Expression of *OsFRDL1*, a MATE gene family member, indicates its involvement in aluminum response in rice

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Abstract – In soils under acidic conditions, Aluminum (Al) is solubilized to its ionic form, which is toxic to plants. Al rapidly inhibits root elongation, water and nutrient uptake, resulting in crop yield reduction. Members of the MATE family are responsible for citrate transport and Al detoxification in different species. In rice, the *OsFRDL1* gene (MATE family) is homologous to the *HvAACT1* and *SbMATE*, which are involved in Al tolerance in barley and sorghum, respectively. Silencing *OsFRDL1* showed that it is not involved in Al tolerance in rice. However, the *OsFRDL1* expression was not accessed in rice genotypes contrasting for Al tolerance. Thus, in this study, four Brazilian rice genotypes were evaluated in response to Al treatment under different times of exposition and *OsFRDL1* expression was analyzed. The cultivars displayed different responses to Al dose x time. Al affected root growth in all analyzed genotypes, however, a minor negative effect that only occurred after 72 and 48 hours of exposure was detected in Farroupilha and BRS Curinga cultivars, respectively. In contrast, BR-IRGA 410 and IAS 12-9 showed a negative effect in root growth from the first hours of exposure to Al. Two cultivars differing in Al tolerance were used for gene expression analysis. The expression of *OsFRDL1* was highly increased in Al-tolerant cultivar Farroupilha when compared to the Al-sensitive cultivar BR IRGA 410. This result indicates that *OsFRDL1* is regulated by Al. This finding suggests that *OsFRDL1* is involved in Al stress response, however seems to be insufficient in controlling Al tolerance.

Index terms: Root development; gene expression; aluminum toxicity; *Oryza sativa*.

Expressão de *OsFRDL1*, um membro da família MATE, indica seu envolvimento na resposta ao alumínio em arroz

Resumo – Em solos em condições de acidez, o alumínio (Al) é solubilizado em sua forma iônica, a qual é tóxica para as plantas. O Al rapidamente inibe o crescimento radicular e a obtenção de água e nutrientes, resultando na redução da produtividade. Membros da família MATE são responsáveis pelo transporte do citrato e detoxificação do Al em diferentes espécies. Em arroz, o gene *OsFRDL1* (da família MATE) é homólogo aos genes *HvAACT1* e *SbMATE*, os quais são envolvidos na tolerância ao Al em cevada e sorgo, respectivamente. O silenciamento de *OsFRDL1* demonstrou que este gene não é envolvido com a tolerância ao Al em arroz. No entanto, a expressão de *OsFRDL1* não foi acessada em genótipos de arroz contrastantes quanto à tolerância ao Al. Assim, neste estudo, quatro genótipos de arroz brasileiros foram avaliados em resposta ao tratamento com Al em diferentes tempos de exposição e a expressão do gene *OsFRDL1* também foi avaliada. Os genótipos analisados apresentaram diferentes respostas ao Al dose x tempo. O Al afetou o crescimento radicular em todos os genótipos avaliados, no entanto, um pequeno efeito negativo que ocorreu após 72 e 48 horas de exposição foi identificado nos genótipos Farroupilha e BRS Curinga, respectivamente. Por outro lado, BR-IRGA 410 e IAS 12-9 apresentaram um efeito negativo no crescimento radicular a partir das primeiras horas de exposição ao Al. Dois genótipos foram utilizados para as análises de expressão gênica. A expressão do gene *OsFRDL1* foi aumentada no genótipo Farroupilha, tolerante ao Al, em relação ao genótipo BR IRGA 410, sensível ao Al. Estes resultados indicam que o gene *OsFRDL1* está envolvido na resposta ao estresse por Al, no entanto, parece que este gene não é suficiente para controlar a tolerância ao Al.

Termos para indexação: Desenvolvimento de raízes; toxidez por alumínio; expressão gênica; *Oryza sativa*.

