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RESEARCH ARTICLE

Root malate efflux and expression of *TaALMT1* in Serbian winter wheat cultivars differing in Al tolerance

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Abstract

Aluminium (Al) toxicity in acid soils is a global problem. Here, we investigated Al tolerance in high yielding winter wheat (*Triticum aestivum* L.) cultivars bred in Serbia. The common relative root length (RRL) test for Al tolerance, and both physiological (malate efflux) and molecular (Aluminium-Activated Malate Transporter 1 [*TaALMT1*] expression) approaches were used for this characterization. Both moderately Al-tolerant cvs. Ljiljana and Arabeska showed significantly higher malate efflux rate from the root tips in comparison to moderately Al-sensitive cv. Pobeda and followed the RRL pattern. Irrespectively of Al supply, moderately Al-tolerant cultivars showed significantly higher relative *TaALMT1* expression than the Al-sensitive ones. A considerably high level of Al tolerance was found in cv. Ljiljana, which showed the highest Al-induced malate efflux along with the highest constitutive expression level of *TaALMT1* transcripts. Our results also demonstrate that Al-tolerance is based on a constitutive trait of high *TaALMT1* expression and malate efflux in wheat roots, resulting in a decrease in root length reduction.

Keywords: Wheat, aluminium tolerance, malate, *TaALMT1*

1. Introduction

Aluminium (Al) toxicity in acid soils affects agriculture production throughout the world, mainly due to the increased solubility of Al^{3+} at a low pH. In addition to the direct impact on plants, high Al concentrations in acid soils also affects phosphorus fractionation (Redel *et al*., 2016). Although mechanisms of Al toxicity still remain unclear, it is known that many plant species have evolved mechanisms as a response

to Al^{3+} stress. There are two broadly accepted strategies to decrease Al damage in plants: (i) Al-resistance mechanisms of Al^{3+} exclusion from the root by the exudation of organic acids and (ii) Al-tolerance mechanisms that chelate Al in subcellular compartments (vacuole) (for reviews see Matsumoto, 2000; Ryan and Delhaize, 2010; Ryan *et al*., 2011). Both mechanisms are related to mitochondrial activity as well as to mitochondrial metabolism and organic acid transport (Nunes-Nesi *et al*., 2014). The Al-resistance mechanisms operate in many common crops such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) (Ryan and Delhaize, 2010); hence research on this topic is important. Under Al-stress conditions, induced root response involves the exudation of organic anions (e.g., malate, citrate, succinate, oxalate and others) from the root apices mediated by the anion efflux transporters (Inostroza-Blancheteau *et al*., 2012; Yang *et al*., 2013). It has been shown that Al^{3+} stimulates the Aluminium-activated Malate Transporter (*TaALMT1*) involved in the secretion of malate from roots (see review by Sharma *et al*., 2016).

The importance of differences between genotypes within species in their ability to cope with Al^{3+} stress has also been recognized (Ulloa-Inostroza *et al*., 2017). This variation was explored by breeders for the development of cultivars better adapted to acid soils (Garvin and Carter, 2003). It was recently shown that the Al-tolerance mechanism of Al-tolerant Chilean wheat cultivars is fully associated with an arbuscular mycorrhizal fungi symbiosis, in contrast to one of recognized Al-tolerance (Atlas 66) (Seguel *et al*., 2016). Overall, wheat is considered as Al^{3+}-sensitive species, and accordingly a large-scale screening of wheat germplasm for Al-tolerance has been performed using physiological and molecular methods (e.g., Sasaki *et al*., 2006; Stodart *et al*., 2007; Martins-Lopes *et al*., 2009; Raman *et al*., 2010). Clear evidence that wheat germplasm collected from the former Yugoslavia consisted of genotypes adapted to various agroecological conditions was reported by Rengel and Jurkic (1992). Large-scale screening for Al-tolerance of bread and durum wheat genotypes originating from different breeding institutions from the Western Balkan region was performed two decades ago (Rengel and Jurkic, 1992, 1993; Cosic *et al*., 1994). However, information based on the physiological and molecular characterization of Serbian wheat genotypes to Al-tolerance is still lacking. Therefore, the aim of the present study was to characterize high-yielding bread wheat cultivars widely grown in Serbia for their tolerance to Al^{3+} toxicity using malate efflux along with the expression of *TaALMT1* efflux transporter as a promising molecular marker for targeted breeding to wheat Al-tolerance (Soto-Cerda *et al*., 2015).

