Metabolic acclimation supports higher aluminium-induced secretion of citrate and malate in an aluminium-tolerant hybrid clone of Eucalyptus

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Abstract

Background

Eucalyptus is the main timber species, most of which are hybrid clones, and usually grow in aluminized acid soil in China. The exudation of organic acids from roots may contribute to detoxification of Al and lead to the Al-tolerance in *Eucalyptus* genotypes. To further understand the organic acid response in Al tolerance in *Eucalyptus*, the Al-tolerant *Eucalyptus grandis × E. urophylla* clone GL-9 (marked as “G9”) and the Al-sensitive *Eucalyptus urophylla* clone GL-4 (marked as “W4”) were used to investigate the secretion and metabolism of citrate and malate in roots.

Results

Eucalyptus seedlings in hydroponics were exposed to the presence or absence of 4.4 mM Al at pH 4.0 for 24 hours. The protein synthesis inhibitor cycloheximide (CHM) and the anion channel blocker phenylglyoxal (PG) were applied to explore possible pathways involved in organic acid secretion. The Al treatments caused higher Al accumulation in roots of both clones. The secretion of malate and citrate was greater in G9 than in W4, corresponding to the relatively higher tolerance in G9 to Al. The peak Al concentration occurred after 1 h in G9 roots and declined afterward, indicating the activation of detoxification to alleviate Al accumulation. After 6 h of Al exposure, the efflux of citrate dramatically increased in G9 after a substantial lag phase, while both peak Al accumulation in roots and peak malate secretion occurred and there was no induction of citrate secretion in W4. Enhanced activity for citrate synthase and phosphoenolpyruvate carboxylase, and reduced activity for NADP-isocitrate dehydrogenase, aconitase and NADP-malic enzyme were closely associated with the greater secretion of citrate in G9. Both PG and CHM were effective inhibitors of citrate and malate secretion in both *Eucalyptus* clones, except the malate secretion in W4 was not affected by CHM.

Conclusions

In two different Al-tolerant *Eucalyptus* clones, both secretion and internal accumulation of citrate and malate in roots were involved in Al detoxification. An anion channel on the plasma membrane could be an important mode of organic acid secretion. Citrate and relevant metabolizing enzymes led more important role in the response to Al in *E. grandis × E. urophylla.*

1 Background

About 50% of the world’s arable soils are acidic, significantly limiting worldwide agricultural production. It is widely recognized that Al^{3+} solubilizes into the soil solution and is rhizotoxic to plants when soil pH is below 5.0 (Kinraide, 1991). The accumulation of Al in root tips usually leads to rapid inhibition of root growth within minutes to hours by affecting the absorption of nutrients and disrupting other

physiological processes. A number of studies have demonstrated that Al tolerance mechanisms have evolved in some higher plants (Matsumoto, 2000; Kochian et al., 2004; Panda et al., 2008; Vendrame et al., 2010; Soares et al., 2014). One mechanism is the Al-induced exudation from roots of organic acids that chelate and inactivate Al^{3+} (Taylor, 1991; Delhaize et al., 1993; Pellet et al., 1995; Zheng et al., 1998). It has been suggested that secreted organic acids have a very important role in the detoxification of external Al by forming Al-linked clathrate around the rhizosphere (Barceló and Poschenrieder, 2002; Kikui et al., 2007). Increasing evidence shows that the ability to tolerate Al varies between genotypes within species, and that Al-tolerant genotypes often secrete higher levels of organic acids from roots than sensitive genotypes (Ryan et al., 2001; Poschenrieder et al., 2008; Kopittke et al., 2017). Many woody species growing in acid soils are well adapted to elevated Al^{3+} conditions through this mechanism (Bruner et al., 2013). Excretion of organic acids from roots is a pivotal defense mechanism for Al detoxification in some tree species, such as citrate secretion in *Betula platyphylla* (Sandnes et al., 2005), *Pinus densiflora* and *Paraserianthes falcataria* (Tahara et al., 2005b; Osawa and Kojima, 2006), citrate and oxalate secretion in *Cunninghamia lanceolate* and *Picea abie*s (Lin et al., 2006; Eldhuset et al., 2007), and citrate, oxalate and malate secretion from *Populus tomentosa* (Qin et al., 2007).

Some reports suggested that *Eucalyptus* has higher tolerance to Al toxicity than other tree species such as *Quercus robur*, *Pinus radiata* and *Melaleuca cajuputi*, and may even benefit from the presence of Al at some concentrations (Álvarez et al., 2005; Tahara et al., 2008; Huang and Bachelard, 1993). There were also differences in Al tolerance and response to Al stress among *Eucalyptus* species and their clones in acidic environments (Tahara et al., 2005a, 2008, 2014; Alcântara et al., 2015; Barros et al., 2016; Sliva et al. 2017). China is one of major producers of *Eucalyptus* with more than 4.5 million hectares under cultivation, accounting for about 2.1% of China's total forest area and 6.3% of its total plantation area. Guangxi province, located in the south of China, has the largest planting area of fast-growing eucalyptus in China. This region is an important contributor to the timber market with an annual output of fast-growing *Eucalyptus* timber exceeding one third of China's total timber output. Much of the soil in Guangxi is acidic and rich in Al. However, no reduction in *Eucalyptus* productivity was found in acid soil with declining pH (Ye et al., 2010). The exudation of low molecular weight organic acids from the roots of several *Eucalyptus* species may be an important mechanism in determining Al tolerance in *Eucalyptus* in such acidic soils (Nguyen et al., 2003; Silva et al., 2004; Tahara et al., 2005a). However, knowledge about the Al^{3+}-induced processes that lead to the secretion of organic acids by *Eucalyptus* roots is limited.

