Aluminum triggers broad changes in microRNA expression in rice roots

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ABSTRACT. MicroRNAs are small 21-nucleotide RNA molecules with regulatory roles in development and in response to stress. Expression of some plant miRNAs has been specifically associated with responses to abiotic stresses caused by cold, light, iron, and copper ions. In acid soils, aluminum solubility increases, thereby causing severe damage to plants. Although physiological aspects of aluminum toxicity in plants have been well characterized, the molecular mediators are not fully elucidated. There have been no reports about miRNA responses to aluminum stress. Modulation of miRNA expression may constitute a key element to explain the mechanisms implicated in aluminum toxicity and tolerance. We examined the expression of at least one miRNA member from each miRNA family in rice roots of *Oryza sativa* spp *indica* cv. Embrapa Taim and *Oryza sativa* spp *japonica* cv. Nipponbare under high concentrations of aluminum. Forty-six miRNA families were effectively detected by quantitative PCR. Among these, 13 were down-regulated and six were up-regulated in roots of the Nipponbare

cultivar after 8 h of aluminum treatment. In roots of the Embrapa Taim cultivar, five miRNAs were down-regulated and three were up-regulated. Analyses of their putative targets suggest that these rice miRNAs are involved in the regulation of various metabolic pathways in response to high concentrations of aluminum.

Key words: Aluminum; MicroRNAs; Abiotic stress; Gene expression

INTRODUCTION

MiRNAs (microRNAs) are small 21-nucleotide-long RNA molecules with a central role in regulating gene expression (Xie et al., 2010). MicroRNAs act similarly to small interfering RNAs (siRNAs) by a mechanism of cleavage of their complementary mRNA targets or by repression of translation. The ancient origin of miRNAs, together with the potential link between miRNAs and development, implies that miRNAs might have participated in the origin and evolution of both plant and animal multicellular life (Reinhart et al., 2002). In plants, hundreds of miRNAs have been characterized by cloning and sequencing. Their putative targets, identified by *in silico* analysis, are mainly associated with developmental processes and stress responses (Chen et al., 2006). A large number of new miRNAs have been identified from *in silico* analyses of *Arabidopsis thaliana*, *Populus trichocarpa*, *Brassica napus*, and *Oryza sativa* (Lindow et al., 2007; Huang CF et al., 2010; Huang SQ et al., 2010) and by in-depth sequencing of small RNA libraries (Sunkar, 2010). In contrast to the discovery of many small RNAs, the biological role of the majority of miRNAs remains to be elucidated.

In plants, there are examples of miRNAs that regulate specific steps in cellular differentiation and organ development, while others have been associated with responses to biotic and abiotic stresses. The GAMYB-like gene, MYB33, which is important during anther development, is down-regulated by miRNA159-directed cleavage in *Arabidopsis* (Millar and Gubler, 2005). Post-transcriptional regulation of the flowering gene APETALA2, controlled by miRNA172 in *Arabidopsis*, incorporates the dual mechanism of mRNA cleavage and inhibition of translation (Zhu and Helliwell, 2011). Challenge of *Arabidopsis* with the flagellin protein contributes to *Pseudomonas syringae* resistance and has been associated with miRNA393a induction and repression of auxin signaling (Navarro et al., 2006). Also, in rice, miRNA393 and miRNA393b genes respond differently to salinity and alkaline stress. *Arabidopsis* cultivated under cold, high salt concentrations or with the plant hormone, abscisic acid, showed differential expression of several miRNAs (Jung and Kang, 2007). Increasing concentrations of sulfate repress the expression of miRNA395 and up-regulated the expression of its target ATP sulfurylase-1. *Arabidopsis* plants overexpressing miRNA395 result in strong down-regulation of ATP sulfurylases and sulfate transporters (Liang et al., 2010). MiRNA399 contributes to the maintenance of phosphate homeostasis by down-regulating the expression of ubiquitin conjugating enzyme (UBC) in plants cultivated under low inorganic phosphate conditions. *Arabidopsis* plants cultivated under high light intensity and high iron or copper concentrations down-regulate miRNA398 and up-regulate its target genes, superoxide dismutases *AtSOD1* and *AtSOD2*, which play a role in the detoxification of reactive oxygen species (ROS). Moreover, new technologies for sequencing small RNA libraries have been showing a huge diversity of miRNAs regulating plant development and in response to biotic

and abiotic stresses. These high-throughput methods for sequencing confirm the occurrence of conserved miRNAs and have accelerated the discovery of new small RNAs as major players in plant biology (Sunkar, 2010; Xie et al., 2010).