2. Materials and Methods

2.1. Plant material, growth conditions and treatments

Winter bread wheat (*Triticum aestivum* L.) cultivars tested in this study were bred at the Institute of Field and Vegetable Crops, Novi Sad, Serbia. All cultivars were released over the past two decades. In our preliminary screening test, 17 Serbian genotypes were compared with the reference cvs. Atlas-66 (Al-tolerant) and Neepawa (Al-sensitive) according to Zhang and Taylor (1989).

Wheat seedlings were grown under controlled conditions in a growth chamber with a dark/light regime of 16/8 h, temperature regime of 24/20 °C, relative humidity of ~60% and photon flux density of 250 μmol m^{-2} s^{-1} at plant height. Seeds were surface sterilized in 5% (v/v) sodium hypochlorite, rinsed with distilled H_2O and germinated on filter paper soaked with saturated $CaSO_4$ solution for three days.

In the first experiment, uniform seedlings of each cultivar were transferred to 3 L pots filled with constantly aerated solutions containing (in mM): 0.4 $CaCl_2$, 0.65 KNO_3, 0.25 $MgCl_2$ and 0.08 NH_4NO_3. Prior to the determination of root length, relative root length (RRL) and Al concentration in roots, wheat cultivars were subjected to +Al/-Al treatments for 4 days. Aluminium was applied in the form of $AlCl_3$ x $6H_2O$ at 50 µM, which gives Al^{3+} ionic activity of 42.5 µM, as calculated by the software GEOCHEM-EZ v. 1.0. The pH of both –Al and +Al treatments was adjusted to 4.1 ± 0.1 and controlled daily with 0.2 M HCl and 0.2 M KOH. For further study, Pobeda and NS Futura were chosen as moderately Al-sensitive cultivars, whereas Arabeska and Ljiljana where chosen as moderately Al-tolerant cultivars. Three replicate pots per treatment (10 plants per replication) were arranged in a randomized block design. For RNA extraction and Real-time quantitative PCR, wheat seedlings were grown in the solutions without (–Al) or with 50 µM $AlCl_3$ (+Al) as described above, for 24 h. To obtain malate content in root apical tissues plants were exposed to Al for 5 h.

In the second experiment, 5-d-old seedlings were precultured in a standard nutrient solution containing: 0.7 mM K_2SO_4, 0.1 mM KCl, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 1.0 µM H_3BO_3, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$ and 20 µM Fe(III)-EDTA. Before exposure to Al, roots were rinsed with distilled water and then transferred to a solution supplied with 50 µM $AlCl_3$ (pH=4.1) for 5 h and malate efflux from root apices was measured.

2.2. Determination of root length

The length of the central seminal roots was determined as the mean of 30 plants per treatment of each wheat cultivar. The relative root length (RRL) was calculated as the ratio between the lengths of central seminal roots in Al-supplied (+Al) and Al-free (–Al) solutions [RRL(%)= +Al/–Al×100].

2.3. Determination of Al in roots

Roots of wheat seedlings previously exposed to 50 µM $AlCl_3$ for 4 d (as described for determination of root length) were washed with distilled H_2O, dried at 70°C for 48 h and digested with 3 mL of HNO_3 + 2 mL of H_2O_2 in a microwave oven (Speedwave MWS-3$^+$; Berghof Products + Instruments GmbH, Eningen, Germany). Samples were then diluted with deionized H_2O in 25 mL plastic flasks, and the volume was adjusted to 25 mL with deionized H_2O. The Al concentrations were determined by inductively coupled plasma optical emission spectroscopy (Spectro-Genesis EOP II, Spectro Analytical Instruments GmbH, Kleve, Germany).

2.4. RNA extraction and Real-time quantitative PCR

Root apical tissues (0.5–1 g FW) were frozen in liquid N_2 and ground thoroughly in a mortar. RNA was isolated using the GeneJET™ RNA Purification kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. DNA removal, cDNA synthesis and real-time PCR were performed as described in Kostic *et al*. (2015). Two sets of primers were used in this study: i) for *Triticum aestivum* L., *TaALMT1* gene (GenBank accession no. AB081803) 5'-TGTTGCAAGTGATGCATGTG-3' and 5'-ATAACCACGTCAGGCAAAGG-3', and ii) for *TaACTIN*, a wheat housekeeping gene (GenBank accession no. AAW78915.1) 5'-CCAGGTATCGCTGACCGTAT-3' and 5'-GCTGAGTGAGGCTAGGATGG-3'. Levels of transcription were calculated with the $2^{-\Delta Ct}$ method using *ACT* as an internal control. Each PCR reaction was done in triplicate and

included no template controls. To determine the amplification efficiency of real-time PCRs, cDNAs were diluted 5, 10, 20, and 40 times. The calculated PCR efficiency [E(%)=(10−1/slope−1)×100] was between 90 and 100% (-3.6 > slope > -3.1).