Asexual hybrids of propagated *Eucalyptus* are currently the main source of stock for plantations. In previous research, we assessed the Al tolerance of four fast-growing *Eucalyptus* clones and ranked them in descending order of tolerance: *E. grandis* × *E. urophylla* GL-9 (marked as “G9”), *E. grandis* × *E. urophylla* G12, *E. urophylla* × *E. camaldulensis* G3 and *E. urophylla* GL-4 (marked as “W4”) (Yang et al., 2011a, 2011b, 2015). The *E. grandis* × *E. urophylla* hybrids are one of the most popular clones for planting throughout south China because of its relative fast growth rate, straight trunk shape and good environmental acclimatization. In this context, the aim of the present study was to characterize the processes and impact of citrate and malate secretion by Al-tolerant *E. grandis* × *E. urophylla* G9 and Al-

sensitive *E. urophylla* W4 clones after exposing their roots to $AlCl_3$. The specific aim was determine the links between organic acid secretion and metabolic enzyme activities in *Eucalyptus* roots. The results further clarify the function of organic acids in tolerance to Al stress. The inclusion of the protein synthesis inhibitor cycloheximide (CHM) and the anion channel blocker phenylglyoxal (PG) helped to reveal the regulation of the citrate and malate secretory pathways in *Eucalyptus* roots that are induced by exposure to Al.

2 Results

2.1 Accumulation of Al in roots of *Eucalyptus*

In our previous research on the inhibitory impact of Al on the growth and physiological characteristics of *Eucalyptus* seedlings, *E. grandis* × *E. urophylla* G9 was more Al tolerant than *E. urophylla* W4 (Yang et al., 2011a, 2011b, 2015). Treatment with Al caused significantly higher Al concentrations in roots of both genotypes compared to the absence of Al. The peak Al concentration and the concentration after 24 h were higher in W4 roots than in G9 roots (Fig. 1). The lower accumulation of Al in G9 roots indicated that the degree of Al exclusion differed between the two *Eucalyptus* clones that had differential tolerance to Al. The Al concentration in roots of hybrid clone G9 peaked after 1 h of Al exposure. This result was similar to that in our previous study on Al accumulation in 7-month-old soil-grown *Eucalyptus* seedlings exposed to Al for 4 months. The Al concentration in roots, stems and leaves of G9 was lower than those of W4 (Yang et al, 2012). Taken together, these results suggest that G9 may have a greater ability to exclude Al from the interior of the root by chelating Al around its rhizosphere, thereby reducing Al absorption.

2.2 Al-induced secretion of organic acids from roots of *Eucalyptus*

To determine whether root organic acid exudation may have a role in the exclusion of external Al, the Al-induced malate and citrate secretion from roots was compared between the contrasting clones over a 24 h time course (Fig. 2). The amount of malate secreted from roots was generally lower than the amount of citrate secreted in both the non-Al-treated control (CK) and the Al-treated clones. Clone G9 had a higher level of organic acid secretion than W4 at all time points after the start of the Al treatment. The accumulation of malate in G9 peaked around 1 h after exposure to Al and then decreased gradually, but was still higher than that in the absence of Al through 24 h. (Fig. 2A). In contrast, the accumulation of malate was slower for W4, peaking at 6 h and at a lower level than in G9 at this time point. Citrate accumulation for G9 was activated after 3 h of Al exposure and peaked by 6 h, while there was no significant change in citrate accumulation in W4 through 24 h (Fig. 2B). These results indicated that the secretion mechanisms of these two organic acids differed between the tolerant and sensitive genotypes of *Eucalyptus*.

2.3 Effect of inhibitors on organic acid secretion by *Eucalyptus* clones contrasting in Al tolerance

To investigate the secretory pathways for citrate and malate from roots after Al treatment, the anion-channel inhibitor PG and the protein-synthesis inhibitor CHM were added at the start of a 24-h Al treatment (Fig. 3). The inhibitory effect of PG on Al-induced malate secretion in both G9 and W4 was 100%. In G9, PG further inhibited the background malate secretion present in the control plants to the lower background level observed in the W4 control plants. The background malate secretion in W4 was not affected by PG. Treatment of G9 with CHM had a similar effect on malate secretion as treatment with PG. However, CHM had no effect on the Al-induced secretion of malate in W4. The Al-induced and background secretion of citrate in both G9 and W4 was inhibited by CHM to the same low level.