The previous examples make evident aspects about how miRNAs are involved in responses to biotic and abiotic stresses. While miRNAs are clearly involved in responses to metal stress caused by Fe and Cu, there are presently no reports on miRNAs responding to aluminum (Al) treatment in plants. Al toxicity has been recognized as a major limiting factor in plant productivity on acidic soils. The primary Al toxicity symptoms occur in roots, causing the inhibition of the elongation process in the apical meristems (Kikui et al., 2005). An extensive review of the effects and mechanisms of Al tolerance point out some important features: i) low pH causes the solubilization of soil Al; ii) impairment of root growth diminishes water and mineral uptake; iii) high Al reactivity could cause the interaction of this metal with many cellular sites; iv) Al can interact with some important Ca^{2+} signaling pathways, and v) cellular contact with Al can elicit ROS. A major gene conferring tolerance to Al, *ALMT1*, was cloned from wheat and also conferred Al tolerance in transgenic rice and tobacco cells. Differential display reverse transcription PCR has been used to identify genes that respond to Al in rice cultivars displaying different sensitivities to this metal. Among the genes identified, several were related to signal transduction, ion transport, cellular metabolism, stress, and cytoskeleton rearrangements (Yang et al., 2007). Two genes, *Os*STAR1 encoding a nucleotide binding domain, and *Os*STAR2, found to be a bacterial-type ABC gene, being expressed mainly in roots, conferred Al tolerance in rice. Also, in *Arabidopsis*, the ortholog of the *Os*STAR2 gene is involved in Al detoxification, indicating a similar functional mechanism (Huang et al., 2010). Recently, a major protein, ART1, was proposed to be a transcriptional factor regulating several genes involved in Al response in rice, including *Os*STAR1 and *Os*STAR2 (Yamaji et al., 2009). The physiological aspects for Al resistance have been well studied, but the molecular regulatory mechanisms controlling the Al response are not completely known. In maize, a monocot plant species like rice, more genes were found in the Al-sensitive genotype than in the Al-tolerant genotype (Maron et al., 2008). However, a citrate-exclusion mechanism was suggested to release toxic Al in maize, which is known to be different for rice (Khan et al., 2009). Since miRNAs have only recently been shown to participate in the cellular regulatory machinery, and since the molecular aspects of plant-aluminum interactions are far from being elucidated, we examined the expression of mature miRNAs in roots of rice seedlings treated with Al.

MATERIAL AND METHODS

Plant material and growth conditions

Rice plants cv. Nipponbare (*O. sativa* ssp *japonica*) and cv. Embrapa Taim (*O. sativa* ssp *indica*) were used in this study. Seeds were sown on Petri dishes and germinated for 4 days (48-h dark/48-h light cycle). Twelve-day-old plants were cultivated in a low-ionic-strength hydroponic medium (Baier et al., 1995), pH 4.5. The composition of the medium was 10 μM $(NH_4)_2SO_4$; 400 μM $CaCl_2.2H_2O$; 250 μM $MgCl_2.6H_2O$; 40 μM NH_4NO_3; 650 μM KNO_3. For the aluminum treatment, plants were cultivated with 450 μM $AlCl_3$ for 4 and 8 h. After treatment, the plants were harvested and the roots were separated from the aerial part and immediately frozen in liquid nitrogen for RNA extraction.

Quantitative real-time PCR (RT-qPCR)

Total RNA from roots was extracted using the Trizol reagent, following manufacturer instructions (Invitrogen). A stem loop primer was used to synthesize the miRNA cDNAs (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACNNNNNN-3'), where the letter *N* represents the sequence of the six nucleotides that hybridizes to the 3' end of the mature miRNA (Chen et al., 2005). Mature miRNAs cDNA was synthesized by adding 0.1 to 2 μg total RNA; 100 U MMLV reverse transcriptase (Promega); 0.05 μM stem loop primer; 1X enzyme buffer; 0.33 mM dNTP mix in a final volume of 30 μL. The cDNA synthesis conditions were 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. The RT-qPCR mix was 10 μL of cDNA (1:2); 0.4 μL 5 mM dNTPs; 0.2 μL of each forward (10 μM) and universal (10 μM) primers; 2 μL 10X PCR buffer; 1.2 μL MgCl$_2$; 2 μL 1X SYBR Green (Molecular Probe); 0.1 μL ROX (1:100 in water) (Invitrogen); 0.05 μL Taq Platinum (Invitrogen) to a final volume of 20 μL. A forward primer (Table 1) and a universal reverse primer (5'-GTGCAGGGTCCGAGGT-3') were used in the quantitative PCR assays. The primers *Os18S*R (5'-ACACTTCACCGGACCATTCAA), *Os18S*F (5'-CTACGTCCCTGCCC TTTGTACA); *OsS27α*R (5'-ACGCCTAAGCCTGCTGGTT), *OsS27α*F (5'-ACCACTTCGA CCGCCACTACT), *OseFα1*R (5'-GACTTCCTTCACGATTTCATCGTAA), *OseFα1*F (5'-TT TCACTCTTGGTGTGAAGCAGAT); *OsFDH*R (5'-TTCCAATGCATTCAAAGCTG), *OsFDH*F (5'-CAAAATCAGCTGGTGCTTCTC); *OssnU6*R (5'-AGGGGCCATGCTAATCTTCT), and *OssnU6*F (5'-GGGGACATCCGATAAAATTG) were used as internal reference genes to normalize the expression of the miRNAs. The RT-qPCR conditions were 94°C for 5 min followed by 40 cycles at 95°C for 15 s, 60°C for 10 s, 72°C for 15 s. Then, the samples were heated from 55° to 99°C with an increase of 0.1°C/s to acquire the denaturing curve of the amplified products. Relative quantifications of amplified products were made by the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the Applied Biosystem 7500 Real-time PCR with the SDS software. SYBR-green (Molecular Probes) was used to detect amplification and estimate C$_T$ values and to determine specificity of amplicons by denaturing curves and melting temperatures (T$_m$).

