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Magnesium deficiency-induced changes in organic acid metabolism of *Citrus sinensis* roots and leaves

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Abstract

Organic acid (OA) metabolisms are of fundamental importance but very limited data are available on the responses of plant OA metabolisms to Mg-deficiency. Seedlings of *Citrus sinensis* (L.) Osbeck cv. Xuegan were irrigated with Mg-deficient (0, 50, or 500 μM $MgSO_4$) or Mg-sufficient (2000 μM $MgSO_4$) nutrient solution every other day for 12 weeks. Thereafter, we investigated the content of Mg, malate, and citrate as well as the activities of acid-metabolizing enzymes in roots and leaves. Root malate content remained stable except for an increase in the highest Mg content and root citrate content increased with increasing root Mg content. As leaf Mg content increased, leaf malate and malate + citrate content decreased whereas leaf citrate content increased. Mg-deficiency decreased or did not affect activities of citrate synthase (CS), aconitase (ACO), phospho*enol*pyruvate carboxylase (PEPC), NADP-isocitrate dehydrogenase (NADP-IDH), NAD-malate dehydrogenase (NAD-MDH), NADP-malic enzyme (NADP-ME), and pyruvate kinase (PK) in roots, whereas phospho*enol*pyruvate phosphatase (PEPP) activity slightly increased. In contrast, Mg-deficient leaves had higher or similar activities of enzymes above mentioned except PEPP, NAD-MDH, and NADP-ME. In conclusion, both glycolysis and tricarboxylic acid (TCA) cycle may be up-regulated in Mg-deficient leaves but down-regulated in Mg-deficient roots.

Additional key words: organic acid-metabolizing enzymes, citrate, glycolysis, malate, tricarboxylic acid cycle.

Introduction

Magnesium is an essential macronutrient required for the normal growth and development of higher plants. Mg plays an important role in photosynthesis, as a central atom of chlorophyll (Chl) molecule, and in many other processes, such as glycolysis, pentose phosphate pathway, tricarboxylic acid (TCA) cycle, and the formation of DNA and RNA (Terry and Ulrich 1974, Salisbury and Ross 1992, Marschner 1995, Li *et al*. 2001a, Shaul 2002, Epstein and Bloom 2004, Cakmak and Kirby 2008, Büchert *et al*. 2011, Guha and Rao 2012). Mg-deficiency is a widespread problem affecting productivity and quality of crops (Hermans *et al*. 2004).

Mg-deficiency decreases photosynthesis in many plant species including *Citrus sinensis* (Ling *et al*. 2009, Yang *et al*. 2012), *Citrus grandis* (Yang *et al*. 2012), *Zea mays* (Peaslee and Moss 1966), *Spinacea oleracea* (Bottrill *et al*. 1970), *Dimocarpus longan* (Li *et al*. 2001b), *Cucumis sativus* (Yang *et al*. 2002), *Pinus radiata* (Laing *et al*. 2000), *Vicia faba* (Hariadi and Shabala 2004), *Beta vulgaris* (Terry and Ulrich 1974), and *Phaseolus vulgaris* (Fischer 1997). Respiration in plants is also affected by Mg-deficiency. Bottrill *et al*. (1970) observed that in whole spinach plants, Mg-deficiency resulted in reduced respiration rate when expressed on an insoluble nitrogen basis, but it had no effect on respiration rate when expressed on a fresh mass basis. Terry and Ulrich (1974) reported that in sugar beet leaves Mg-deficiency decreased the rate of photorespiration but increased the rate of dark respiration. In addition, Mg-deficiency resulted in accumulation of amino acids, which were made from intermediates of TCA cycle and other major pathways, in source and sink leaves (Fischer *et al*. 1998). An important function of Mg is its involvement in the export of sugars from source to sink. Mg-deficiency results in accumulation of sugars in source leaves, especially of sucrose and starch (Cakmak *et al*.

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Abbreviations: ACO - aconitase; CS - citrate synthase; NAD-MDH - NAD-malate dehydrogenase; NADP-IDH - NADP-isocitrate dehydrogenase; NADP-ME - NADP-malic enzyme; OA - organic acid; PEPC - phospho*enol*pyruvate carboxylase; PEPP - phospho*enol*pyruvate phosphatase; PK - pyruvate kinase.