2.4 Organic acid concentrations in root tips of *Eucalyptus* clones with contrasting Al tolerance

No significant genotypic differences were found in the internal concentrations of malate and citrate in root tips in the absence of Al (Fig. 4). After 24-h exposure to Al, the citrate and malate concentrations in G9 increased by about 50% and 25%, respectively, compared to the control. Thus, G9 roots acclimated to Al stress by accumulating citrate and malate, consistent with their secretion from the root tips. Meanwhile, the concentration of malate in W4 roots decreased by about 70%, but the concentration of citrate did not change with Al exposure. The presence of PG or CHM significantly inhibited the accumulation of both malate and citrate in the roots of both clones to below background levels. The inhibition was $\geq$ 90%, except for the inhibition of malate accumulation in W4, which was 76% from a lower starting level.

2.5 Activities of acid-metabolizing enzymes in root tips of Eucalyptus clones with contrasting Al tolerance

The activities of PEPC, MDH, ME, IDH, ACO and CS were examined in root apices of plants exposed to Al and inhibitors (Fig. 5). PEPC, MDH and ME are important enzymes associated with the metabolism of malate. In the absence of Al, the activity of PEPC was higher in roots of G9 than in W4. There was a significant increase in PEPC activity in roots of both clones after Al treatment, but the activity was below non-treated control levels when either PG or CHM were included in the Al treatment. In G9, the addition of inhibitors resulted in 80% lower PEPC activity compared to the addition of Al alone. MDH and ME activities were significantly lower in G9 after Al treatment than in the absence of Al treatment, as was the activity of ME in W4. In contrast, the activity of MDH in W4 was unaffected by Al treatment. Interestingly, compared with the treatment with Al alone, the activity of MDH in G9 increased significantly after the addition of both PG and CHM, while the activity of MDH in W4 and ME in both clones remained unchanged by the addition of these inhibitors.

IDH, ACO and CS are closely associated with citric acid metabolism. In W4 roots exposed to Al, IDH and ACO activities were significantly higher while CS activity was unchanged compared to the untreated control. In contrast, in roots of Al-treated G9, IDH activity was unchanged, ACO activity was much lower and CS activity was higher compared to the minus Al control. The increased activity of CS may be one of the main reasons for the higher Al-induced accumulation and secretion of citric acid in G9. In Al-treated G9 roots, the inhibitors PG and CHM had no impact on the low activity of IDH in root apices, repressed the Al-induced increase in CS activity and relieved the Al-induced loss of ACO activity. Interestingly, CHM even stimulated ACO activity above that in the untreated control. In Al-treated W4 roots, the two inhibitors abolished the Al-induced increase in IDH and ACO, and lowered the CS activity to below that in the untreated control.

3 Discussion

3.1 An Al-tolerant *Eucalyptus* hybrid clone has enhanced accumulation and exudation of malate and citrate

The available evidence indicates that the Al-induced secretion of organic acids from roots may lead to the detoxification of Al in higher plants (Ma, 2000, 2005). A role for organic acids leading in Al tolerance in *Eucalyptus* has been observed previously (Nguyen et al., 2003; Silva et al., 2004; Tahara et al., 2008). The lower root concentration of Al coupled with the higher root secretion of citrate and malate in Al-tolerant *E. grandis* × *E. urophylla* clone G9 than that in Al-sensitive *E. urophylla* clone W4 suggested that secretion of these two organic acids was involved in increased tolerance to Al in G9. This trait was consistent with the report in Al-tolerant *E. camaldulensis* (Nguyen et al, 2003; Tahara et al., 2008; Ikka et al., 2013). Tahara et al. (2008) documented with *E. camaldulensis* that citrate showed the strongest Al-binding capacity among citrate, oxalate, malate and phosphate. It was likely that the Al-stimulated accumulation and secretion of citrate may be the main underlying mechanism contributing to detoxification of Al in *Eucalyptus* roots, particularly in Al-tolerant genotypes. However, Silva et al. (2004) put forward the hypothesis that Al tolerance was due to the internal detoxification of Al by malate complexation. These conflicting results made the role of malate in conferring Al tolerance on *Eucalyptus* unclear. Adding to the complexity, Ikka et al (2013) inferred that the types of organic acids in response to Al may vary among *Eucalyptus* species. The features of malate and citrate accumulation and secretion in Al-tolerant hybrid clone G9 and Al-sensitive parental clone W4 have provided clues for further exploring Al-induced genes or proteins.

3.2 The transport pathways of citrate and malate secretion are potentially regulated by carrier proteins or an anion channel

The rapid release of organic acid in response to exposure to Al suggested that pre-existing anion transporters on the plasma membrane quickly initiated organic acid secretion without the need to produce new proteins, but a lag in release suggests a requirement for relevant gene expression and / or protein synthesis (Ma, 2000; Kollmeier et al.,2001; Ryan et al., 2003). Clone G9 had no significant delay in the malate secretion followed by later citrate secretion, while W4 had a significant lag in malate secretion and did not activate citrate secretion. On the other hand, both PG and CHM significantly reduced the Al-induced secretion and internal concentration of citrate in both *Eucalyptus* clones roots as well as the malate in G9 roots, but CHM had no impact on malate secretion in W4 root, which may indicate that there is genetic variation in this response within the genus. Generally, Al-tolerant species or varieties had higher activation and quantities of carrier proteins and anion channel proteins than Al-sensitive genotypes (Piñeros et al., 2002; Li et al., 2009; Yang et al., 2011; Yu et al., 2012). Therefore, it may be inferred that the citrate and malate secreted by the Al-tolerant G9 may require both an anion channel and synthesis of new proteins. Generally, if protein synthesis is required for the production of a carrier protein, then one would expect an obvious lag of several hours before secretion becomes apparent, as observed for the putative citrate channel in G9. However, the secretion of malate by G9 rapidly increased after exposure to Al, suggesting there could be other factors responsible for the rapid secretion of malate in Al-tolerant genotypes.