In silico analysis of miRNA targets

Putative miRNA targets were checked *in silico* using the miRU tool (Zhang, 2005). All miRNA sequences were retrieved from the MiRBase (http://microrna.sanger.ac.uk/). Based on the rules described to identify microRNA targets, we were able to find more than one putative target for some of the miRNAs. Although we could have missed real targets, we adopted the following stringent criteria to choose putative targets: a limit score of 3.0 for the kind of base pairs (Wooble and/or Watson Crick base pair) and a maximum of three mismatches between miRNAs and their putative targets (Brennecke et al., 2005).

RESULTS

In rice, several miRNA families have been described *in silico*. Among these, some families are represented by more than one gene member, and others are represented by only one gene member (Griffiths-Jones et al., 2006; Zhang et al., 2010). A PCR quantitative analysis was conducted to have a general view of miRNA expression in response to Al treatment.

Table 1. List of forward primers used for the RT-qPCR analysis in rice roots.

miRNA	Forward primer 5'-3'
miRNA156l	GCCCGCGACAGAAGAGAGTG
miRNA159d	GGCCGGATTGGATTGAAGGGA
miRNA160e	GGTGCCTGGCTCCCTGT
miRNA162a	GGCCGGTCGATAAACCTCTGC
miRNA164e	GGCTGGAGAAGCAGGGCA
miRNA166k	GGCTCGGACCAGGCTTCA
miRNA167a	GGCTGAAGCTGCCAGCAT
miRNA168a	GCGTCGCTTGGTGCAGAT
miRNA169g	GGCUAGCCAAGGAUGACUUGCCUA
miRNA171a	GGTGATTGAGCCGCGCC
miRNA172a	GGCCGGGAGAATCTTGATGATG
miRNA390	AAGCUCAGGAGGGAUAGCGCC
miRNA393	GGCGTCCAAAGGGATCGCA
miRNA393b	GGCGTCCAAAGGGATCGCAT
miRNA395a	GGU GAA GUG CUU GGG GGA ACU C
miRNA396e	GGCCGTCCACAGGCTTTCTT
miRNA397a	GGCCTCATTGAGTGCAGCG
miRNA398a	GGCGGTGTGTTCTCAGGTCA
miRNA398b	GGCGGTGTGTTCTCAGGTCG
miRNA399d	UGCCAAAGGAGAGUUGCCCUG
miRNA408	CCGCUGCACUGCCUCUUC
miRNA413	GGCCCUAGUUUCACUUGUUCUGCAC
miRNA414	GGCCUCAUCCUCAUCAUCAUCGUCC
miRNA415	GGCCGCAACAGAACAGAAGCA
miRNA416	GGCCTGTTCGTCCGTACAC
miRNA417	GGGCCCGGAATGTAGTGAATTT
miRNA418	GGCCGGUAAUGUGAUGAUGAAAUGACG
miRNA419	GGCCGTGATGAATGCTGACG
miRNA420	GCGCGCTAAATTAATCACGGA
miRNA426	GCGCGGTTTTGGAAGTTTGTC
miRNA435	GGCCGUUAUCCGGUAUUGGAGUUGA
miRNA437	GGGCCGGAAAGTTAGAGAAGTT
miRNA439	GGTGTCGAACCGCGGTT
miRNA440	GGCAGTGTCTCCTGATGATCG
miRNA441a	GGCCGGGTACCATCAATATAAATGT
miRNA442	GGCGUGACGUGUAAAUUGCGAG
miRNA443	GGCGGATCACAATACAATAAA
miRNA444	UUGCUGCCUCAAGCUUGCUGC
miRNA445	GGCCGGUAAAUUAGUGUAUAAACAUCCGAU
miRNA446	GGCCGCAUCAAUAUGAAUAUGGGAAAUGG
miRNA528	CCTGGAAGGGGCATGCA
miRNA530	GGCAGGTGCAGAGGCAGA
miRNA531	CTCGCCGGGGCTGCGTGC
miRNA535	GGCCCTGACAACGAGAGAGA
miRNA806	AUGUGCUAAAAAGUCAACGGUG
miRNA807a	GGCCGTCATCTCACAGGT
miRNA808	GGCCGGATGAATGTGGGAAATG
miRNA809a	GGCCGGTGAATGTGAGAAATGT
miRNA811a	GGCCACCGTTAGATCGAGAAAT
miRNA812	GGCGACGGACGGTTAAACG
miRNA813	GGCGGGTTATGGAATGGGT
miRNA814a	GGCCGGCACTTCATAGTACAAC
miRNA815a	GGCCAAGGGGATTGAGGAG
miRNA817	GGCTCCAACTTGAGGCCC
miRNA818a	GGCCGGCCAATCCCTTATATTATG
miRNA819b	GGCCGUCAGGUUAUAAGACUUUCUAGC
miRNA820a	GCGGCCTCGTGGATGG
miRNA821a	GGCCGGGAAGTCATCAACAAAAAAG

