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Differential activation of genes related to aluminium tolerance in two contrasting rice cultivars

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ABSTRACT

Rice (*Oryza sativa*) is a highly Al-tolerant crop. Among other mechanisms, a higher expression of *STAR1*/*STAR2* (*sensitive to Al rhizotoxicity1*/*2*) genes and of *Nrat1* (*NRAMP Aluminium Transporter 1*), and *ALS1* (*Aluminium sensitive 1*) can at least in part be responsible for the inducible Al tolerance in this species. Here we analysed the responses to Al in two contrasting rice varieties. All analysed toxicity/tolerance markers (root elongation, Evans blue, morin and haematoxylin staining) indicated higher Al-tolerance in variety Nipponbare, than in variety Modan. Nipponbare accumulated much less Al in the roots than Modan. Aluminium supply caused stronger expression of *STAR1* in Nipponbare than in Modan. A distinctively higher increase of Al-induced abscisic acid (ABA) accumulation was found in the roots of Nipponbare than in Modan. Highest ABA levels were observed in Nipponbare after 48 h exposure to Al. This ABA peak was coincident in time with the highest expression level of *STAR1*. It is proposed that ABA may be required for cell wall remodulation facilitated by the enhanced UDP-glucose transport to the walls through STAR1/STAR2. Contrastingly, in the roots of Modan the expression of both *Nrat1* coding for a plasma membrane Al-transporter and of *ALS1* coding for a tonoplast-localized Al transporter was considerably enhanced. Moreover, Modan had a higher Al-induced expression of *ASR1* a gene that has been proposed to code for a reactive oxygen scavenging protein. In conclusion, the Al-exclusion strategy of Nipponbare, at least in part mediated by STAR1 and probably regulated by ABA, provided better protection against Al toxicity than the accumulation and internal detoxification strategy of Modan mediated by Nrat1, ALS1 and ARS1.

1. Introduction

The mechanisms of Al-induced inhibition of root growth in sensitive plant species and varieties are rather well established. Aluminium primarily targets the root tip transition zone [1] causing fast inhibition of both cell expansion [2,3] and cell division [4,5]. A direct interaction of Al with the cell walls crosslinking the negative charges of pectates may lead to cell wall stiffening and lower cell expansion [3]. In fact, the Al exposure causes a fast enhancement of the elastic modulus, i.e. a decrease in elasticity in the outermost cell layer in Al-sensitive maize roots [2]. The fast inhibition of cell division in the root apical meristem is not due to a general cytotoxic effect of Al, but due to a change in cell patterning leading to stimulation of cell division in the distal transition zone and the outgrowth of new lateral roots. This adaptive root growth that may help the plant to grow into less toxic soil zones is regulated by phytohormones. In Al-sensitive species fast changes in endogenous cytokinin and ethylene levels precede Al-induced inhibition of root elongation [6,7] and a local increase of auxin concentration is brought about both by reduced auxin transport [8,9] and enhanced auxin biosynthesis [10]. Furthermore, Al exposure reduces the endogenous concentrations of abscisic acid (ABA) in roots of Al-sensitive bean plants, rendering plants more drought sensitive [11].

Considerably less information on the signalling of Al stress in Al resistant species is available. The best documented response to Al in resistant species is the Al-induced exudation of organic acids that can be induced either immediately (pattern 1 type) or after a lag time of hours or even days upon exposure (pattern 2 type) [12]. Enhanced malate and/or citrate efflux has been observed in Al-resistant genotypes of wheat [13,14], sorghum [15], and bean [16], among other important crop species. Oxalate seems to be the most important Al chelator in root exudates of some highly Al-tolerant species like buckwheat [17]. However, there is now increasing evidence that in the extremely high Al resistance observed in some *Poaceae* such as *Brachiaria decumbens* and rice the release of organic acids by the root tips is not the only or even the main factor responsible for the high level of resistance [18,19]. A characteristic feature of the Al hyperresistant *B. decumbens* is the development of root hairs and the development of an exodermis close to the tip [20]. Aluminium accumulates in these root hair cells. This may reduce the Al binding in the apoplast. Higher levels of chlorogenic acid a well-known inducer of root hairs may contribute to

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this [21]. Recent investigations revealed that the transcription factor ASR5 (**A**bscisic acid **S**tress **R**ipening induced) is required for the expression of Al-responsive genes and Al resistance in rice [22]. Among others *ASR5* promotes the expression of *STAR1* (***S**ensitive to **Al R**hizotoxicity 1*) which together with *STAR2* (***S**ensitive to **Al R**hizotoxicity 2*) is responsible for UDP-glucose export to the cell walls [23].