The beneficial effect of Al on growth of plants adapted to low pH soils was ascribed to increased uptake of nutrients by roots (Osaki et al, 1997). Similarly, Al tolerance in *Eucalyptus* was explained by the maintenance of nutrients and photosynthesis (Silva et al, 2010, 2017). Another study found that a new low-molecular-weight Al-binding ligand, oenothein b, from roots contributed to Al tolerance in *E. camaldulensis* (Tahara et al., 2008, 2017), encouraging further exploration to find other mechanisms that help fast-growing *Eucalyptus* clones to manage excessive soluble Al in acidic soil. Thus, further work is necessary for a wider understanding of other factors that might be responsible for Al tolerance in hybrid *Eucalyptus* clones and how associated molecular mechanisms might be activated through hybridization.

3.3 The effects of Al on enzyme activities involved in the root metabolism of citrate and malate

The Al-induced decrease in root internal malate concentration in response to the increase in malate secretion in W4 contrasted with the increase in both internal root concentration and secretion of malate in G9. This contrast suggested that there could be another factor involved in the regulation of malate secretion. As in other plants, the secretion and accumulation of citrate and malate in *Eucalyptus* were closely linked with changes in the activities of several enzymes involved in organic acid metabolism. We observed that in both clones CS and PEPC activities were markedly induced by Al, while ME activity significantly decreased. In G9, ACO and MDH activities were dramatically decreased in the presence of Al, while IDH activity was unaffected. In contrast, in W4, ACO and IDH activities were significantly increased by Al exposure, while MDH activity was unchanged. Apparently, the accumulation and secretion of citrate in G9 was achieved by stimulating CS activity and suppressing citrate catabolism. Citrate accumulation

in G9 may have been supported by decreased flux through ACO. Previous studies also found increased CS activity and decreased ACO activity were linked with accumulation and secretion of citrate from roots of Al-tolerant species, such as rye (Li et al., 2000), *Paraserianthes facataria* (Osawa and Kojima, 2006), and soybean (Xu et al., 2010). However, Ikka et al. (2013) reported that the Al-induced increase in citrate concentration in roots of *E. camaldulensis* was not caused by a change in CS activity, but only by reduced ACO activity to suppress citrate catabolism. The increased activity of PEPC in both clones upon exposure to Al may contribute to the increased biosynthesis of organic acids by feeding oxaloacetate carbon skeletons into the TCA cycle (Dong et al., 2004). In G9, the apparent synergistic effect of PEPC and CS on the synthesis of citrate was greater than that of other enzymes, such as ACO and IDH, on citrate catabolism, resulting in the production and secretion of more citrate. In the case of W4, the increased ME activity may have been responsible for the lower malate accumulation in this clone. Both PG and CHM seemed likely to enhance the activity of enzymes that were beneficial to citrate synthesis and decreased the activity of enzymes that metabolized citrate. The Al-induced synthesis or catabolism of citrate and malate was regulated by the balance among the various organic acid-metabolizing enzymes, which served to produce differential secretion of these two organic acids in *Eucalyptus* clones differing in their Al tolerance.

4 Conclusion

Taken together, the results presented here addressed questions regarding the differences in citrate and malate secretion and the metabolic responses underlying these differences in two *Eucalyptus* clones with differential Al tolerance. The results suggested that both citrate and malate contributed to Al tolerance in *Eucalyptus* and that both internal accumulation and secretion of the two organic acids were involved in Al detoxification. Citrate had a more important role in the response to Al in *E. grandis* × *E. urophylla* than in *E. urophylla*. In addition, PG and CHM treatments indicated that both anion channel proteins and carrier protein synthesis was involved in Al-induced secretion of citrate in Al-tolerant *E. grandis* × *E. urophylla*, but the secretory pathway for malate remained unclear. The enhanced activities of CS and PEPC and the reduced activities of IDH, ACO and ME resulted in Al-induced accumulation and subsequent secretion of citrate and malate, which appeared to be a general metabolic adaptation in Al-tolerant *E. grandis* × *E. urophylla*. It will be worth determining the variation in the metabolism and secretion of citrate in other *Eucalyptus* genotypes to further identify mechanisms of Al tolerance.