MiRNAs recently identified *in silico* and by high-throughput methods were not included in this analysis (Zhang et al., 2010). The group composed of miRNA319, miRNA394, miRNA438,

miRNA529, and miRNA816 was also excluded from analysis, since no putative mRNA target was found in the rice genome using the miRU tool.

An initial kinetics of Al effect on miRNA expression was provided by measuring mature miRNA393 levels in Nipponbare rice roots after 4 and 8 h of incubation with 450 μM Al. miRNA393 was chosen because it has been identified in several plant systems and has been indubitably associated with plant responses to other abiotic stresses. The kinetics study was kept to a short 8-h time window to assure that direct effects of aluminum on miRNA expression would be evaluated, since long-term incubations could trigger secondary metabolic and structural effects that could also change miRNA levels. Analyses were focused on roots as they are the primary contact site with aluminum under natural field conditions. Also, roots are the organs where the toxic effects of Al are most obvious.

We found that miRNA393b expression was slightly down-regulated compared to control plants after 4 h of Al treatment. However, after 8 h a stronger down-regulation of miRNA393b was observed in response to Al in Nipponbare rice roots (Figure 1). Based on these results, the expression of all other miRNAs was evaluated after 8 h of Al treatment. We detected expression of 44 mature miRNAs. Among these, 13 were down-regulated and six were up-regulated in roots of rice seedlings treated with 450 μM Al for 8 h in a hydroponic culture under low pH (Figure 2). For the six up-regulated miRNAs, the level of expression varied from approximately 1.5-fold to more than 2.0-fold, that of miRNA528 being the highest (Figure 2). For the 13 down-regulated miRNAs, the level of expression was reduced approximately 50 to 95%. Compared to control plants, miRNA415 expression was the most reduced in rice roots treated with Al for 8 h (Figure 2). As far as we know, this is the first time that expression of miRNA415 and miRNA426 has been detected. Moreover, they were differentially expressed in rice roots treated with Al for 8 h (Figure 2).
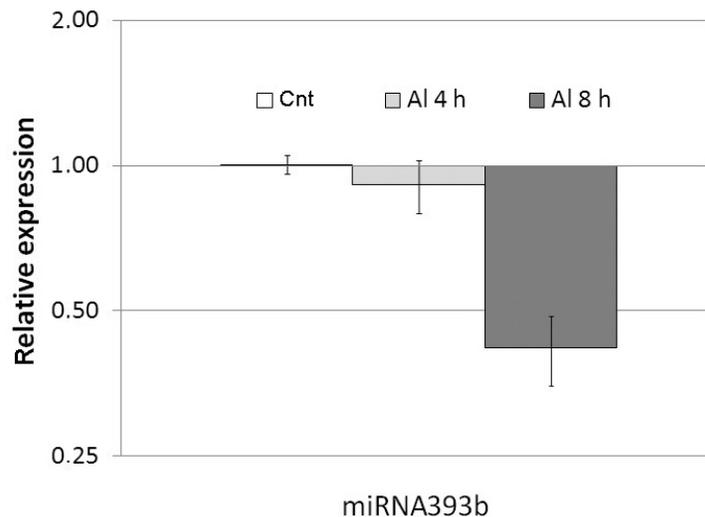