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1994a,b, Fischer *et al*. 1998, Hermans *et al*. 2004, 2005, Hermans and Verbruggen 2005). Yang *et al*. (2012) showed that Mg-deficiency increased the content of glucose, fructose, and sucrose in *C. sinensis* leaves but decreased glucose and fructose content in roots. Hoffland *et al*. (1992) suggested that part of malate accumulated in phosphorus-deficient rape roots was probably newly synthesized from sugars imported from leaves to roots. These results indicate that sugar utilizations for synthesis of organic acids (OAs) may be increased in Mg-deficient leaves but decreased in Mg-deficient roots. Although OA metabolisms are of fundamental importance (López-Bucio *et al*. 2000), very little is known about the responses of plant OA metabolism to Mg-deficiency. Besford (1978) reported that pyruvate kinase (PK, EC 2.7.1.40) activity in tomato leaves correlated with Mg content proposing that its activity was a suitable indicator of Mg disorder. In the xylem sap of beech roots, there was a significant positive correlation between malate content and Mg content (Schell 1997).

Citrus spp. belong to evergreen fruit trees and are commercially grown in many countries. In China, Mg-deficiency is frequently observed in *Citrus* plantations and is responsible for loss of productivity and fruit quality (Ling *et al*. 2009). Previous studies showed that malate and citrate were the major OAs in both leaves (Achituv and Bar-Akiva 1978) and fruit juice (Yamaki 1989) of *Citrus*. In plant cells, malate synthesis mainly occurs in the cytosol and is catalyzed by phospho-*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) and NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) (Moing *et al*. 2000). Malate can be decarboxylated by NADP-malic enzyme (NADP-ME, EC 1.1.1.40) in cytosol to form pyruvate and CO_2 (Chen *et al*. 2002, Lin *et al*. 2011). The synthesis and degradation of citrate are mediated by citrate synthase (CS, EC 4.1.3.7), NADP-isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42), and aconitase (ACO, EC 4.2.1.3). Phospho*enol*pyruvate phosphatase (PEPP, EC 3.1.3.60) and PK are also involved in the metabolism of malate and citrate (Sadka *et al*. 2000, Lin *et al*. 2011). In this study, we investigated the effects of Mg-deficiency on the content of Mg, malate, and citrate as well as the activities of acid-metabolizing enzymes in *C. sinensis* leaves and roots with the aim to elucidate how Mg-deficiency affects OA metabolisms.

Materials and methods

Plant culture, Mg treatments, and sampling were performed according to Yang *et al*. (2012). Briefly, 6-week-old seedlings of *Citrus sinensis* (L.) Osbeck cv. Xuegan were transplanted to 6 dm^3 pots containing sand. Seedlings, two per pot, were grown outdoors at Fujian Agriculture and Forestry University. Each pot was supplied with 500 cm^3 of nutrient solution every other day. The nutrient solution contained the following macronutrients [mM]: KNO_3, 5; $Ca(NO_3)_2$, 5; KH_2PO_4, 1; and $MgSO_4$, 2; and micronutrients [μM]: H_3BO_3, 10; $MnCl_2$, 2; $ZnSO_4$, 2; $CuSO_4$, 0.5; $(NH_4)_6Mo_7O_{24}$, 0.065; and Fe-EDTA, 20. Ten weeks after transplanting, each pot was supplied every other day with Mg-deficient (0, 50, or 500 μM $MgSO_4$) or Mg-sufficient (2 000 μM $MgSO_4$) nutrient solution (approx. 500 cm^3) for 12 weeks. Four concentration of Mg was created by withholding the supply of $MgSO_4$ and replacing it with equivalent moles of Na_2SO_4 in order to maintain the supply of sulfur and the osmotic potential of the nutrient solution. There were five replications per Mg treatment with 4 pots each in a completely randomized design. At the end of the experiment, fully-expanded leaves were used for all the measurements. Leaf discs (0.608 cm^2 in size) were collected at noon under full sun and immediately frozen in liquid nitrogen. Approximately 5-mm-long root apices were frozen immediately in liquid nitrogen after they were excised from the same seedlings used for sampling leaves. Both leaf and root samples were stored at -80 °C until extraction.