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Introduction

Aluminum (Al) is the most abundant metal on earth crust. In acid soils, Al is solubilized to its ionic form (Al^{3+}), which is toxic to the plants (FOY, 1988; ARENHART et al., 2014). The presence of Al in soil solution quickly inhibits the root growth as well as water and nutrient uptake by the plant, resulting in a significant reduction in the crop production under these conditions (CHANG et al., 2015). It is estimated that acidic soils comprise 40 and 50% of the world's arable soil (KOCHIAN et al., 2015; RAHMAN et al., 2018). Some plant species evolved mechanisms to deal with Al toxicity (MA et al., 2001; RYAN & DELHAIZE, 2001; KOCHIAN et al., 2005; ARENHART et al., 2014). The most studied mechanism of tolerance is related to the secretion of organic acid anions from roots (MA, 2000; MA et al., 2001; RYAN & DELHAIZE, 2001; KOCHIAN et al., 2005; KOCHIAN et al., 2015). These anions include citrate, oxalate and/or malate (YOKOSHO et al., 2011; KOCHIAN et al., 2015) and are able to complex with Al, preventing Al from entering the plant (FURUKAWA et al., 2007). Many efforts have been applied to understand the nature of organic acid secretion induced by Al (MA et al., 2001; RYAN & DELHAIZE, 2001; KOCHIAN et al., 2005; ZHANG et al., 2019a). Plants differ from each other related to the type of secreted organic acid, temporal secretion patterns, sensitivity to temperature and responses to different Al doses (MA, 2000; YANG et al., 2013).

Two different patterns (I and II) can be identified in organic acids release based on secretion rhythm. In type I, the secretion occurs almost immediately after the addition of Al, which suggests that the metal activates a pre-existing ion channel in the plasma membrane and that it is not necessary to induce gene expression. Plants that display type II, the release of organic acids is delayed for several hours after exposure to Al, suggesting that there is a need of gene expression induction. Some inducible proteins may be involved in the metabolism of organic acids or in the transport of their anions (MA, 2000; YANG et al., 2013). Previous studies demonstrated that the secretion of organic acids is performed through anionic channels or transporters. In maize plants, Al has been shown to activate Cl^- efflux, as well as the citrate-permeable anionic channel (PIÑEROS & KOCHIAN, 2001).

The molecular control of the anion secretion has been unveiled. The first gene directly related to Al tolerance in plants, *ALMT1* (*Al-Activated Malate Transporter 1*), was identified to be responsible for malate release in wheat (SASAKI et al., 2004; RAHMAN et al., 2018). Some proteins belonging to the MATE (Multidrug and Toxic Compound Extrusion) family are involved in the transport of citrate into plants and required for iron (Fe) translocation or Al detoxification (YOKOSHO et al., 2009). Differential gene expression analysis in soybean demonstrate that MATE genes, specially *GmMATE75*, are involved in Al tolerance and increased transcript accumulation 12 and 24 hours after exposed to Al treatment (LIU et al., 2016). Two MATE members were also characterized in maize, *ZmMATE1* and *ZmMATE2*, which co-localize to a major Al tolerance QTL (MARON et al., 2010). In addition, many transporters, including members of MATE and ABC families, were involved in the process of Al-citrate complex transport in *Hydrangea macrophylla* roots (CHEN et al., 2015). In *A. thaliana,* it has been shown that the FRD3 (Ferric Reductase Defective3) protein acts in citrate transport, which is required for translocation of Fe from roots to shoots (DURRETT et al., 2007). Two other studies showed that the release of citrate induced by Al in barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*) is mediated by transporters from MATE family (FURUKAWA et al., 2007; MAGALHÃES et al., 2007). In barley, the gene *HvAACT1* (*Aluminum Citrate-Activated Transporter1*), encodes the carrier protein placed in plasma membrane of the root epidermal cells and is able to realize citrate secretion under Al toxicity condition (FURUKAWA et al., 2007). In sorghum, the *SbMATE* gene is also involved in the citrate efflux leading to Al tolerance (MAGALHÃES et al., 2007).

