2 and excessive at Ca3.

In conclusion, the increased application of Ca^{2+} diminished the foliar levels of K^+ and Mg^{2+}, and decreased the activity of PK. The decreased PK activity in the Ca3 treatment could be due to: *1*) strong competition between Ca^{2+} and the cations K^+ and Mg^{2+}, the presence of which is essential for the PK activity; *2*) a increase in the formation

of complexes between Ca^{2+} and the substrates of PK (Ca^{2+}-PEP and Ca^{2+}-ADP), which would diminish the affinity of these with the enzyme and therefore its activity; and *3*), finally, a decline in this treatment of nitrogenous metabolism (Ruiz *et al.* 1999 b). In view of our results, the comparison between the basal PK activity and the PK activity in the presence of Ca^{2+}, Mg^{2+} and K^+ could enable us to establish the optimal balance between these cations principally in their free forms.

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