2.5. Collection of root exudates

Root exudates were collected according to Kostic *et al*. (2015), using sample application papers for electrophoresis (10 x 5 mm; SERVA Electrophoresis GmbH, Heidelberg, Germany) previously washed in methanol and deionized water and subsequently dried. After 5 h of exposure to Al, three intact roots per plant were removed from the solution, and moistened paper pieces were fixed onto root tips (0-20 mm) between two small attached plastic sheets. The remaining parts of the roots were covered with filter paper moistened with deionized water to prevent drying. After 1 h, paper pieces with absorbed root exudates were extracted in a methanol:deionized water (1:3 v/v) mixture, filtered through 0.22 μm pore size nylon syringe filters (Phenomenex, Torrance, CA, USA) and stored at -80ºC prior to HPLC analyses.

2.6. Root tissue extraction

Root tissue extracts were prepared according to Pavlovic *et al*. (2013). Root tips (0-20 mm; 20 tips per cultivar) were cut, immediately frozen in liquid N_2, ground thoroughly and extracted in 1 mL of methanol:deionized H_2O (3:1, v/v) mixture, filtered through 0.22 μm pore size nylon syringe filters, and stored at -80 ºC prior to HPLC analyses.

2.7. HPLC determination of malic acid

Quantification of malic acid was performed using an HPLC system (Waters, Milford, MA, USA) consisting of 1525 binary pumps, thermostat, and 717+ autosampler connected to the Waters 2996 diode array detector (DAD; Waters) adjusted at 210 nm. The ion exclusion Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), which was 300 x 7.8 mm with appropriate guard column, was used with 5 mM H_2SO_4 as a mobile phase. Isocratic elution was performed with a flow rate of 0.6 mL min^{-1} at 40°C. The detected malic acid peak was quantified by the external standard method using pure malic acid standard (Sigma-Aldrich, St. Louis, MO, USA) as reference for concentration, retention time and characteristic UV spectra, respectively. Data acquisition and spectral evaluation of the peaks was processed by the Empower 2 Software (Waters, Milford, MA, USA). The results were expressed as μmol root tip^{-1} for malate content and μmol root tip^{-1} h^{-1} for malate exudation rate.

2.8. Statistical analysis

Data were subjected to analysis of variance using the statistical software Statistica 6 (StatSoft Inc., Tulsa, OK, USA) and means were compared using Tukey's test.

3. Results

3.1. Relative root length and Al accumulation in roots

Root elongation was decreased in all examined cultivars exposed to 50 μM of Al (Table 1). Apart from the referent Al-sensitive (Neepawa) and Al-tolerant (Atlas-66) cultivars (RRL 29% and 90%, respectively) the range of RRL was relatively narrow; cvs. Pobeda and NS Futura were ranked as moderately sensitive due to much lower RRL (49%) in comparison to cvs. Arabeska, Etida, Rapsodija, Gordana and Ljiljana (RRL of 70-74%) ranked as moderately tolerant. Root length of 4-d-old plants not exposed to Al differed significantly between cultivars (Table 1).

Table 1. Root length, relative root length (RRL, -Al/+Al) and Al concentration in roots of wheat cultivars subjected to 50 μM $AlCl_3$ for 4 days. RRL values (means of 30 plants per cultivar) were divided into four Al-tolerance ranks: VS-very sensitive, VT-very tolerant, MS–moderately sensitive, and MT-moderately tolerant. Different letters denote significant differences at $p \leq 0.05$; data are means±SD (n=3).