The rice genome presents six *AtFRD3*, *HvAACT* and *SbMATE* homologous genes, which were identified as *OsFRDL* (*Ferric Reductase Defective-like*). *OsFRDL1* (Os03g0216700), a citrate transporter, is close to *HvAACT* gene (FURUKAWA et al., 2007; YOKOSHO et al., 2009). The silencing of *OsFRDL1* indicates that it is not involved with citrate secretion induced by Al, but with the efficient translocation of Fe to the shoot (YOKOSHO et al., 2009). In addition, the authors observed in a specific genotype that *OsFRDL1* expression was not affected by Al treatment. However, the expression profile of *OsFRDL1* in response to exposure to Al in rice genotypes with different levels of tolerance has not been evaluated. Since rice roots secrete citrate in response to Al, a difference in the expression of genes involved in this process can be expected between tolerant and sensitive genotypes. The identification of differences in gene expression may contribute to the elucidation of the mechanisms involved in rice Al response. In this sense, this work aimed to evaluate the *OsFRDL1* expression in rice genotypes with contrasting Al response.

Material and methods

Plant material and growth conditions

The rice genotypes BRS Curinga, Farroupilha, BR-IRGA 410 and IAS 12-9 Formosa were grown in hydroponic system, under controlled environmental conditions. Rice seeds were germinated on nylon screens adapted to pots

containing complete nutrient solution (CAMARGO & OLIVEIRA, 1981) composed by 4mM $Ca(NO_3)_2$; 2mM $MgSO_4$; 4mM KNO_3; 0.435mM $(NH_4)_2SO_4$; 0.5mM KH_2PO_4; 2mM $MnSO_4$; 0.3µM $CuSO_4$; 0.8µM $ZnSO_4$; 30µM NaCl; 10µM Fe-EDTA; 0,10µM $NaMoSO_4$ and 10 µM H_3BO_3. After four days in the dark, the genotypes were subjected to a photoperiod of 12 hours of light / 12 hours of dark to the light intensity of 7,000 lux. A half part of the total of plants in V3 stage (SOSBAI, 2018) were transferred to aluminum excess treatment, which consisted of one-tenth of the total solution (without addition of phosphate to avoid possible precipitation of Al^{3+}) containing concentrations of 0 and 14 mg L^{-1} of aluminum, provided in the form of $Al_2(SO_4)_3$. The rest of the plants were kept in a standard solution (control condition). Control and Al stressed plants were kept in hydroponic system at 26°C. Plant solutions were continuously aerated and its pH adjusted to 4.0 ± 0.3 by addition of 1N HCl, as described by Camargo & Oliveira (1981). The main root length of ten plants in each treatment were morphologically evaluated at 0, 2, 6, 12, 24, 48, 72 and 96 hours after exposure to treatment. Root samples for gene expression analysis were collected at 0, 12, 24 and 48 hours after exposure to treatment. The samples were washed with autoclaved ultrapure water and stored at -80°C until RNA extraction.

RNA Extraction, cDNA synthesis and Real-time quantitative reverse transcription-PCR (RT-qPCR) analysis

To evaluate the expression of *OsFRDL1* (Os03g0216700) in response to aluminum treatment in rice roots, two genotypes, one tolerant (Farroupilha) and one sensitive (BR-IRGA 410), were used. For gene expression analyses, the total RNA was extracted from root samples using TRIzol reagent (Invitrogen, CA, USA). The RNA quality and integrity were assessed by spectrophotometry (Hitachi spectrophotometer, model U-1800) and electrophoresis in agarose gel. Subsequently, the RNA samples were treated with DNase I (Amplification Grade Dnase I, Invitrogen) in order to remove remaining genomic DNA. The cDNA synthesis was performed using SuperScript II RT (Invitrogen) and Oligo(dT) according to the manufactured recommendations. The RT-qPCR experiment was performed according to MIQE guidelines (BUSTIN et al., 2009). Oligonucleotides for the target gene *OsFRDL1* (Forward primer - 5'-TGCTGAAAAGACCAGGAAGACA-3' and Reverse primer - 5'-GTTGGCTCATTTCTTGGGCTAC-3') were designed from sequences deposited in The Rice Annotation Project Data Base (RAPDB), using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Oligonucleotides for the housekeeping gene *Ubiquitin5* (*UBQ5*) (Forward primer – 5'-ACCACTTCGACCGCCACTACT-3' and Reverse primer 5'-ACGCCTAAGCCTGCTGGTT-3') were obtained from JAIN et al. (2006). The RT-qPCR assay was conducted in triplicate in an ABI RT PCR 7500 (Applied Biosystems) thermocycler using SYBR Green (Applied Biosystems, California, USA) detection system. The relative expression of the target gene was calculated through the ΔΔCt method (LIVAK & SCHIMITTGEN, 2001).