Figure 1. Quantification of mature miRNA393b in rice roots treated with aluminum (Al) for 4 and 8 h compared to control (Cnt). The histograms are means of three replicates. The values for the relative expression of miRNAs are expressed in logarithms of base 2. Expression of miRNA was normalized by comparison with expression of *Os18S* and *OssnU6* housekeeping RNAs.
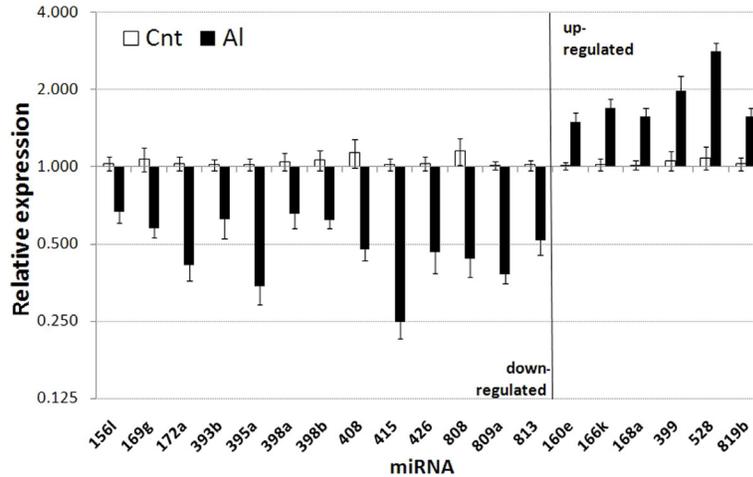
Figure 2. Comparative expression levels of miRNAs down- and up-regulated in Nipponbare rice roots after 8-h treatment with aluminum (Al) in respect to control (Cnt). The histograms are means of three replicates. The values for the relative expression of miRNAs are expressed in logarithms of base 2. Relative miRNA expression was normalized based on an average of five independent housekeeping genes and expressed on a logarithmic scale.

To compare microRNA expression pattern in rice roots of *indica* and *japonica* Nipponbare cultivars cultivated under high Al concentrations, we used Embrapa Taim, which is a Brazilian rice cultivar planted in waterlogged soils. For this analysis, we ran RT-qPCR only for the differentially expressed microRNAs found in the Nipponbare rice roots. We found that miRNA393b, miRNA395a, miRNA398a, miRNA398b, and miRNA408 were significantly down-regulated after 8 h of Al treatment (Figure 3). The expression of these miRNAs was
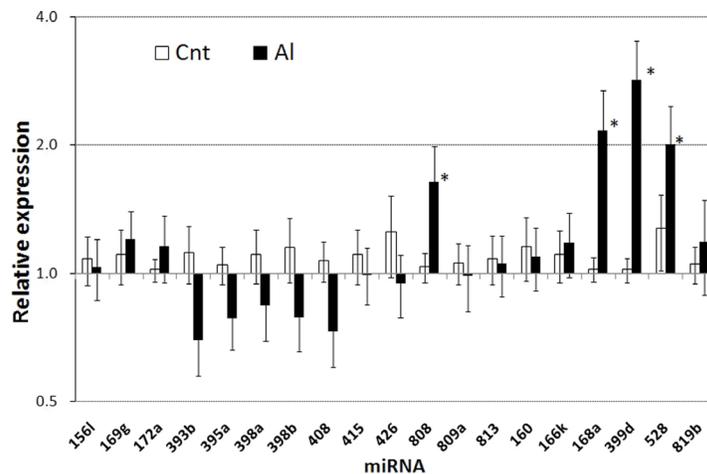

Figure 3. Comparative expression levels of miRNAs down- and up-regulated in Embrapa Taim rice roots after 8-h treatment with aluminum (Al) in respect to control (Cnt). The histograms are means of four replicates. The values for the relative expression of miRNAs are expressed in logarithms of base 2. Relative miRNA expression was normalized based on an average of three independent housekeeping genes and expressed on a logarithmic scale. Asterisks correspond to miRNA with different expression levels between Cnt and Al ($P < 0.05$).

reduced by 40%. Only miRNA168a, miRNA399d, and miRNA528 were significantly up-regulated, approximately 2.0-fold after 8 h of Al treatment (Figure 3). To confirm the inverse correlation between the targets and the expression of the miRNAs, we ran an RT-qPCR for three miRNA528 targets; a slight down-regulation was observed for two of the targets (Figure 4 and Table 2).
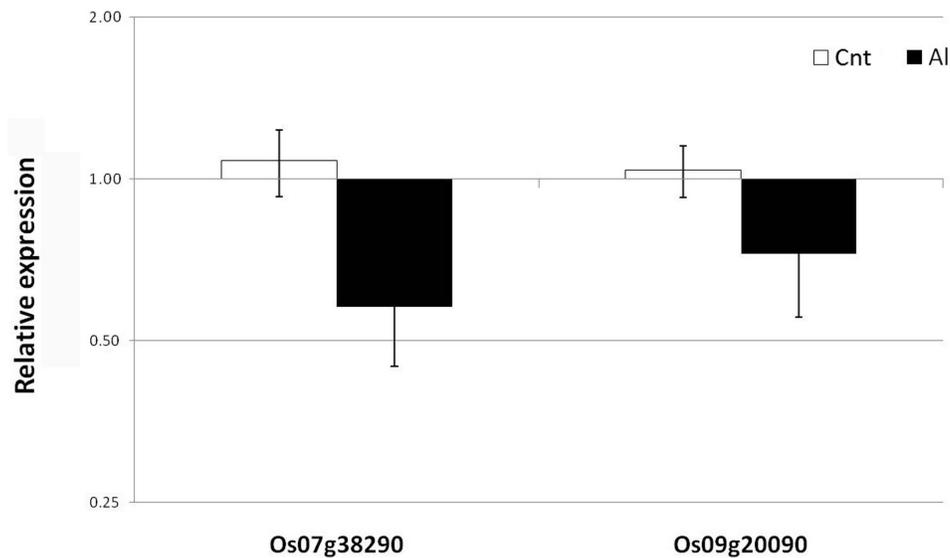