Genotype	Root length (mm)		RRL	Tolerance	Root Al concentration
	-Al	+Al	(%)	ranking	(mg g^{-1} DW)
Neepawa	120a	35	29h	VS	1.25±0.18a
Atlas-66	70hi	63	90a	VT	0.82±0.16c
Pobeda	103bc	50	49g	MS	1.52±0.03a
NS Futura	94cd	46	49g	MS	1.30±0.29a
Milijana	89de	49	55fg	MT	1.07±0.08bc
Zvezdana	78fgh	44	56efg	MT	1.23±0.09a
Gora	78fgh	47	60def	MT	1.09±0.11bc
NS 40S	70hi	43	61def	MT	0.99±0.22bc
NS Enigma	76gh	46	61def	MT	1.15±0.22b
NS Dika	108b	69	64cde	MT	1.21±0.21b
Arija	82efg	53	65cd	MT	1.22±0.19a
Dragana	92d	60	65cd	MT	1.11±0.07bc
Natalija	87def	58	67bcd	MT	1.21±0.21b
Simonida	65i	44	68bcd	MT	0.94±0.05bc
Arabeska	107b	75	70bc	MT	1.00±0.11bc
Etida	77gh	54	70bc	MT	1.11±0.13bc
Rapsodija	85d-g	60	70bc	MT	1.21±0.22b
Gordana	84d-g	60	72bc	MT	1.14±0.22b
Ljiljana	72hi	53	74b	MT	0.97±0.15bc

The concentration of Al in the whole roots of moderately sensitive cvs. Pobeda and NS Futura was 1.52 and 1.30 mg g^{-1} DW, respectively. In moderately tolerant cultivars, the concentration of Al ranged from 0.94 to 1.23 mg g^{-1} DW (Table 1). The lowest Al concentration was obtained in Al-resistant Atlas-66 (0.82 mg g^{-1} DW).

3.2. Root malate content, efflux and relative expression of TaALMT1

There were no significant differences in the malate contents of the root tips (0-20 mm) among the examined wheat cultivars exposed to 50 μM $AlCl_3$ (Figure 1A). Both moderately Al-tolerant cvs. Ljiljana and Arabeska showed significantly higher malate efflux rate from the root tips in comparison to Al-sensitive cv. Pobeda (Figure 1B). Compared to the Serbian genotypes tested in our study, roots of referent cultivars exhibited much stronger differential response to Al toxicity (6-fold higher malate exudation in Al-tolerant Atlas-66 compared to Al sensitive Neepawa).

The four Serbian wheat cultivars differing in Al tolerance along with benchmark cultivars were further subjected to gene expression analysis of *TaALMT1* coding for malate exporter after 24 h exposure to 50 μM $AlCl_3$. Cultivars Arabeska and Ljiljana (moderately Al-tolerant) as well

as Al-tolerant Atlas-66 showed significantly higher relative *TaALMT1* expression than the moderately Al-sensitive ones (cvs. Pobeda and NS Futura) and Al-sensitive Neepawa (Figure 1c). *TaALMT1* expression in all examined cultivars was not up-regulated by Al, but the level of constitutive expression of this gene differed significantly between Al-sensitive and Al-tolerant cultivars (Figure 1C).

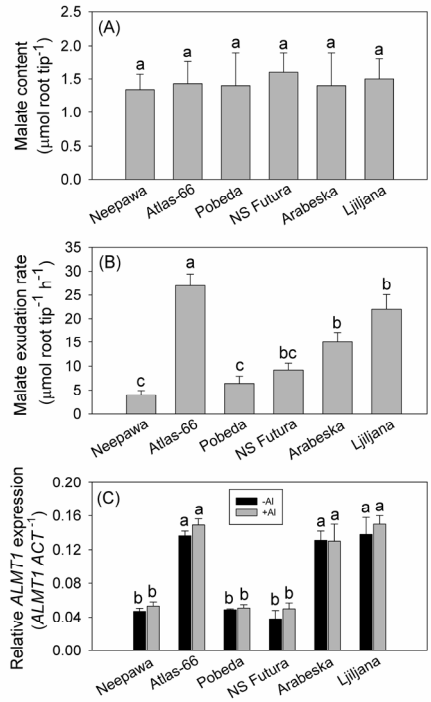

Figure 1. Root content (A), exudation rate of malate (B) and effect of Al on the relative expression level of *TaALMT1* (C) in the apical root tissues of wheat cultivars. For determination of malate content, seedlings were exposed to 50 µM Al for 24 h. Root exudates were collected during 1 h from root tips (0-20 mm) of 5-d old seedlings previously exposed to 50 µM $AlCl_3$ for 5 h. Relative expression level of *TaALMT1* was determined in root apical tissues of seedlings grown in the nutrient solution without (–Al) or with 50 µM $AlCl_3$ (+Al) for 24 h. Different letters denote significant differences at $p \leq 0.05$; error bars indicate standard deviation (n=4).

