Title

Connection between aluminium exclusion and accumulation in the aluminium accumulator buckwheat (Fagopyrum esculentum Moench)

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Introduction

Aluminium accumulation, Al exclusion and Al tolerance in buckwheat is not yet well understood (for further details see: Ma et al. 1998; Zheng et al., 1998; Ma and Hiradate 2000). Little is known about the membrane transport of Al in this Al accumulating plant. The free Al^{3+} is hypothesized to be the form which is taken up (Ma & Hiradate, 2000). The site of Al uptake is not proposed to be at the root apex (Ma & Hiradate, 2000), because the secretion of oxalate in the same root zone is supposed to exclude Al from the Al-sensitive root tip (Zheng et al. 1998). The aim of this study was to explore the interrelationships between Al exclusion and Al accumulation in the root apex of the Al accumulator, buckwheat

Material & Methods

Plant material and growing conditions:

Rooted shoot cuttings of buckwheat (*Fagopyrum esculentum* Moench, cv. Lifago) were low-pH adapted before the beginning of Al treatments in simplified nutrient solution containing 500 µM $CaCl_2$, 8 µM H_3BO_3; 100 µM K_2SO_4 at pH 4.3 and +/- 75 µM $AlCl_3$.

Fractionation of Al and organic acid anions:

A fractionated extraction procedure was applied following the methodology suggested by Yu et al. (1999) and modified by Wang et al. (2004). Briefly, 30 10 mm-root tips were excised on an ice-cooled graduated glass cutting plate. The water free-space fluid (WFSF) was extracted at 4000 *g* for 15 min. Subsequently the root tips were frozen at -20 °C, thawed, and again centrifuged at 4000 g for 15 min yielding the symplastic fraction. To obtain cell-wall material, the root tips were transferred to an E-cup and homogenised in 500 µL EtOH (96 % w/v) in a swing mill (MM200; Retsch; Haan; Germany) at 30 strokes min^{-1} for 3 min. The homogenate was pelleted by centrifugation at 23000 *g* for 15 min. The pellet was resolubilized and centrifuged three times. The pellet was digested using concentrated HNO_3. Organic acids were determined in the same samples used for Al determination.

Al determination:

Aluminium was determined by GF-AAS (Unicam 939 QZ; Analytical Technologies Inc.; Cambridge; UK) after digestion of root tips over night in 500 µL double distilled ultra-pure nitric acid.

Determination of organic acids:

The OAs concentrations in the extracts of root tissue were measured by HPLC (Kroma System 3000, Kontron Instruments, Munich, Germany). Prior to the analysis of exuded organic acid the nutrient solution samples were run through a cation exchange column (Hydrochloric form) (AG® 50W-X8; Biorad; Hercules; CA) followed by concentration to dryness via centrifugal evaporation.

Al loading and unloading of root tips:

Roots of pH-adapted cuttings were excised 10 mm behind the root tip. 30 root tips per sample were collected in net trays in ice cold simplified nutrient solution at pH 4.3. This nutrient solution was replaced 3 times. All root tips, with exception of the control treatment, were transferred in their net trays to either a warm or a cold Al-containing solution (75 µM $AlCl_3$, pH

4.3). Only warm Al loaded root tips were transferred to either warm or cold unloading minimal nutrient solution without Al for 10 or 30 min.

Treatment with an anion-channel inhibitor and oxalate:
Buckwheat cuttings were exposed to Al (0, 75 or 200 µM Al), phenylglyoxal (PG) (+/-10 µM) and oxalic acid (0, 75 or 200 µM] in different ratios in simplified nutrient solution. Plants were pre-treated with 75 µM Al for 15 min prior to the Al/oxalate treatment in order to trigger Al-induced oxalate exudation. Root-growth rate and xylem-sap Al (centrifugation of stem segments) was determined after 24 h of treatment.
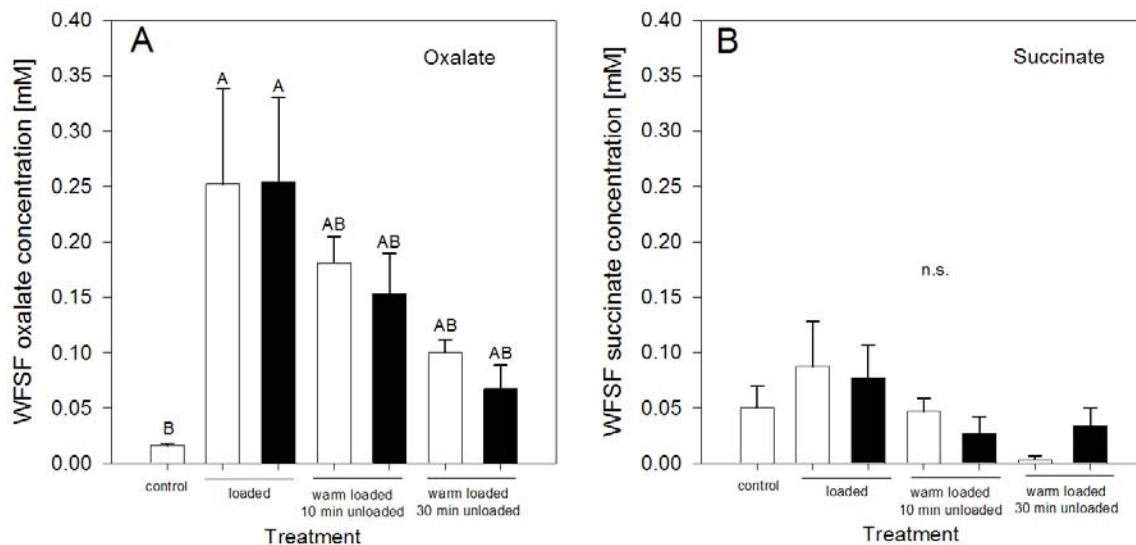
Results