Figure 4. Comparative expression levels of miRNA528 targets in Embrapa Taim rice roots after 8-h treatment with aluminum (Al) in respect to control (Cnt). The histograms are means of four replicates. The values for the relative expression of miRNA targets are expressed in logarithms of base 2. Relative miRNA target expression was normalized based on an average of two independent housekeeping genes and expressed on a logarithmic scale.

Table 2. List of primers used for the target genes in rice roots.

Locus	Forward	Reverse
Os07g38290	GCCACCACTACTTCCTCTGC	GACGGAGAGCACGGACAC
Os09g20090	TAACAAGCTCTGCAGCCTCA	CGACGTGAACTGGTAGCTGA

Identification of putative targets for rice miRNAs differentially expressed in response to Al treatment may contribute to our understanding of the involvement of post-transcriptional gene regulation in plant aluminum tolerance. In order to reduce the number of false-positive targets, we adopted a limit score of 3.0 for mismatches between the miRNA and its putative target and a limit score of 3.0 for the kind of base pair. Using the miRU tool to search for miRNA targets, we found at least one putative gene target for each miRNA (Tables 3 and 4). There are several genes involved in various cellular processes, like cell wall formation, stress responses, auxin signaling, and secondary metabolites. Recently, another study, confirmed by degradome sequencing, the cleavage of several rice miRNA targets, including some that we identified as putative ones in this report (Li et al., 2010).

Table 3. Aluminum up-regulated microRNAs in rice roots and their putative targets.

miRNA	miRNA (seq 5'-3')	Target loci	Score	Mm	Target function	Orthologs
528*	UGGAAGGGGCAUGCAGAGGAG	Os06g06050	1.5	3	F-box/LRR-repeat MAX2	NI
		Os07g38290	2	1	Copper ion binding protein	
		Os04g39120	2	3	Copper amine oxidase-like	
		Os06g37150	2.5	2	L-ascorbate oxidase	
		Os01g62600	2.5	3	Laccase	
		Os09g20090	2.5	3	L-ascorbate oxidase	
		Os08g44770	2.5	4	Superoxide dismutase	
		Os01g44330	2.5	5	L-ascorbate oxidase	
160e**	UGCCUGGCUCCCUGUAUGCCG	Os02g41800	0	1	Auxin response factor 16	zma, sbi, ppt, ptc, vvi
		Os04g43910	0	1	Auxin response factor 16	
		Os06g47150	0	1	Auxin response factor 16	
166k,l**	UCGGACCAGGCUUCAAUCCCU	Os03g01890	2.5	3	Rolled leaf 1	zma, ppt, ptc
		Os03g43930	2.5	3	Class III HD-Zip protein 4	
		Os10g33960	2.5	3	HB1	
		Os12g41860	2.5	3	Class III HD-Zip protein 4	
168a**	UCGCUUGGUGCAGAUCGGGAC	Os11g07160	2.5	4	Receptor-like protein kinase 5	ath, sbi, zma, ptc, mtr
		Os10g08022	2.5	5	Fructose-bisphosphate aldolase	
399d**	UGCCAAAGGAGAGUUGCCCUG	Os08g45000	0.5	2	Inorganic phosphate transporter 1-7	ath, sbi, zma, ptc, mtr
		Os05g48390	1	2	Ubiquitin conjugating enzyme	
819a,b, c,d,e,f,g,h,i,j,k**	UCAGGUUAUAAAGACUUUCUAGC	Os03g31180	0	0	Diacylglycerol kinase 1	NI
		Os12g02520	0	0	O-glycosyl hydrolase	
		Os09g39910	2	3	ABC protein sub-family F	
		Os06g43660	2.5	3	PPi-vacuolar membrane proton pump	
		Os11g41860	2.5	3	Ubiquitin-protein ligase	

ath = *Arabidopsis thaliana*; zma = *Zea mays*; sbi = *Sorgum bicolor*; ppt = *Populus trichocarpa*; ptc = *Phycomitrella patens*; vvi = *Vitis vinifera*; mtr = *Medicago truncatula*; NI = non-identified. *These miRNAs have only one copy in the rice genome. **Different letters for the same miRNA indicate that they have the same mature sequence and one letter indicates that the miRNA mature sequence is different from that of the other members of the family.