4. Discussion

Inhibition of root growth is one of the primary symptoms of excess-Al, as is demonstrated in various crops (e.g., Silva *et al*., 2001; Ali *et al*., 2008, Singh and Choudhary, 2010). Relative root length has previously been considered a better indicator of Al tolerance than root dry weight (for the review see Little, 1988). In comparison to previously released Serbian bread wheat genotypes bred at the Institute of Field and Vegetable Crops, Novi Sad, which showed very high variation of RRL (7 to 85%) under excess-Al (Rengel and Jurkic 1992), Serbian cultivars examined in the present study had a much narrower range of RRL

(49-74%). Al concentration in roots was significantly higher in cv. Pobeda compared to all moderately tolerant cultivars, in accordance with the typical response pattern to Al toxicity (Zhang and Taylor 1989; Zheng *et al*. 2004). Higher Al accumulation in the roots of low RRL compared to high RRL cultivars was correlated with the inhibition of root growth, including referent cultivars, as shown for different wheat cultivars grown at high Al supply (Silva *et al*., 2010).

While it was demonstrated that Al is accumulated mainly in the tissue of the apical root region (Rincón and Gonzales, 1992; Carver *et al*., 1988) and that root tips of Al-sensitive wheat genotype showed higher Al accumulation than the tolerant one (Delhaize *et al*., 1993a), endogenous malate content in wheat root apical tissue has been shown to be independent from Al tolerance (Delhaize *et al*., 1993b). However, the correlation between overall plant Al tolerance and Al-activated efflux of malate from the root apices among wheat genotypes has been well documented (Ryan *et al*., 1995; Tang *et al*., 2002). Both moderately Al-tolerant cvs. Ljiljana and Arabeska showed significantly higher malate efflux rate from the root tips in comparison to Al-sensitive cv. Pobeda (Figure 1B). On the other hand, roots of referent cultivars exhibited much stronger differential response to Al toxicity. A similar response was recorded in some near isogenic wheat lines (5 to 10-fold higher malate exudation in Al-resistant compared to Al sensitive genotypes) (Delhaize *et al*., 1993b).

There was no delay observed between the addition of Al and the onset of carboxylate anion efflux in wheat roots, suggesting that Al may activate pre-existing transporters in the plasma membrane to initiate anion exudation, and that the induction of genes is not required (Yang *et al*., 2013). Accordingly, in the present study, *TaALMT1* expression in the roots of all examined cultivars is not up-regulated by Al. However, the level of constitutive expression of this gene differs significantly between Al-sensitive and Al-tolerant cultivars. A similar relation between Al tolerance and the *TaALMT1* expression level has also been found in other wheat cultivars (Sasaki *et al*., 2006). Therefore, cultivars with a constitutively high expression of *TaALMT1* transcripts also showed high RRL and slightly decreased total root Al concentrations (Table 1; Figure 1C). The high levels of constitutive *TaALMT1* expression in the moderately Al-tolerant genotypes Arabeska and Ljiljana suggest an important role of malate efflux in wheat tolerance to Al^{3+}. In contrast to our findings, Sasaki *et al*. (2006) found only a weak correlation between *TaALMT1* expression and Al tolerance among Japanese wheat lines in comparison to a large number of lines of different origins, whereas these authors reported a significant correlation between Al-activated malate efflux and Al tolerance in Japanese cultivars. Moreover, Eagles *et al*. (2014) showed that *ALMT1* significantly interacts with some environmental parameters, which might mask plant response to Al toxicity. Thus, using this gene as a promising marker for Al tolerance needs establishing a standard protocol for plant growing conditions.

5. Conclusions

Different responses to Al toxicity were observed in high-yielding Serbian winter wheat cultivars. In addition to the common RRL test for Al tolerance, both physiological (malate efflux) and molecular (*TaALMT1* expression) approaches were used for this characterization. Cultivars Pobeda and NS Futura showed moderate sensitivity to excess Al and cannot be recommended for cultivation in acid soils. A considerably high level of Al tolerance was found in cv. Ljiljana, which showed the highest Al-induced malate efflux along with the highest expression level of *TaALMT1* transcripts. However, field trials are

required before cv. Ljiljana is recommended for the breeding program and/or growing in acid soils. These results also demonstrate that Al-tolerance is based on a constitutive trait of high *TaALMT1* expression and malate efflux in wheat roots. Moreover, these physiological and molecular parameters may be used in wheat breeding for low P soils (both acid and calcareous), since P-deficient wheat roots not subjected to Al stress maintain high efflux of malate along with the enhanced expression of anion transporter (Kostic *et al*., 2015).

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