Experimental design and statistical analysis

Three replicates in a random design were used. Morphological data from root evaluation were subjected to analysis of variance (ANOVA) and a regression analysis was performed since interaction between dose and exposure time was detected in ANOVA. Both analyses were performed using SAS statistical software (SAS, 2013). Expression data are shown in bar graphics and error bars represent standard deviation from three independent biological replicates.

Results and discussion

Farroupilha roots are less affected by aluminum excess

Al toxicity is the major factor limiting plant growth in acid soils. Small Al concentrations (micromolar) can inhibit root elongation in minutes or hours, inhibiting the water and nutrient uptake, resulting in reduced growth and yield (MA & FURUKAWA, 2003; RAHMAN et al., 2018). Since roots are strongly affected by Al, many reports have shown the evaluation of traits related to the growth of the root system (CHANG et al., 2015). Here, to understand the Al toxicity effects on rice Brazilian genotypes, we evaluated the root length trait in BR-IRGA 410, BRS Curinga, IAS 12-9 Formosa and Farroupilha genotypes in response to 14mg L^{-1} of Al during 96 hours (Figure 1). To understand the effect of Al on root growth, an analysis of variance was performed (Table 1) and interactions between the treatments and exposure time was detected. In this sense, a regression analysis was performed.

Table 1. Analysis of variance for root length (RL) of rice seedlings under aluminum excess

Tabela 1. Análise de variância para comprimento de raiz (RL) de plântulas de arroz submetidas a excesso de alumínio

		Mean square
FV	DF	RL
Dose	1	46.216*
Time	7	32.368*
Dose*Time	7	5.955*
Residue	128	0.168
Mean		6.704
CV		6.117

*Significant by the F test (P ≤ 0.05).