Figure 1. Oxalate (A) and succinate (B) concentrations in the water WFSF obtained by centrifugal extraction of excised adventitious buckwheat root tips (10 mm) after warm (25°C; white bars) or cold (0°C; black bars) Al loading (75 µM for 30 min), and subsequent unloading in simplified nutrient solution (500 µM $CaCl_2$, 8 µM H_3BO_3; 100 µM K_2SO_4) at pH 4.3. Bars represent means +/- standard error. Different letters denote significant differences between treatments at $\alpha = 5\%$, n = 5.

Aluminium-loaded root tips showed the highest apoplastic oxalate concentrations without any temperature effect (Figure 1A). During the unloading period in Al-free solution, cold treatment consistently but not significantly slightly enhanced the release of oxalate from the root apoplast. The effect of Al on the oxalate concentrations in the WFS were specific for oxalate because succinate (Fig. 1B and other organic acid anions [not shown]) were not affected. The oxalate and succinate concentrations in the WFS were representative also for the exudation rates (data not shown).

The uptake of Al into the root symplast was temperature-dependent (Figure 2). Cold treatment decreased the Al uptake into the symplast. Aluminium accumulated in the symplast at 25 °C was released during the unloading period only at high but not at low temperatures.
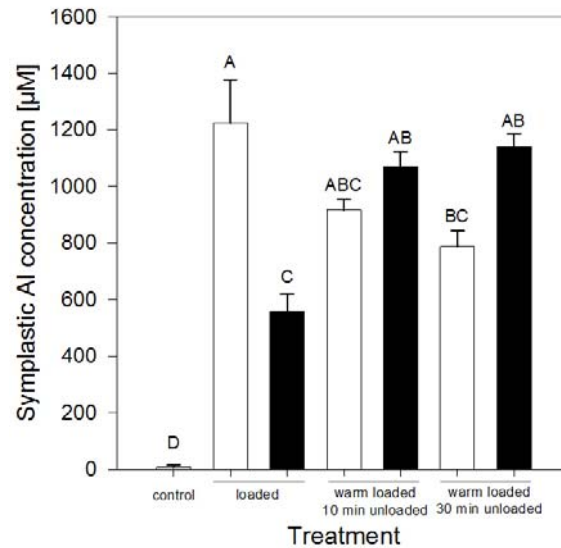
Figure 2. Aluminium accumulation in the symplast (fractionated extraction) of excised adventitious buckwheat root tips (10 mm) after warm (25°C; white bars) and cold (0°C; black bars) Al loading (75 µM) for 30 min, and subsequent unloading in simplified nutrient solution (500 µM $CaCl_2$, 8 µM H_3BO_3; 100 µM K_2SO_4) at pH 4.3. n=5; bars represent means +/- standard error. Different letters denote significant differences at $\alpha = 5\%$