5 Methods

5.1 Plant material

The two clones of *Eucalyptus*,Al-tolerant clone *E. grandis* × *E. urophylla* GL-9 (Deposition numbers:S-SC-EGU-023-2011; marked as “G9”) and Al-sensitive clone *E. urophylla* GL-4 (Deposition numbers:S-SC-EU-022-2011; marked as “W4”) used in this study. The 2 months old tissue cultured seedlings of two clones were provided by Guangxi forestry research institute. Guo dongqiang, a senior engineer and researcher formally identified the two clones [see Additional file 1, 2 and 3]. The seedlings of *E. grandis* × *E. urophylla*

G9 (Al-tolerant) and *E. urophylla* W4 (Al-sensitive) with similar appearance and size were placed in 2L plastic buckets containing nutrient solution with 10 seedlings per bucket. All solutions were prepared with deionized water. For acclimating plants to the hydroponic culture system before Al treatment, seedlings were pre-cultured in 20% nutrient solution, pH 5.0 for 3 days, then in 50% nutrient solution at pH 4.5 for a further 3 days. Acclimated seedlings were transferred to 100% nutrient solution, pH 4.0 for 7 days. The composition of 100% nutrient solution was 6 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 2 mM $NH_4H_2PO_4$, 20 μM Fe-EDTA, 31.25 μM H_3BO_3, 2 μM mg/L $MnCl_2$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$ and 0.065 μM $(NH_4)_6Mo_7O_{24}$. The pH was adjusted with 2 mM HCl. All nutrient solutions were renewed every 2 days. Prior to solution replacement, seedlings were sterilized with 0.1% (v/v) carbendazim for 20 min to inhibit microorganisms. Air pumps were used to continuously aerate the seedlings in solution at 50 L air h^{-1}.

5.2 Aluminum and inhibitor treatments

After 7 days of culture in complete nutrient solution, pH 4.0, seedlings were transferred to fresh nutrient solution, pH 4.0, supplemented with or lacking 4.44 mM Al^{3+} from $AlCl_3$•$6H_2O$ with ten seedlings per pot. Each treatment was carried out in triplicate (3 × 10 plants). Both the incubation solution and roots in three pots were harvested at each time point after 0.5 h, 1 h, 3 h, 6 h, 12 h, and 24 h of exposure to Al^{3+} to determine organic acid secretion. The concentrations of citrate and malate and the activities of organic acid-metabolizing enzymes inside the roots were determined after 24 h of Al treatment.

To identify potential factors involved in organic acid secretion, the impact of the protein synthesis inhibitor CHM, and the anion channel blocker PG were determined by cultivating the seedlings as above in solutions supplemented with 0.5 mg L^{-1} CHM and 0.5 mg L^{-1} PG with 0 or 4.44 mM Al^{3+} for 24 hours. The secretion of citrate and malate from the roots was determined after 24 h.

5.3 Determination of organic acids in root exudates

The collected growth solution was filtered through a mixed fiber membrane to obtain 50 mL filtrate. The filtrate was passed through a cation exchange column (15 mm × 11 cm, 5 g Amerlite IR-120 resin), followed by an anion exchange column (2 g Dowex 1-X8 resin). The organic acids bound to the anion exchange column were eluted with 2 M HCl. The eluent was condensed to dryness at 40℃ by rotary evaporation (R215, Buchi, Switzerland). The dried eluent was re-dissolved into 1 mL of Milli-Q water, and filtered (0.45 μm membrane filter). The filtered solution was analyzed for citrate and malate using ion chromatography (ICS-5000 Ion Chromatography system with 4 × 250 mm AS11-HC analytical column and 4 × 50 mm AS11-HC guard column, Dionex, USA).

5.4 Analysis of malate and citrate in roots

After rinsing harvested roots with Milli-Q water, 2 cm was excised at the root apices to determine the internal concentration of citrate and malate. A total weight of 0.2 g for each sample was ground under liquid nitrogen before adding 1.5 mL ice-cold 4% (v/v) $HClO_4$ into the powder and gently homogenizing. The mixture was thawed slowly on ice into a suspension and allowed to stand for 30 min, followed by centrifugation at 20,000 × g at 4 °C for 10 min. The supernatant was first passed through an ion

exchange column (l5mm × 11 cm) filled with cation exchange resin (Amerlite IR-120 resin, H^+ form, USA) to remove cations, and was then passed through the a pretreatment column (RP18 column, Dionex, USA) to absorb plant pigments before malate and citrate were determined by ICS as described above.

5.5 Determination of Al in roots

Roots were rinsed with Milli-Q water three times. The 3 cm at root apices were excised and dried at 80 °C before grinding to a fine powder. Powdered roots (100 mg) were digested in 10 mL HNO_3 : $HClO_4$ (5 : 1 v/v) until the solution was clear. The digest was diluted to 25 mL with Milli-Q water and the Al concentration in the solution was immediately determined by inductively coupled plasma atomic emission spectroscopy (5100 ICP- OES, Agilent Technologies, USA).

5.6 Activity measurement of organic acid-metabolizing enzymes

After plants were cultured for 24 h in the presence or absence of Al, the activities of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), NADP-isocitrate dehydrogenase (NADP-IDH), NADP-malic enzyme (NADP-ME) and aconitase (ACO) were measured. Based on the methods of Chen et al. (2009) and Yu et al. (2012), 200 mg of fresh root apices were homogenized in ice-cold extraction buffer containing 50 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1% (w/v) PVPP and 5 mM DTT. After clarification by centrifugation at 15,000 × g for 15 min at 4 °C, the supernatant was used for enzyme activity determination. The activities were measured in a 3 mL reaction mixture using spectrophotometric assays described by Jenner et al. (2001), Chen et al. (2009) and Ikka et al. (2013). The activities of all enzymes were determined at 340 nm, except CS activity was determined at 412 nm.

