BIOLOGIA PLANTARUM 54 (4): 766-768, 2010

BRIEF COMMUNICATION

Citrate transporters play an important role in regulating aluminum-induced citrate secretion in *Glycine max*

J.F. YOU, N.N. HOU, M.Y. XU, H.M. ZHANG and Z.M. YANG*

College of Plant Science, Jilin University, Changchun, 130062, P.R. China

Abstract

To further understand the process of Al-induced citrate secretion from soybean roots, the effect of protein synthesis inhibitor, anion channel blockers, and citrate carrier inhibitors on Al-induced citrate exudation was investigated in Al-resistant soybean cultivar PI 416937. Citrate exudation from roots increased with the increase of Al concentration from 10 to 50 µM and initiated after 4 h of Al exposure. Protein synthesis inhibitor, cycloheximide (CHM; 25 µM) completely inhibited Al-induced citrate secretion during 12-h exposure, suggesting that novel protein synthesis was necessary in Al-induced citrate efflux. Also both anion channel blocker anthracene-9-carboxylic acid (A-9-C) and citrate carrier inhibitor mersalyl acid (Mersalyl) significantly reduced citrate secretion, suggesting that both anion channels in plasma membrane and citrate carriers in mitochondria membrane were the rate limiting factors of Al dependent citrate release. However, Al-induced citrate secretion was insensitive to anion channel blockers phenylglyoxal (PG), 4,4'-diisothiocyanostibene-2,2'-disulfonat (DIDS) and citrate carrier inhibitor pyridoxal 5'-P (PP).

Additional key words: aluminum stress, soybean, anion channel blockers, citrate carrier inhibitors.

Aluminum (Al) toxicity is one of the main constraints of plant growth and crop production in acid soils (pH ≤ 5.5) (Kochian *et al.* 2004). Many Al-resistant plant species and genotypes have been described and some Al-resistance mechanisms have been reported in the previous studies (Kochian *et al.* 2004). Organic acid exudation contributes to Al resistance by excluding Al from the root apex (Ryan and Delhaize 2001). In pattern I plants species, exudation is switched on without a delay after exposure to Al and kept constant over time. In pattern II plants species, organic acid secretion is induced by the addition of Al after a lag phase of several hours and the rate of release increases over time. Ma (2000) has suggested that different processes are involved in the two secretion patterns. In pattern I, Al might activate a preexisting transporter (anion channel) in the plasma membrane to initiate organic anion efflux, and the induction of novel protein synthesis is not required. Whereas in pattern II, the induction of genes and synthesis of proteins involved in organic acid metabolism (biosynthesis and decomposition) or transport (anion channel on plasma membrane and/or tonoplast, or transport of organic acids from mitochondria) is required.

Soybean (*Glycine max* L.) is cultivated in the acidic soils world-widely, including the Southeast USA, Cerrados in Brazil and the South China. Al caused marked reduction in the soybean growth (both root and shoot), chlorophyll content and net photosynthetic rate (Shamsi *et al.* 2008). Citrate exudation from roots has been suggested as an important Al exclusion mechanism in soybean (Yang *et al.* 2000, 2001, Silva *et al.* 2001), which needs at least 4 h of Al exposure (Yang *et al.* 2001). Therefore, it was classified into pattern II, and gene induction and following novel protein synthesis were supposed to involve in the process of citrate secretion. However, to our knowledge, there is no experimental evidence to support the above speculation.

Yang *et al.* (2001) reported that Al-induced secretion of citrate in soybean was poorly associated with the activities of enzymes involved in the synthesis of citrate in the root cells. Also, the relationship of citrate secretion and the transporting systems sited on plasma membrane and/or mitochondria membrane is not clear. In the present study,

---

Received 29 April 2009, *accepted* 11 October 2009.

Abbreviations: A-9-C - anthracene-9-carboxylic acid; CHM - cycloheximide; DIDS - 4,4'-diisothiocyanostibene-2,2'-disulfonat; PG - phenylglyoxal; PP - pyridoxal 5'-P.

Acknowledgements: This research was supported by Natural Science Foundation of China (No. 30671241) for Z.M. Yang and New Teacher Research Fund of the Doctoral Program of Higher Education of China (20070183163) for J.F. You.