Malate and citrate were extracted and assayed according to Chen *et al*. (2002) with some modifications (Yang *et al*. 2011). Acid-metabolizing enzymes (CS, ACO, PEPC, NADP-IDH, PEPP, NAD-MDH, NADP-ME, and PK) in roots and leaves were extracted and measured according to Yang *et al*. (2011).

Differences among treatments were separated by the least significant difference (LSD) test at $P < 0.05$ level using a software named *Data Processing System* (*DPS*, Zhejiang University, Hangzhou, China) developed by Tang and Feng (2002).

Results

Root content of malate, citrate, and malate + citrate did not change over the range of Mg supply except for an increase under 2 000 μM Mg. Mg-deficiency decreased leaf content of malate and malate + citrate whereas leaf citrate content was higher under 2 000 μM Mg than under 0, 50, and 500 μM Mg (Table 1).

Root malate content did not significantly correlate with root Mg content (Fig. 1*A*) whereas root citrate and malate + citrate content increased with increasing root Mg content (Fig. 1*B-C*). As leaf Mg content increased, leaf malate and malate + citrate content decreased whereas leaf citrate content increased (Fig. 1*D-F*).

Root activities of CS and PEPC decreased as Mg supply decreased from 2 000 to 50 μM and then kept unchanged under 0 μM Mg. The activity of ACO was higher in roots of 2 000 μM Mg-treated plants than in

Table 1. Effects of Mg supply on malate and citrate content [μmol g^{-1}(f.m.)] in roots and leaves. Means ± SE, n = 5. The same letters within a column means no significant difference at 95 % probability level.

Mg treatments [μM]	Roots malate	citrate	malate + citrate	Leaves malate	citrate	malate + citrate
0	17.9 ± 0.9 b	3.6 ± 0.2 b	21.5 ± 0.7 b	61.4 ± 1.8 a	4.0 ± 0.4 b	65.4 ± 1.8 a
50	17.4 ± 1.4 b	4.7 ± 0.4 ab	22.1 ± 1.3 b	57.1 ± 3.0 a	5.2 ± 0.3 b	62.3 ± 2.8 a
500	17.4 ± 0.7 b	5.3 ± 0.7 ab	22.7 ± 1.3 b	42.2 ± 3.3 b	6.8 ± 0.3 b	49.0 ± 3.4 b
2000	22.8 ± 1.7 a	6.3 ± 0.8 a	29.1 ± 2.2 a	25.8 ± 4.0 c	17.2 ± 2.9 a	43.0 ± 3.4 b

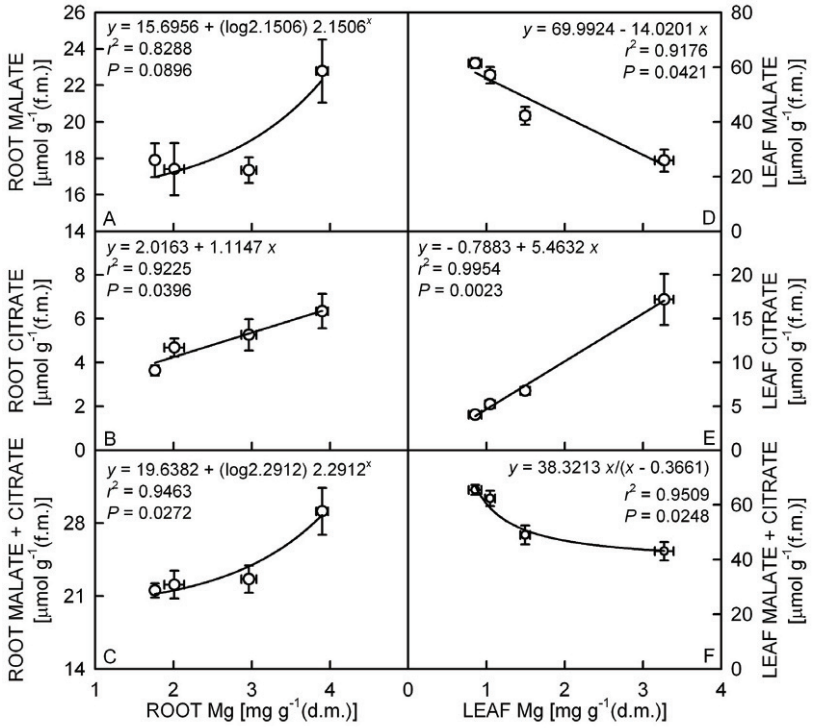

Fig. 1. Malate and citrate content in relation to Mg content in roots and leaves. Data of Mg content in roots and leaves cited here are from a previous report (Yang *et al*. 2012).