5.7 Data analysis

Each treatment was done with three biological replicates of 10 plants each. Statistical analysis was performed using SPSS software package. All data between different treatments was compared using one-way analysis of variance with LSD test and significant differences between the means of two treatments were determined using the Duncan test at $P \leq 0.05$.

Abbreviations

CHM: cycloheximide; PG:phenylglyoxal; NADP:nicotinamide adenine dinucleotide phosphate; CK:control check; PEPC:phosphoenolpyruvate carboxylase; MDH:malate dehydrogenase; ME:malic enzyme; IDH:isocitrate dehydrogenase; ACO:aconitase; CS:citrate synthase; TCA:tricarboxylic acid; NADP-IDH:NADP-isocitrate dehydrogenase; NADP-ME:NADP-malic enzyme; EDTA:ethylene diamine tetraacetic acid; PVPP:crosslinked polyvinylpyrrolidone; DTT:Dithiothreitol.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data used in the analysis are included within the article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

WNL, QD and MY conceived and designed the research. WNL and QD collected, analyzed the data, and prepared the manuscript. MY and PF discussed the results and revised the manuscript. All of the authors read and approved the manuscript.

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Figures

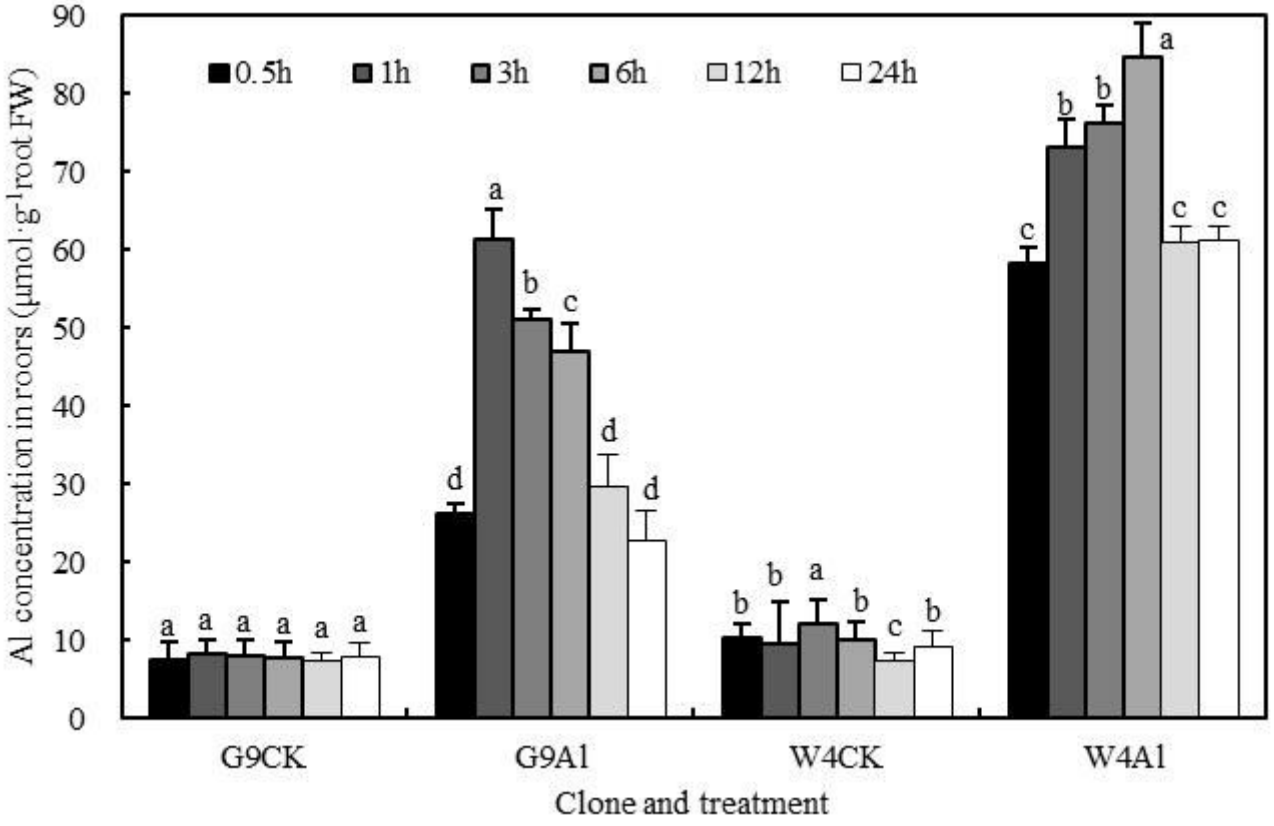

Figure 1

Aluminum concentration in roots of E. grandis × E. urophylla G9 and E. urophylla W4 at the indicated times after addition of Al. Bars represent means ± standard errors (n = 3). Different letters above the bars indicate a significant difference at P< 0.05. CK, non-Al-treated control; Al, aluminium treated