* Corresponding author; fax: (+86) 431 87836029, email: zmyang@jlu.edu.cn

protein synthesis inhibitor, anion channel blockers, and citrate carrier inhibitors were used to investigate the transport of citrate during its secretion period in the presence of Al.

Soybean (*Glycine max* L.) seedlings of Al-resistant cultivar PI 416937 were grown in 1-dm^3 plastic pots (7 seedlings per pot) containing the nutrient solution (pH 4.5) with the following composition (Horst *et al.* 1992) [µM]: KNO_3 - 750, $Ca(NO_3)_2$ - 250, $MgSO_4$ - 325, KH_2PO_4 - 10, Fe-EDTA - 20, H_3BO_3 - 8, $MnCl_2$ - 0.2, $CuSO_4$ - 0.2, $ZnSO_4$ - 0.2, $(NH_4)_6Mo_7O_{24}$ - 0.2. The nutrient solution was renewed every other day. Soybean seedlings were grown in a growth room with a 14-h photoperiod (irradiance of 300 µmol m^{-2} s^{-1}), day/night temperature of 25/20 ºC and relative humidity 70 %.

14-d-old seedlings were pre-cultured in $CaCl_2$ (0.5 mM) solution (pH 4.5) for 12 h and then following treatments were applied. *1*) Plants were subjected to $CaCl_2$ (0.5 mM) solution (pH 4.5) containing different concentrations of $AlCl_3$ (0, 10, 20, 30, 40, 50 µM) and after 12 h, the root exudates were collected to analyze the organic acid anion secretion. *2*) Plants were subjected to 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 µM $AlCl_3$ in the absence or presence of cycloheximide (CHM; 25 µM). Root exudates were collected at a 4-h interval for 12 h. *3*) Plants were subjected to 0.5 mM $CaCl_2$ solution containing different concentration of anion channel blockers [anthracene-9-carboxylic acid (A-9-C; 10, 20, 30 µM), phenylglyoxal (PG; 5, 10, 20, 30 µM), 4,4'-diisothio-cyanostibene-2,2'-disulfonat (DIDS; 5, 10, 20, 30 µM)] or citrate carrier inhibitors [pyridoxal 5'-P (PP; 10, 20, 30 µM), Mersalyl (10, 20, 30 µM)] in the presence or absence of 50 µM $AlCl_3$. After 12 h, the root exudates were collected for determination of organic acid.

The composition of root exudates was determined according to Yang *et al.* (2000). Briefly, the collected root exudates were passed through a cation-exchange column (16 mm × 14 cm) filled with 5 g *Amerlite IR-120B* resin (H^+ form, *Murocachi Chemical*, Tokyo, Japan), and then through an anion-exchange column (16 mm × 14 cm) filled with 2.0 g *AG 1-X8* resin (100 - 200 mesh, formate form, *Bio-Rad*, Hercules, USA). The organic acid anions retained in the anion exchange resin were eluted with 10 cm^3 of 2 M HCl. The eluent was concentrated to dryness by a rotary evaporator at 40 ºC. The residue was re-dissolved in 1 cm^3 *Milli-Q* water (*Bio-Rad*, Hercules, USA). The organic acid anions were detected by high performance liquid chromatography (HPLC) equipped with a C18 column (300 × 8 mm; *Kromasil*, Dalian, China). The mobile phase was 0.55 % KH_2PO_4 buffer solution (adjusted pH to 2.7 by diluted H_3PO_4) with a flow rate of 0.55 cm^3 min^{-1} at 30 ºC. The peaks at 214 nm were recorded. Statistical analysis was carried out using *SPSS* version *16.0*. Means were compared using Tukey test at $P < 0.05$.

In the present study, citrate was the only organic acid anion detected in the root exudates in the presence of Al, which increased with the increase of Al concentration (10 - 50 µM) (Fig. 1), and 50 µM Al was used for the other studies.