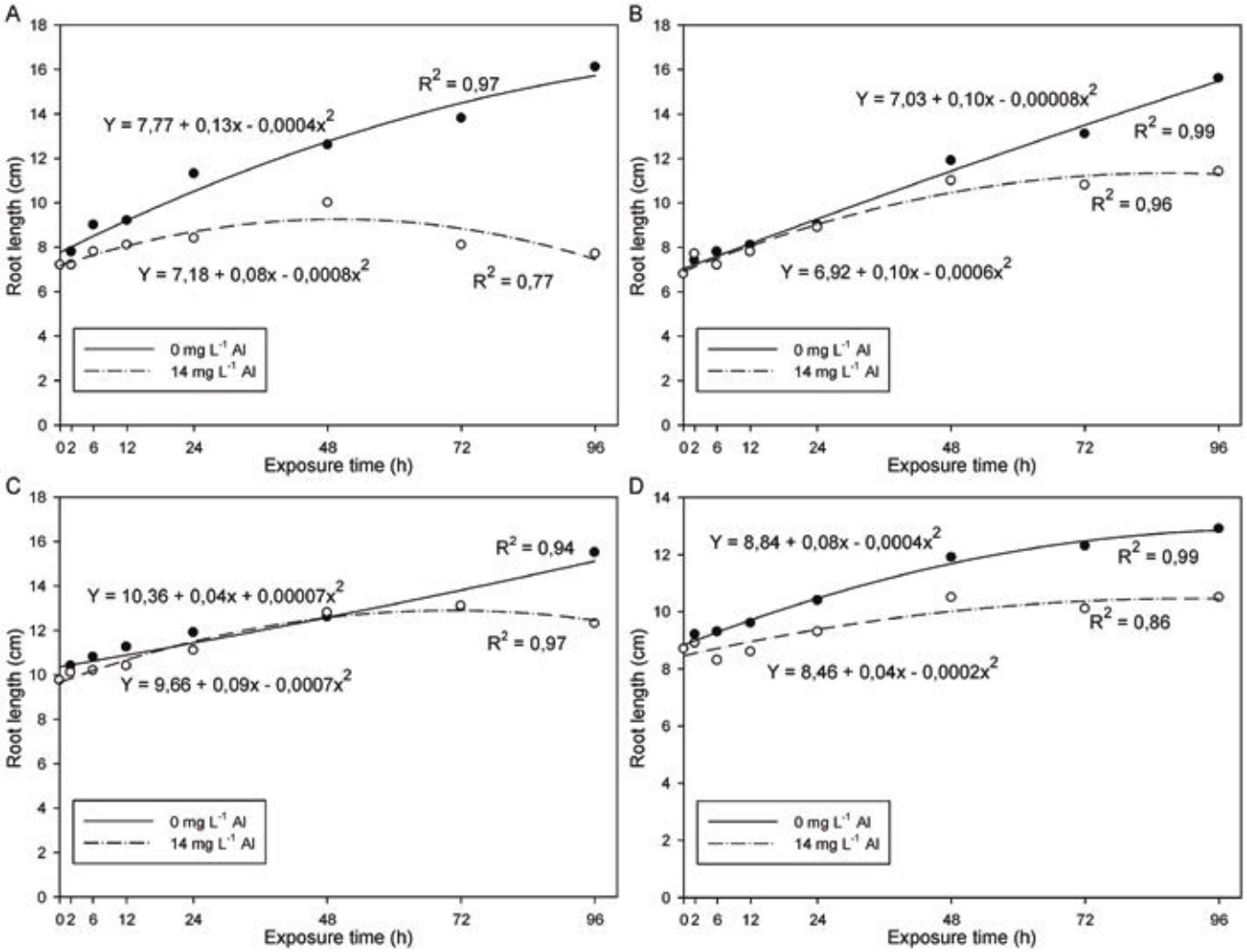

Figure 1. Root length of rice plants under aluminum treatment in different time of exposition. A: BR IRGA 410; B: BRS Curinga; C: Farroupilha; D: IAS 12-9 Formosa

Figura 1. Comprimento da raiz de plantas de arroz sob tratamento de alumínio com diferentes tempos de exposição. A: BR IRGA 410; B: BRS Curinga; C: Farroupilha; D: IAS 12-9 Formosa

The increase in exposure time to Al resulted in a reduction in root length of the BR-IRGA 410 cultivar when compared to the control. The root length was highly reduced after 24 hours of exposure, showing a 50% reduction at 96 hours (Figure 1A). The negative effects in the root development of BRS Curinga were observed at 48 hours after Al treatment and were intensified at 72 hours (Figure 1B). Al treatment negatively affected Farroupilha roots only after 72 hours of exposure (Figure 1C). The presence of Al in the growing media was also harmful to IAS 12-9 Formosa root growth (Figure 1D) as well as to BR IRGA 410, demonstrating sensitivity since the first hours of Al treatment. It can be also noticed that the Al effect in IAS 12-9 Formosa was less intense as the time of exposure to the metal increased.

One of the major constraints to evaluate plants related to Al tolerance is the correct setting of the stress level, which needs to achieve a significant reduction in root growth in the sensitive and a limited effect in the tolerant genotype. On top of this, the exposure time is also an important factor to be considered (MACEDO et al., 1997). The Al dose used here (14mg L^{-1}) as well as the exposure time, seems to be useful to characterize different genetic materials. In addition, the morphological difference found here showed a negative effect of Al over all genotypes analyzed at 96 hours, although in different magnitudes, indicating that 96 hours is not a suitable treatment for gene expression analysis, since the molecular signaling responsible for the phenotype was activated before 96 hours. Taking into account the phenotype observed in the morphological analysis, we chose