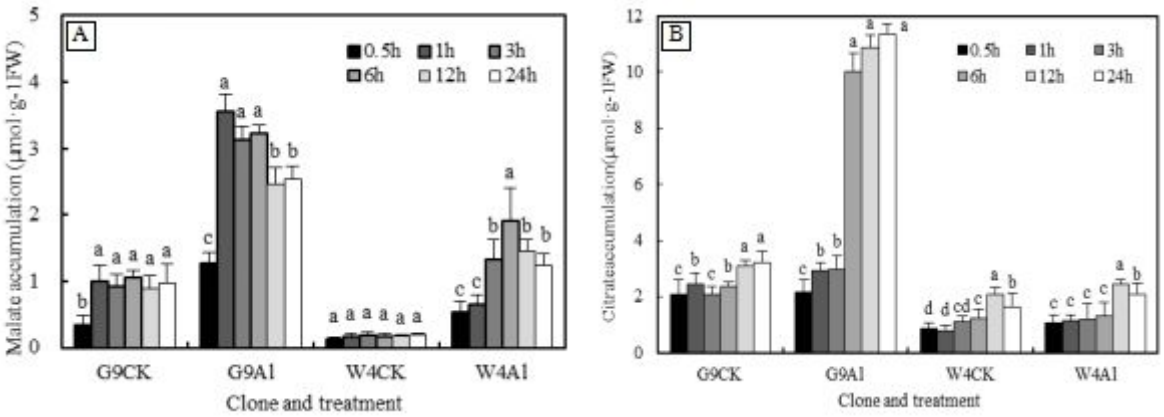

Figure 2

Aluminium-induced malate (A) and citrate (B) excretion from roots of E. grandis × E. urophylla G9 and E. urophylla W4 into growth media at the indicated times after initiation of Al treatment. Growth media containing root exudates was sampled at each time point after the addition of Al. Data are means ± standard error (n = 3). Different letters above the bars indicate a significant difference at P< 0.05. CK, non-Al-treated control; Al, aluminium treated

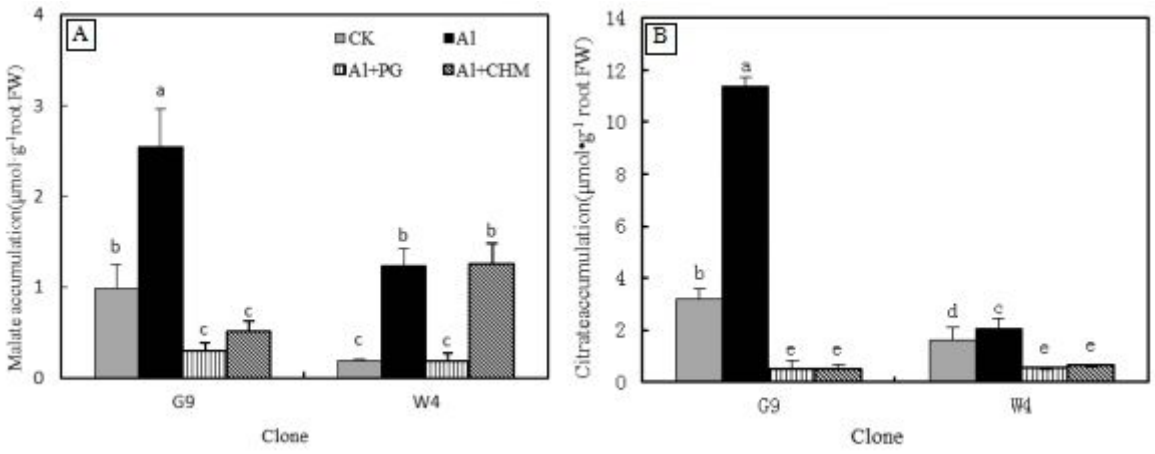

Figure 3

Malate (A) and citrate (B) excreted from the roots of E. grandis × E. urophylla G9 and E. urophylla upon exposure to Aluminium in the absence and presence of the anion channel inhibitor PG or the protein synthesis inhibitor CHM for 24 hours. Bars represent means ± standard errors (n = 3). Different letters above the bars indicate a significant difference at P < 0.05. CK, non-Al-treated control; PG, phenylglyoxyl; CHM, cycloheximide