In a next step we studied the role of the anion-channel inhibitor phenylglyoxal (PG) and externally applied oxalate on Al-inhibited root growth and the uptake and xylem transport of Al (Figure 3). Application of PG in absence of Al did not negatively affect root elongation (Fig. 3A) and the xylem Al concentration (Fig. 3B) during 24 h of treatment. Application of Al inhibited root elongation, and the combined application of 75 µM Al and 10 µM PG led to an additional reduction in root-growth rate (Fig. 3A). This can be explained by an inhibition of the Al-induced oxalate exudation by PG (data not shown). Application of 200 µM but not of 75 µM oxalate improved Al-inhibited root elongation. Xylem-sap Al concentration steeply increased in Al-treated plants (Fig. 3B). This increase was not affected by PG. However, the Al concentration in the xylem sap was lower with the addition of oxalate to the external medium. If oxalate was present in excess compared with Al, the xylem Al concentration decreased to the control levels.
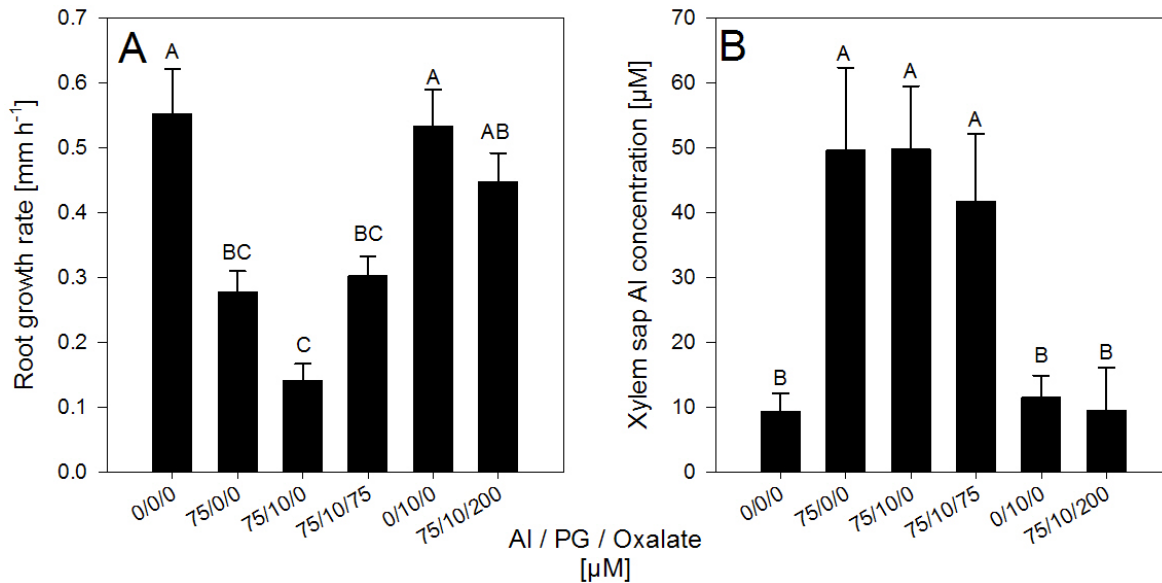
Figure 3. Xylem-sap Al concentration (**A**) and root-growth rate (**B**) as effected by anion channel inhibitor, oxalic acid and Al supply for 24 h in simplified nutrient solution (500 µM CaCl$_2$, 8 µM H$_3$BO$_3$; 100 µM K$_2$SO$_4$) at pH 4.3. n = 5; bars represent means +/- standard deviation. Different letters denote significant differences at α = 5%

Discussion

Little is known about the membrane flux of Al in the Al accumulator, buckwheat. Ma and Hiradate (2000) showed that hydroxylamine, an unspecific inhibitor of respiration did not affect root Al uptake rates, suggesting a passive uptake process. However, we showed here a reduction in Al accumulation into the symplast by 50% at 4°C compared to 25 °C (Fig. 2). This indicates a metabolism-dependent Al uptake process because low temperature reduction of ion transport is more closely related to an inhibition of an active transport system than to respiration or membrane permeability (Bravo-F. and Uribe, 1981). Additionally, Al release from the symplast (unloading) appears to also be an active process since the Al concentration in the symplast did change at 25°C but not at 4 °C (Fig. 2). The unloading of the symplast is a necessary step for the transport of Al into the xylem. In analogy to studies with heavy metal hyperaccumulators (Klein et al., 2008; Verbrüggen et al., 2009), we thus conclude that the loading of Al into the xylem in buckwheat requires a second energy-requiring step.

It could be confirmed that the exudation of oxalate and particularly the supply of oxalate in excess to Al in the external solution reduced Al-induced inhibition of root elongation (Ma et al., 2001, Fig. 3) by reducing the accumulation of Al in the root apoplast (not shown). Aluminium triggers the release of oxalate in a metabolism-independent way, since the Al-induced oxalate concentration in the root WFSF was the same at 4°C and 25 °C (Fig. 1). Application of 75 µM Al increased the oxalate concentration in the root WFSF to about 250 µM, equimolar to the Al concentration (not shown). Thus Al in the WFSF is likely to be present as the 1:1 (Al-oxalate)$^{+}$ complex. This might be circumstantial evidence that Al is transported into the symplast as the Al-Ox$_1^{+}$ complex. This could reconcile the oxalate root growth inhibition ameliorating effect through reducing Al-binding in the root apoplast with Al accumulation in the same root zone (5 mm apex). However, the inhibition of oxalate exudation by the anion-channel inhibitor PG enhanced Al-induced inhibition of root elongation (Fig. 3A), but did not reduce Al uptake and

translocation (Fig. 3B). Present research focuses on further elucidation of the form of Al transported into the symplast and the xylem.

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