Table 4. MicroRNAs down-regulated in rice roots by aluminum and their putative targets.

miRNA	miRNA (seq 5'-3')	Target loci	Score	Mm	Target function	Orthologs
miRNA156l**	cGACAGAAGAGAGUGAGCAUa	Os01g69830	1.5	3	Teosinte glume architecture 1	NI
		Os08g39890	1.5	3	Squamosa promoter-binding-like protein 9	
miRNA169f,g**	UAGCCAAGGAUGACUUGCCUA	Os02g53620	1	2	Nuclear transcription factor Y subunit A-3	ath, sbi, zma, ptc, bna, vvi
		Os03g44540	1.5	3	Nuclear transcription factor Y subunit A-10	
miRNA172a,d**	AGAAUCUUGAUGAUGCUGCAU	Os03g60430	0.5	2	Floral homeotic protein	ath, zma, sbi gma, ptc, vvi
		Os05g03040	0.5	2	Floral homeotic protein APETALA2	
miRNA393b**	UCCAAAGGGAUCGCAUUGAUCU	Os04g32460	1.5	4	Transport inhibitor response 1 protein	ath, ptc, vvi
		Os05g05800	1.5	4	Transport inhibitor response 1 protein	
miRNA395a**	GUGAAGUGCUUGGGGGAACUC	Os03g53230	0.5	2	3-Padenosine 5-phosphosulfate synthetase	ath, sbi, mtr, zma, ptc, vvi
		Os03g09940	1	2	Low affinity sulfate transporter 3	
		Os03g09930	2	3	Sulfate transporter 2.1	
miRNA398a**	UGUGUUCUCAGGUCACCCCUU	Os07g46990	1.5	3	Superoxide dismutase 2	ath, gma, ptc, vvi
miRNA398b**	UGUGUUCUCAGGUCGCCCCUg	Os07g46990	2	3	Superoxide dismutase 2	ath, gma, ptc
miRNA408*	CUGCACUGCCUCUUCCCUGGC	Os03g15340	0	1	Chemocyanin precursor	ath, sof, zma, ptc, ppt, pta, smo, tae, vvi
miRNA415*	AACAGAACAGAAGCAGAGCAG	Os12g42280	0	0	Viviparous-14	ath
		Os02g18320	1.5	3	Brassinosteroid insensitive 1	
miRNA426*	UUUUGGAAGUUUGUCCUUACG	Os02g17940	3	5	Leucoanthocyanidin dioxygenase	ath
miRNA808*	AUGAAUGUGGGAAAUGUAAGAA	Os08g36840	1	2	Glycoprotein 3-alpha-L-flucosyltransferase A	NI
miRNA809a,b,d, e,f,g,h***	UGAAUGUGAGAAAUGUUAGAAU	Os09g34250	0	1	Indole-3-acetate beta-glucosyltransferase	NI
		Os01g34620	0.5	1	OsGrx_S15.1 - glutaredoxin subgroup II	
miRNA813*	GGGUUAUGGAAUGGGUUUUACC	Os02g49720	2.5	4	Aldehyde dehydrogenase	NI

ath = *Arabidopsis thaliana*; gma = *Glycine max*; zma = *Zea mays*; sbi = *Sorgum bicolor*; ppt = *Populus trichocarpa*; ptc = *Phycomitrella patens*; vvi = *Vitis vinifera*; sof = *Saccharum officinarum*; mtr = *Medicago truncatula*; bna = *Brassica napus*; pta = *Pinus taeda*; smo = *Selginella moellendorffi*; tae = *Triticum aestivum*; NI = non-identified. *These miRNAs have only one copy in the rice genome. **Different letters for the same miRNA indicate that they have the same mature sequence and one letter indicates that the miRNA mature sequence is different from the other members of the family.