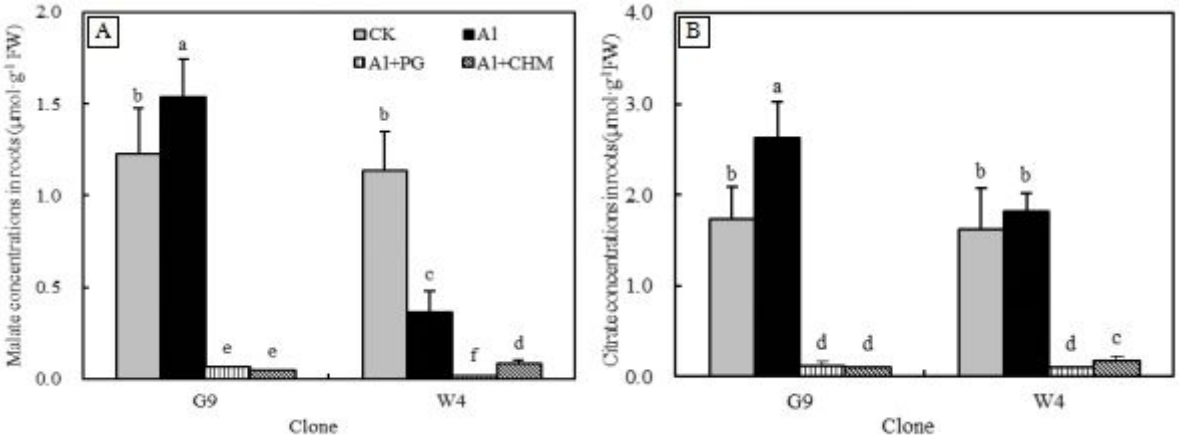

Figure 4

Malate (A) and citrate (B) concentrations in roots of E. grandis × E. urophylla G9 and E. urophylla W4 treated with Al in the absence and presence of the anion channel inhibitor PG or the protein synthesis inhibitor CHM for 24 hours. Bars represent means ± standard errors (n = 3). Different letters above the bars indicate significant differences at P < 0.05. CK, non-Al-treated control; PG, phenylglyoxyl; CHM, cycloheximide

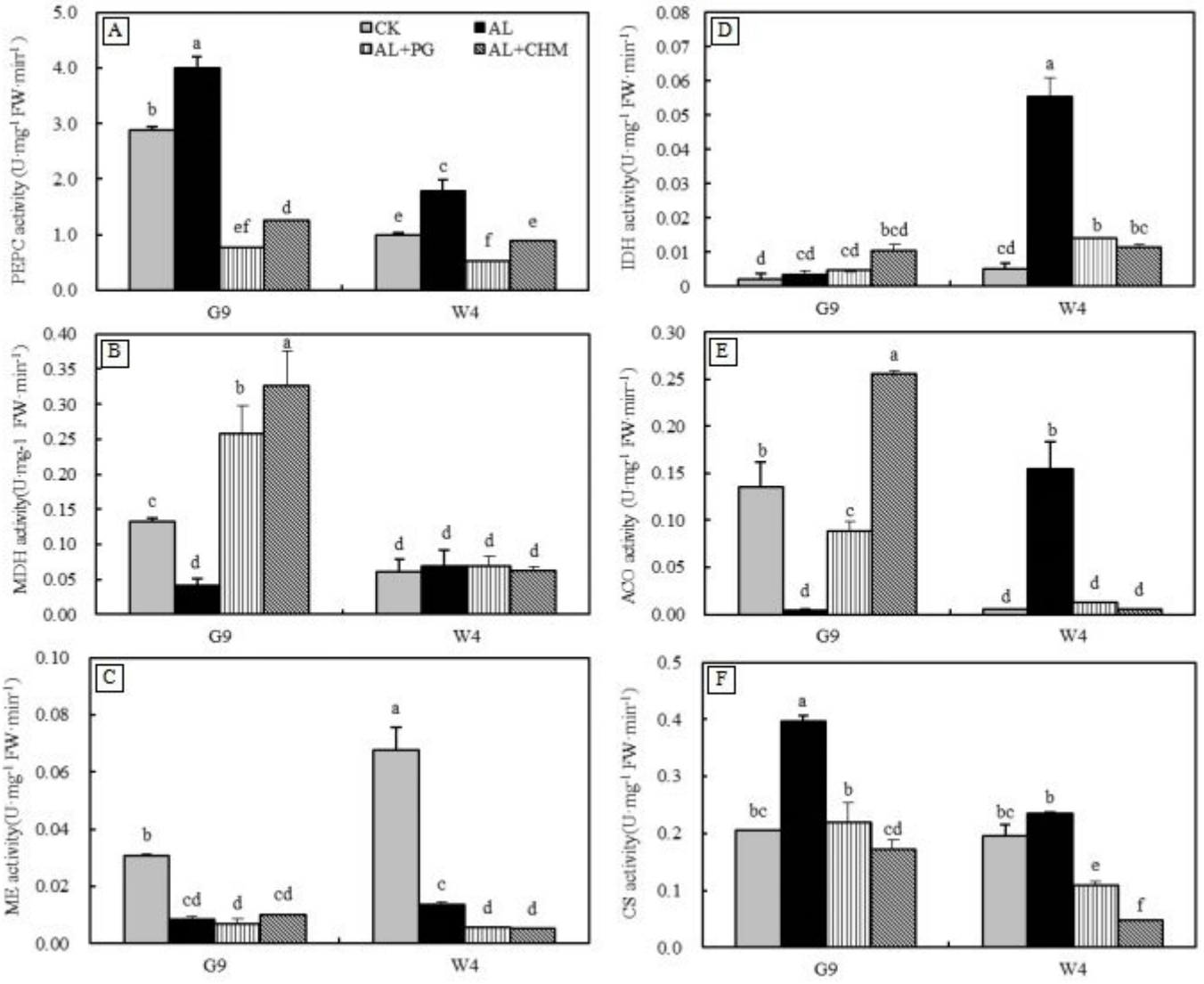

Figure 5

Activities of PEPC (A), MDH (B), ME (C), IDH (D), ACO (E) and CS (F) in the roots of E. grandis × E. urophylla G9 and E. urophylla W4 treated with aluminium in the absence and presence of the anion-channel inhibitor PG or the protein-synthesis inhibitor CHM for 24 hours. Bars represent means ± standard errors (n = 3). Different letters above the bars indicate significant differences at $P < 0.05$. CK, non-Al-treated control; PG, phenylglyoxyl; CHM, cycloheximide