Table 2. Effects of Mg supply on the activities of citrate synthase (CS), aconitase (ACO), phospho*enol*pyruvate carboxylase (PEPC), NADP-isocitrate dehydrogenase (NADP-IDH), phospho*enol*pyruvate phosphatase (PEPP), NAD-malate dehydrogenase (NAD-MDH), NADP-malic enzyme (NADP-ME), and pyruvate kinase (PK) in roots. Means ± SE, n = 5. The same letters within a column means no significant difference at 95 % probability level.

Mg [μM]	Root activities of acid-metabolizing enzymes [nmol g^{-1}(f.m.) s^{-1}] CS	ACO	PEPC	NADP-IDH	PEPP	NAD-MDH	NADP-ME	PK
0	4.5 ± 0.6 c	22.5 ± 0.7 b	11.1 ± 0.7 c	16.8 ± 1.6 a	16.6 ± 1.1 ab	2634 ± 168 a	19.6 ± 0.8 a	16.9 ± 2.0 a
50	6.5 ± 0.6 c	22.8 ± 1.0 b	10.5 ± 0.3 c	18.5 ± 0.8 a	18.5 ± 1.0 a	2822 ± 122 a	21.2 ± 0.7 a	20.5 ± 1.8 a
500	10.1 ± 0.2 b	21.9 ± 1.2 b	12.9 ± 0.6 b	16.7 ± 1.3 a	15.3 ± 0.4 b	2667 ± 173 a	21.2 ± 1.0 a	19.6 ± 3.3 a
2000	13.1 ± 0.7 a	31.1 ± 1.2 a	16.6 ± 0.5 a	18.2 ± 1.5 a	14.7 ± 1.4 b	2648 ± 117 a	20.6 ± 1.3 a	21.5 ± 1.0 a

0, 50, and 500 μM Mg-treated ones. Root activities of other acid-metabolizing enzymes did not change in response to Mg supply except for a higher PEPP activity at 50 μM Mg (Table 2).

The activity of CS was higher in leaves of 50 and 500 μM Mg-treated plants than in 0 and 2 000 Mg-treated ones. Mg-deficiency increased leaf activities of ACO, PEPC, NADP-IDH, and PK, but decreased leaf activities of PEPP, NAD-MDH, and NADP-ME (Table 3).

Root malate and citrate content increased with

Table 3. Effects of Mg supply on the activities of citrate synthase (CS), aconitase (ACO), phospho*enol*pyruvate carboxylase (PEPC), NADP-isocitrate dehydrogenase (NADP-IDH), phospho*enol*pyruvate phosphatase (PEPP), NAD-malate dehydrogenase (NAD-MDH), NADP-malic enzyme (NADP-ME), and pyruvate kinase (PK) in leaves. Means ± SE, $n = 4$. The same letters within a column means no significant difference at 95 % probability level.

Mg [μM]	Leaf activities of acid-metabolizing enzymes [nmol g^{-1}(f.m.) s^{-1}]							
	CS	ACO	PEPC	NADP-IDH	PEPP	NAD-MDH	NADP-ME	PK
0	22.9 ± 1.9 b	18.6 ± 0.7 a	21.9 ± 1.2 a	19.6 ± 0.2 a	22.7 ± 1.1 b	3886 ± 159 c	9.1 ± 0.3 c	49.4 ± 1.4 a
50	31.9 ± 0.9 a	14.6 ± 0.6 b	21.9 ± 0.7 a	18.1 ± 0.5 b	26.7 ± 2.2 ab	4199 ± 262 bc	10.5 ± 0.2 bc	48.9 ± 0.8 a
500	30.5 ± 1.5 a	13.5 ± 0.9 b	18.2 ± 0.7 b	13.8 ± 0.5 c	25.1 ± 1.0 ab	4982 ± 213 b	11.8 ± 0.7 b	41.5 ± 2.4 b
2000	26.1 ± 1.0 b	8.2 ± 0.7 c	14.6 ± 0.6 c	9.5 ± 0.5 d	28.5 ± 1.6 a	7007 ± 361 a	14.3 ± 0.3 a	33.7 ± 2.3 c