Taken together these observations indicate a central role for phytohormones in the early response of Al-resistant rice varieties to Al. However, the influence of Al exposure on these regulatory substances has poorly been investigated in Al-tolerant varieties. The objective of this work was to see the possible relationship between the Al-induced changes in the endogenous root levels of stress-related phytohormones and the expression of key genes involved in Al resistance in rice.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of rice varieties Nipponbare (Al-tolerant) and Modan (Al-sensitive) were germinated during four days in filter paper rolls moistened with tap water at 27 °C in the dark. Uniform seedlings were transferred to continuously aerated nutrient solution at pH 4.2 [24]; the solution was completely renewed at four-day intervals. After 15 days of pre-culture half of the plants of each variety received nutrient solution supplemented with 500 μM Al in the form of $AlCl_3$. The rest of the plants received control solution without Al. The plants were grown in a growth chamber for 24, 48 and 72 h under controlled-environment conditions of the following characteristics: 12 h light, 12 h darkness photoperiod, photon fluency rate 310 μE m^{-2} s^{-1}, night and day temperature 25 and 27 °C, respectively.

2.2. Aluminium toxicity indicators

Inhibition of root elongation, Al root and shoot accumulation, haematoxylin and Evans blue staining of root tips and root malondialdehyde concentrations were used as indicators for Al toxicity. All parameters were determined at 24-hour intervals during 72 h comparing control plants without Al supply to those receiving Al.

Root elongation of the longest root of each plant was measured with a ruler before and after the different treatment times. For the assessment of Al accumulation, after plant harvest the roots were carefully rinsed with distilled water, followed by 10 min treatment with 1 mM citric acid and a further wash with distilled water. The plants were divided into roots and shoots and the material was oven dried (60 °C). Acid digestion (69% HNO_3:30% H_2O_2, 5:2) was done in a hot block system (model 154-240, Environmental Express, Charleston, SC, USA). Aluminium concentrations were analysed by ICP-OES (model Elan 6000, Perkin Elmer, Massachusetts, USA). For quality assurance certified *Olea europea* leaves (BCR 62) were analysed in parallel. The percent recovery rate of the certified Al concentration was 104 ± 3.

Haematoxylin staining of the entire root tips of plants from the different Al-exposure times was performed according to Polle et al. (1987) [25]. Additionally, after 24 h of Al exposure longitudinal 10 mm root tips were embedded in 6% Bacto Agar (Difco, BD Company, Madrid, Spain) and sectioned (100 μm) with a vibratome (Vibratome Series 1000, TPI Inc., St. Louis, MO, USA). After staining with morin [26] the sections were visualized in a fluorescence microscope (Nikon Optiphot-2, Nikon Instruments, Amsterdam, The Netherlands).

Cell viability loss was assessed by Evans blue staining [27]. Uptake of Evans blue stain into the cells is a marker for damaged plasma membranes. This can be quantitatively assessed by spectrophotometry after desorbing the stain from the roots. For this purpose roots of intact plants were stained during 15 min with a 0.25% Evans blue solution. After careful washing with distilled water (three washes 10 min each) the root tips (10 mm) were excised and submerged for stain desorption during 1 h in N, N-dimethylformamide [27]. The optical density of the desorbing solution was measured at 600 nm in a spectrophotometer (Shimadzu-UV2450, Kyoto, Japan).

The concentrations of malondialdehyde in roots of control and Al treated plants were analysed according to Lin et al. [28].