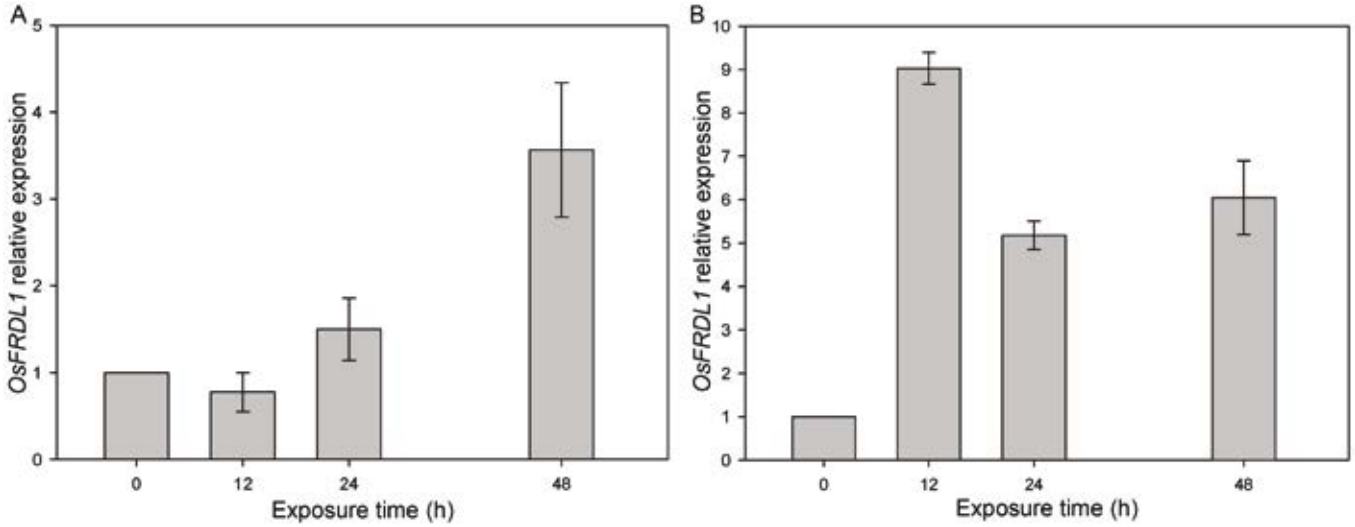

Figure 2. Relative expression of OsFRDL1 in root tissue of rice plants during different exposition time under aluminum treatment. A: BR IRGA 410 and B: Farroupilha. Samples in control condition were used as baseline. Error bars represents mean ± standard deviation (n=3)
Figura 2. Expressão relativa de OsFRDL1 no tecido radicular de plantas de arroz durante diferentes tempos de exposição sob tratamento com alumínio. A: BR IRGA 410 e B: Farroupilha. Amostras em condição de controle foram utilizadas como linha de base. As barras de erro representam a média ± desvio padrão (n = 3)

two contrasting genotypes as models for Al tolerance and sensitivity to perform gene expression analyses. In this sense, Farroupilha, as a tolerant and BR IRGA 410 as a sensitive genotype were chosen for *OsFRDL1* transcriptional analyses at 0, 12, 24 and 48 hours.

OsFDRL1 is activated in response to Al toxicity in different backgrounds

To evaluate the effects of the increased Al exposure (0, 12, 24 and 48 hours) on the expression of *OsFRDL1,* a RT-qPCR assay was performed. Treatment with 14mg L^{-1} of Al for 12 hours did not cause increases in the expression of *OsFRDL1* in BR-IRGA 410, however, resulted in a 9-fold increase in Farroupilha. On the other hand, for 24 and 48 hours of treatment, 1.5 and 3.6-fold increases in expression were observed for BR-IRGA 410, respectively (Figure 2A). In Farroupilha, 5.2 and 6.1-fold increases were detected at 24 and 48 hours, respectively (Figure 2B). Overall, Farroupilha showed a higher increase and a rapid activation of *OsFRDL1* expression in response to Al.