Al-induced citrate secretion begins at least 4 h after Al exposure (Fig. 1). The lag between Al treatment and citrate secretion suggested that intermediate steps occurred between the stimulus reception and citrate secretion. Non-specific protein synthesis inhibitor CHM has been reported to effectively inhibit the *de novo* protein synthesis in the root tips of buckwheat and *Cassia tora* (Yang *et al.* 2006). In present study, 25 µM CHM completely inhibited Al-induced citrate secretion from soybean roots during the whole 12-h treatment period (Fig. 1), suggesting that novel protein synthesis was involved in the process of Al-induced citrate secretion from soybean roots. This supports the previous suggestion that gene induction and novel protein synthesis are involved in the Al induced organic acid anions efflux in pattern II (Ma 2000).

Anion channel blockers (A-9-C, PG and DIDS) were used to investigate Al-induced organic acid anions secretion from roots in this study. A-9-C, PG or DIDS (10 µM) did not affect the growth and citrate exudation in roots of soybean in the absence of Al (data not shown). However, in the presence of Al, A-9-C (10, 20, 30 µM) completely inhibited, while PG (5 - 30 µM) and DIDS (5 - 30 µM) did not affect Al-induced citrate secretion from roots (Fig. 2). The anion channel blockers have been used to characterize Al-induced organic acid secretion in different plant species (Ryan *et al.* 1995, Pellet *et al.* 1995, Zheng *et al.* 1998). For example, Ryan *et al.* (1995)

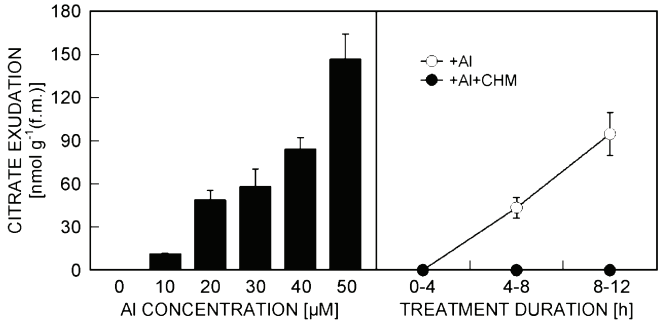

Fig. 1. Effect of different Al concentration (*left*) and Al treatment duration (*right*) on citrate secretion from soybean roots. Means of three independent experiments ± SD.

indicated that Al-induced malate secretion in roots of wheat was sensitive to A-9-C and niflumic (NIF), but insensitive to DIDS. Yang *et al.* (2006) reported that Al-induced citrate secretion in *Cassia tora* was sensitive to A-9-C and NIF, but insensitive to PG. Zheng *et al.* (1998) found that Al-induced oxalate secretion in buckwheat was inhibited by PG, not affected by A-9-C and DIDS, but was stimulated by NIF. Also in maize, Al-induced citrate secretion from roots was inhibited by DIDS and NIF (Kollmeier *et al.* 2001). These results suggested that the opening of anion channels might be involved in Al-induced organic acid secretion including citrate secretion in soybean.

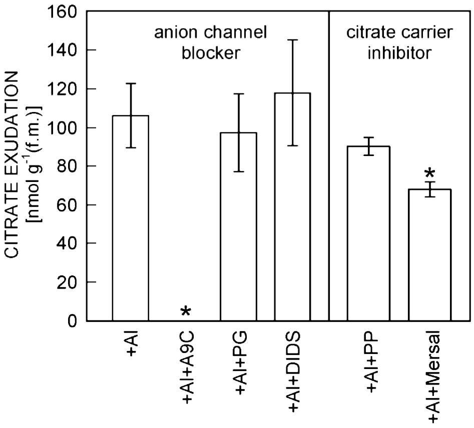

Fig. 2. Effect of different anion channel blockers and citrate carrier inhibitors on Al-induced citrate secretion from soybean roots. Means of three independent experiments ± SD. The *asterisk* marks statistically different means ($P < 0.05$).

Mersalyl and PP have been reported to be inhibitors of citrate carrier located in mitochondria membrane and specific to cysteine and lysine, respectively (Genchi *et al.* 1999). In the present study, PP and Mersalyl (10 µM) did not affect the growth and citrate secretion in roots of soybean in the absence of Al (data not shown). Al-induced citrate secretion from roots was not affected by supply of PP (10 µM), but was significantly inhibited by Mersalyl (10 µM) (Fig. 2). The inhibitory effect of Mersalyl on citrate exudation suggested that citrate carrier on mitochondria membrane also participated in Al-dependent citrate efflux. However, PP and phenylisothiocyanate were reported to inhibit Al-induced citrate secretion from rye roots (Li *et al.* 2000).