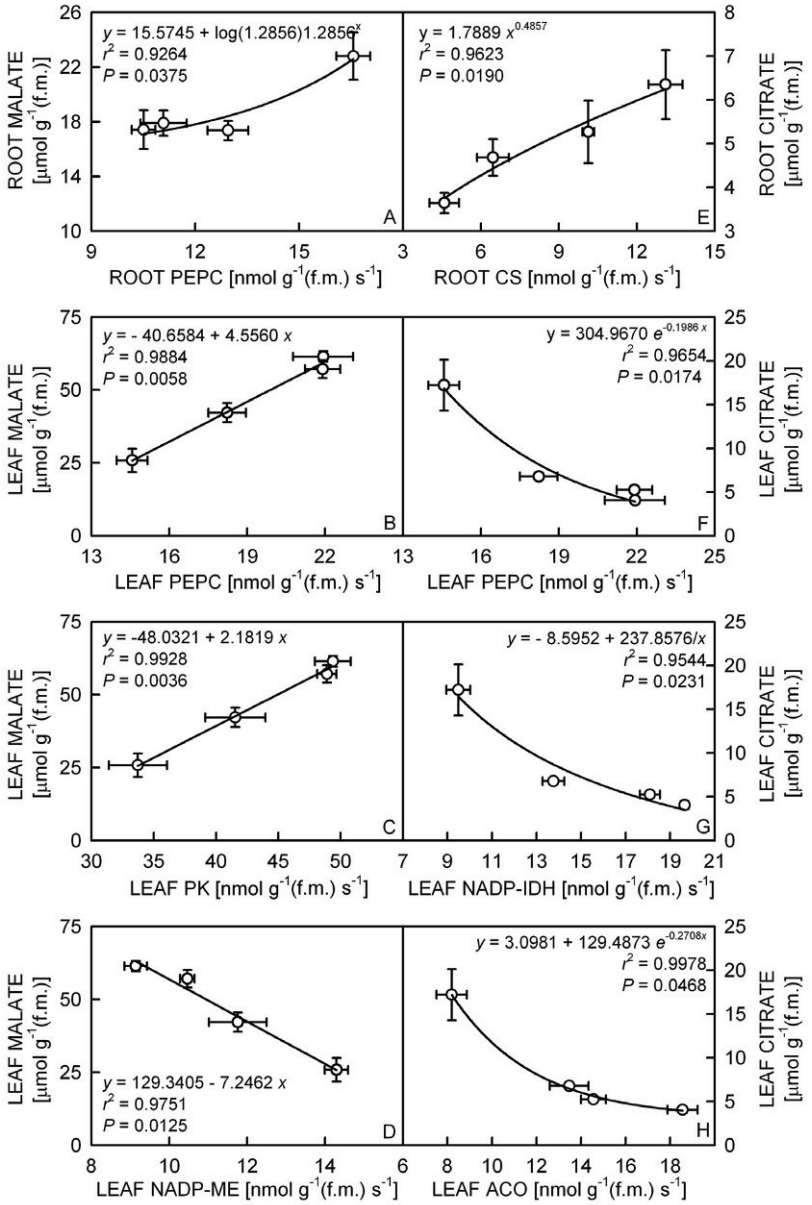

Fig. 2. Relationships between malate and citrate content in roots and leaves and activities of acid-metabolizing enzymes [phospho*enol*pyruvate carboxylase (PEPC), aconitase (ACO), citrate synthase (CS), pyruvate kinase (PK), NADP-malic enzyme (NADP-ME), and NADP-isocitrate dehydrogenase (NADP-IDH)].

increasing PEPC (Fig. 2*A*) and CS (Fig. 2*E*) activities, respectively. Leaf malate content increased with increasing PEPC (Fig. 2*B*) or PK (Fig. 2*C*) activities and decreased with increasing NADP-ME activity (Fig. 2*D*). Leaf citrate content decreased with increasing PEPC (Fig. 2*F*), NADP-IDH (Fig. 2*G*) or ACO (Fig. 2*H*) activities.