Barley and sorghum, two other members of Poaceae, as is rice, present OsFRDL1 homolog genes, *HvAACT1* in barley and *SbMATE* in sorghum (FURUKAWA et al., 2007; MAGALHÃES et al., 2007). In barley exposed to 0 and 30µM of Al for 6 hours, an increased expression of *HvAACT1*, that encodes a citrate carrier membrane protein, were detected in roots and shoots. However, higher transcript accumulation was detected in roots (FURUKAWA et al., 2007). The amount of transcript was 26 times higher in the Al tolerant cultivar than in the Al sensitive, but the level of expression was not induced by Al in none of them. The authors suggested that *HvAACT1* is constitutively expressed in roots of barley and that secretion of citrate is mediated by the activation of HvAACT1 protein. Expression of *SbMATE* gene in sorghum, which is also related to citrate secretion, was increased in roots of resistant Al plants and was induced by the Al treatment. An increased expression was also detected with the increase of exposure time (MAGALHÃES et al., 2007).

When considering the amino acid sequence homology, *OsFRDL1* shows 87% identity with HvAACT1 and 57% with AtFRD3 (present in *Arabidopsis*) (YOKOSHO et al., 2009). HvAACT1 is involved in the citrate secretion induced by Al (FURUKAWA et al., 2007), while AtFRD3 releases citrate that participates in the transport of iron from the roots to the shoots (DURRETT et al., 2007). Therefore, it is expected that this protein in rice membrane is functionally related to citrate release to extracellular environment in response to Al, which represents one of the major mechanisms of plant tolerance to this stress (KOCHIAN et al., 2005; ZHANG et al., 2019a). In the rice genome, there are six homologous genes close to *AtFRD3*, *HvAACT1* and *SbMATE*. Previous reports showed that *OsFRDL1,* closed related to *HvAACT1,* was not affected by exposure to 50µM Al for 3 hours (YOKOSHO et al., 2009). In addition, no difference in ▸

citrate secretion was detected between the knockout *OsFRDL1* line and the cultivar Nipponbare in the presence of Al. On the other hand, here we identified differences in expression levels of *OsFRDL1* in both BR-IRGA 410 (sensitive) and Farroupilha (tolerant) when control and Al treatments are compared (Figure 2).

Farroupilha showed a higher increase in *OsFRDL1* expression in response to Al. As *OsFRDL1* expression was observed at 12 hours after the treatment, probably the expression was initiated before, in a period between 0 and 12 hours. It may be associated to the absence of difference in root growth observed when comparing the control condition and the shorter times of Al exposure (Figure 1C). BR IRGA 410 showed a different profile, an increase in *OsFRDL1* transcripts was observed after 48 hours of Al treatment, and at lower levels when compared to Farroupilha. A tendency in root length reduction was observed in the first hours of Al treatment in BR IRGA 410. That reduction is probably related to the non-activation of *OsFRDL1,* responsible for citrate transport to extracellular medium. However, other genes can be involved in this process. Citrate displays a chelating role and neutralizes Al^{3+}, the most toxic form of Al, preventing Al entering in root cells which can have negative effect on root growth in low pH conditions (KOCHIAN, 1995; ZHANG et al., 2019b). On the other hand, it was verified that both genotypes showed *OsFRDL1* expression in absence of Al (data not shown), agreeing with the response to *HvAACT1* gene in barley (FURUKAWA et al., 2007). However, when exposed to Al, the *OsFRDL1* transcriptional activation was more efficient in Farroupilha, which may explain in part, the observed phenotype, whereas BR IRGA 410 seems not to be able to overcome the Al presence through this mechanism.

Conclusion

-Farroupilha (Al tolerant) showed an increased expression of *OsFRDL1* when compared to BR-IRGA 410 (Al sensitive).

-The differences found in expression levels may be associated with the morphological responses observed in genotypes in response to Al exposure, suggesting that *OsFRDL1* is involved in response to Al in rice.

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