In conclusion, in the present study, the pattern of citrate exudation from roots can be classified into pattern II with a 4-h lag phase (Fig. 1), which was in agreement to our previous studies with soybean (Yang *et al.* 2001). Protein synthesis inhibitor CHM completely inhibited Al-induced citrate efflux (Fig. 1), suggesting that novel protein synthesis was necessary in the Al induced citrate secretion in soybean. One of anion channel blockers and one of citrate carrier inhibitors tested inhibited the Al induced citrate secretion (Fig. 2), suggesting that both anion channels in plasma membrane and citrate carrier on mitochondria membrane are involved in Al dependent citrate release. Citrate transport probably plays critical role in regulating the Al induced citrate efflux in soybean.

References

Genchi, G., Spagnoletta, A., Palmieri, F.: Purification and characterization of the reconstitutively active citrate carrier from maize mitochondria. - Plant Physiol. **120**: 841-847, 1999.

Horst, W.J., Asher, C.J., Cakmak, I., Szulkieiez, P., Wissemeier, A.H.: Short-term responses of soybean roots to aluminium. - J. Plant Physiol. **140**: 174-178, 1992.

Kochian, L.V., Hoekenga, O.A., Piñeros, M.A.: How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. - Annu. Rev. Plant Biol. **55**: 459-493, 2004.

Kollmeier, M., Kietrich, P., Bauer, C.S., Horst, W.J., Hedrich, R.: Aluminum activates a citrate-permeable anion channel in the aluminum-sensitive zone of the maize root apex. A comparison between an aluminum sensitive and an aluminum-resistant cultivar. - Plant Physiol. **126**: 397-410, 2001.

Li, X.F., Ma, J.F., Matsumoto, H.: Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. -Plant Physiol. **123**: 1537-1543, 2000.

Ma, J.F.: Role of organic acids in detoxification of aluminum in higher plants. - Plant Cell Physiol. **41**: 383-390, 2000.

Pellet, D.M., Grunes, K.L., Kochian, L.V.: Organic acid exudation as an aluminium-tolerance mechanism in maize (*Zea mays* L). - Planta **196**: 788-195, 1995.

Ryan, P.R., Delhaize, E.: Function and mechanism of organic anion exudation from plant roots. - Annu. Rev. Plant Physiol. Plant mol. Biol. **52**: 527-560, 2001.

Ryan, P.R., Delhaize, E., Randall, P.J.: Characterization of Al-stimulated efflux of malate from the apices of Al-tolerant wheat roots. - Planta **196**:103-110, 1995.

Shamsi, I.H., Wei, K., Zhang, G.P., Jilani, G.H., Hassan, M.J.: Interactive effects of cadmium and aluminum on growth and antioxidative enzymes in soybean. - Biol. Plant. **52**: 165-169, 2008.

Silva, I.R., Smyth, T.J., Raper, C.D., Carter, T.E., Rufty, T.W.: Differential aluminum tolerance in soybean: an evaluation of the role of organic acids. - Physiol. Plant. **112**: 200-210, 2001.

Yang, J.L., Zheng, S.J., He, Y.F., You, J.F., Zhang, L., Yu, X.H.: Comparative studies on the efflux of a protein-synthesis inhibitor on aluminum-induced secretion of organic acids from *Fagopyrum esculentum* Moench and *Cassia tora* L roots. - Plant Cell Environ. **29**: 240-246, 2006.

Yang, Z.M., Nian, H., Sivaguru, M., Tanakamaru, S., Matsumoto, H.: Characterization of aluminum-induced citrate secretion in aluminum-tolerant soybean (*Glycine max*) plants. - Physiol. Plant. **113**: 64-71, 2001.

Yang, Z.M., Sivaguru, M., Horst, W.J., Matsumoto, H.: Aluminum tolerance is achieved by exudation of citric acid from roots of soybean (*Glycine max*). - Physiol Plant. **110**: 72-77, 2000.

Zheng, S.J., Ma, J.F., Matsumoto, H.: High aluminum resistance in buckwheat I. Al-induced specific secretion of oxalic acid from root tips. - Plant Physiol. **117**: 745-751, 1998.