2.3. Root concentrations of abscisic acid, salicylic acid, jasmonate and 1-aminocyclopropane-1- carboxylic acid

Analyses of abscisic acid (ABA), 1-aminocyclopropane-1- carboxylic acid (ACC), salicylic acid (SA), and jasmonic acid (JA) extracted from shoot and root material were carried out by injection of samples into the LC–ESI–MS/MS as previously described [29]. Briefly, frozen material (0.25 g) was ground under cold conditions and was repeatedly extracted with 750 μL MeOH:H_2O:HOAc (90:9:1 v/v/v) and centrifuged for 1 min at 10,600 *g*. Pooled supernatants were dried under N_2, resuspended in 200 μL of 0.05% HOAc in H_2O:MeCN (85:15 v/v), and finally filtered through a cellulose acetate membrane with a pore size of 0.45 μm (Corning Costar Spin-X — centrifuge tube filter from Sigma). Deuterated salicylic acid and 1-aminocyclopropane-1- carboxylic acid (both 98 atom% D -Sigma-Aldrich, Steinheim, Germany) at 500 ppb were used as internal standards in all the samples.

Quantification was done using a standard addition calibration curve spiking control plant samples with SA (99% Fluka, Buchs, Switzerland) and ACC (Sigma-Aldrich, Steinheim, Germany) ranging from 50 to 1500 ppb, and ABA and (±)-JA (Sigma-Aldrich, Steinheim, Germany) solutions ranging from 5 to 150 ppb and extracting as described above.

Hormones were separated using HPLC Agilent 1100 (Waldrom, Germany) on a Discovery C18 2.1 × 150 mm ID, 5 μm column (Supelco, Bellefonte, USA). MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). The analyses were performed using the Turbo Ion spray source in negative ion mode (137/93 for SA, 209/59 for JA, 263/153 for ABA, and 141/97 for d6SA) and positive mode (102/56 for ACC and 106/60 for d4ACC) and quantification was performed by injection of extracted and spiked samples in multiple reaction monitoring (MRM) mode, because many compounds could present the same nominal molecular mass. Identification was done on the basis of retention time and presence of peak in the MRM trace compared with those of the standards. Given values are means from 3 individual samples per each time sample and treatment factor.

2.4. Quantitative real-time PCR (RT-qPCR)

Rice roots were collected and immediately frozen in liquid nitrogen; total RNA was isolated using Trizol reagent (Invitrogen®) following the manufacturer's instructions and the cDNAs were obtained using the iScript™ cDNA Synthesis Kit (Bio-Rad). 3 μL of 5-fold dilution cDNA from each simple was used for the quantitative analysis of gene expression with SYBR Green (Bio-Rad). The three-step protocol for RT-qPCR is summarized as follows: A first step of 3 min at 98 °C followed by 40 cycles of 15 s at 95 °C and 10 s at 58 °C. Samples were maintained for 10 s at 72 °C. A slow ramp rate was used with 2 °C increments per second. Samples were then warmed to 95 °C for 10 s followed by melt curve from 65 °C to 95 °C with 0.5 °C increments for 5 s. All reactions were carried out in three technical replications with 10 μL of the diluted cDNA, and expression gene calculations were performed using the 2-DDCt method [10]. Actin1 gen was used as an internal control. The sequences of the used primers were as follows: Actin1, 5′-GACTC TGGTGATGGTGT CAGC-3′ (forward) and 5′-GGCTGGAAGAG GACCTCAGG-3′ (reverse); STAR1, 5′-TCGCATTGGCTCG CACC CT-3′ (forward) and 5′-TCGTCTTCTT CAGCCGCACGAT-3′ (reverse) [30]; ARS5, 5′-CCAGGACGAGTACGAGAG GT (forward) and 5′-CGATCTCCTCCGTGATCTTG (reverse); ARS1 5′-TGGTGGACTACGACAAGGAGA (forward) and 5′-GCCACCTCCTCCTTCA CC (reverse) [22], Nrat1, 5′-GAGGCC GTCTGCAGGAGAGG-3′ (forward) and 5′-GGAAGTATCTGCAAGCA GCTCTGATGC-3′ (reverse) [31]; ALS1, 5′-GGTCGTCAGTCTCTGCCTTGTC-3′ (forward) and 5′-CCTCCCCATCAT

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