Discussion

Our results shows that Mg-deficient leaves had higher content of malate and malate + citrate (Table 1) and activities of several key enzymes involved in glycolysis and TCA cycle, including ACO, PEPC, NADP-IDH, and PK (Table 3), indicating that both glycolysis and TCA cycle may be up-regulated in Mg-deficient leaves. This is consistent with previous reports that Mg-deficiency increased the rate of dark respiration in sugar beet (Terry and Ulrich 1974) and bean (Fischer and Bremer 1993) leaves. However, Mg-deficiency decreased PK activity in tomato leaves (Besford 1978). Because Mg-deficient leaves accumulated more glucose, fructose, and sucrose (Yang *et al*. 2012), the up-regulation of both glycolysis and TCA cycle agreed with the increased requirement for consuming the excessive sugars. This is also supported by the results of Wang *et al*. (2010) who reported that both glycolysis and TCA cycle, as well as content of citrate and malate + citrate were enhanced in the chlorotic leaves of apple with excessive accumulation of sugars and drastically decreased CO_2 assimilation. Although enhanced rate of dark respiration and content of malate in Mg-deficient leaves reduce the net C balance, it is possible that enhanced respiration and malate provide the energy to maintain basic metabolic processes in Mg-deficient leaves that have lower photosynthetic rate.

Our finding that Mg-deficiency-induced decrease in leaf citrate content (Table 1) may be caused by its increased degradation due to increasing ACO or NADP-IDH activities (Fig. 2*G,H*) rather than by its decreased synthesis because Mg-deficient leaves had higher or similar CS and PEPC (Table 3) activities. Increased malate content in leaves with decreased Mg supply (Table 1) may result from its increased synthesis due to increasing PEPC (Fig. 2*B*) and PK (Fig. 2*C*) activities and its decreased degradation due to increasing NADP-ME activity (Fig. 2*D*). In addition, more sugars may be utilized to synthesize malate due to the decreased sugar export from Mg-deficient leaves, as indicated by increased accumulation of sugars (Yang *et al*. 2012). In Mg-deficient leaves, the higher malate content was likely related to the lower citrate content (Table 1). Most citrate and malate are preferentially stored in the vacuoles and tonoplast transporters are responsible for their accumulation (López-Bucio *et al*. 2000, Hurth *et al*. 2005). Rentsch and Martinola (1991) and Emmerlich *et al*. (2003) showed that malate transport into vacuoles is competitively inhibited by citrate and *vice versa*. The inhibition of citrate on malate transport in Mg-deficient leaves may be decreased. Thus, more malate may be accumulated in the vacuoles.

In Mg-deficient plants, roots had lower or similar content of malate and citrate and activities of acid-metabolizing enzymes comparing with those in Mg-sufficient ones (Tables 1 and 2) meaning that both glycolysis and TCA cycle may be down-regulated in roots. As Mg-deficient plants accumulated less glucose, fructose, and sucrose in roots (Yang *et al*. 2012), the down-regulation of both glycolysis and TCA cycle and the decrease in malate and citrate content provided an obvious advantage to the net C balance in roots.

Decreased content of malate and citrate in Mg-deficient roots (Table 1) may be associated with their decreased synthesis due to decreasing PEPC (Fig. 2*A*) and CS (Fig. 2*E*) activities, respectively, and with reduced amount of sugars available for OA syntheses due to decreased translocation of sugars from leaves to roots. Mg-deficiency increased the content of glucose, fructose, and sucrose in leaves but decreased their content in roots, except for similar sucrose content among Mg treatments (Yang *et al*. 2012). The decrease in root malate content in response to Mg-deficiency may be also caused by Mg-deficiency-induced alteration in the partitioning of malate between roots and leaves as leaf malate content increased (Table 1). Schell (1997) suggested that the positive correlation between malate content and Mg content in the xylem sap of beech roots could be an indicator of Mg-malate formation. This may explain why root malate content increased with increasing root Mg content (Fig. 1*A*).

In conclusion, both glycolysis and TCA cycle might be up-regulated in Mg-deficient leaves but down-regulated in Mg-deficient roots.

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