DISCUSSION

Root growth responses

Auxin is an important hormone involved in root development. The first symptom of Al stress in rice plants is root growth inhibition (Kikui et al., 2005). It has also been suggested that Al inhibits the basipetal transport of auxin in maize roots because exogenous applications of indole-3-acetic acid reverted this inhibition. In the *japonica* Nipponbare cultivar, miRNA393b down-regulated the TIR1 gene (Navarro et al., 2006). In *Arabidopsis*, TIR1 is an auxin-binding protein and mediates auxin signaling during lateral root formation by participating in protein degradation via SCF complexes (Xie et al., 2000). On the other hand, miRNA160e was up-regulated by Al treatment. Since miRNA160e regulates auxin response factors, it may be playing a counter-balancing role through the inverse regulation of miRNA393b in the auxin-signaling pathway in response to Al. miRNA528, which was found to be up-regulated in our experiment, has as a potential target an F-box/LRR repeat MAX2 gene. This gene encodes a protein that participates in an SCF complex that is involved in the regulation of shoot branching in *Arabidopsis* (Stirnberg et al., 2007). Also, it was suggested recently that another miRNA528 target, L-ascorbate oxidase, might have a role in regulating cell division during early developmental stages in rice seeds (Xue et al., 2009). MiRNA166k was also up-regulated. Our *in silico* analysis showed that this miRNA regulates genes from the III HD-ZIP family. In *Arabidopsis*, class III HD-ZIP genes are related to lateral root formation. It was also shown that miRNA166 is involved in the regulation of class III HD-ZIP genes in shoot apical meristem formation in *Arabidopsis* and in shoot meristem initiation in rice (Nagasaki et al., 2007). Hence, we suggest that up-regulation of miRNA528, miRNA160e, and miRNA166k, and down-regulation of miRNA393b could be contributing to fine control of root responses in rice plants in response to high concentrations of Al. Also, aluminum-induced stress can generate ROS, causing oxidized proteins to be degraded. Since miRNAs 528, 160e and 393b could be involved in the regulation of genes important in degradation pathways, these miRNAs may also play a role in protein quality control via the proteasome in response to Al stress.

The pectin methyl esters in cell wall polysaccharides have been found to be specifically involved in the exclusion of Al from the root apex. The fucosyl transferase gene is associated with the biosynthesis of cell wall sugars (Vanzin et al., 2002) and was identified as the primary target of miRNA808 (Table 4). The down-regulation of miRNA808 on Al-treated roots, associated with up-regulation of the fucosyl transferase gene, could cause structural modifications in the cells of rice plants (Figure 2).

Metabolism of nutrients and carbon

Mineral nutrients are essential for plant growth, and mineral deficiency can inhibit growth and damage the plants. Plants have evolved complex molecular mechanisms for cellular uptake, movement and metabolism of minerals (Grotz and Guerinot, 2002). In *Arabidopsis*, microRNAs such as miRNA399 and miRNA395 play a role in plant nutrient metabolism (Liang et al., 2010). MiRNA399d was up-regulated in our experiments. It regulates the PHO2 protein (UBC) that is involved in phosphate homeostasis in *Arabidopsis*. Northern blot and RT-qPCR analyses revealed elevated expression of members of the miRNA399 family, especially miRNA399d, in rice plants

grown without inorganic phosphate (Bari et al., 2006). Because the primary symptom of Al stress is root growth impairment, causing inhibition of water and mineral uptake, accumulation of miRNA399d in rice roots treated with Al could be the reason for a lack of mineral uptake by the roots.

A proteomic approach in roots of rice seedlings grown under high Al and low phosphorus concentrations indicated alterations in carbon and nucleotide metabolism (Fukuda et al., 2007). These authors found that the gene encoding fructose bisphosphate aldolase, which is involved in carbon metabolism, was down-regulated 6 h after Al treatment. In our study, miRNA168a was up-regulated after 8 h of Al treatment. This suggests that miRNA168a could regulate the FBPA structural gene, its putative target.

MiRNA395 is reported to be a regulator of a low-affinity sulfate transporter and of ATP sulfurylases, key proteins in sulfur homeostasis (Liang et al., 2010). In roots of rice seedlings, expression of genes related to sulfur metabolism, including ATP sulfurylases, are up-regulated in response to Al (Yang et al., 2007). This response is possibly connected to down-regulation of miRNA395, since our analysis showed that miRNA395 expression was reduced after 8 h of Al treatment (Figures 2 and 3).

Reactive oxygen species detoxification

ROS are reactive radicals produced in aerobic cellular processes and can cause severe damage to cells. Superoxide dismutases (SOD) are important enzymes involved in detoxifying superoxide radicals (O_2^-), catalyzing their dismutation to hydrogen peroxide and O_2. MiRNA398a and miRNA398b were down-regulated in response to Al treatment. It has already been shown that this family of microRNAs is involved in the regulation of SOD genes in *Arabidopsis* plants cultivated under high iron and copper concentrations. Under these stress conditions, increased expression of *AtSOD1* and *AtSOD2* genes is in contrast to the down-regulation of miRNA398. In rice, increased activity of SOD in seedlings treated with high Al concentrations was also found (Sharma and Dubey, 2007). Based on our results and those in the literature, miRNAs398a and b are involved in the regulation of SOD genes in plants under Al and other stress conditions.

Secondary messengers and metabolites

There is evidence that phosphatidic acid is an important lipid signaling molecule. In a suspension of cells of *Coffea arabica*, it was shown that Al blocks phosphatidic acid generation through the phospholipase C/diacylglycerol kinase route. We found up-regulation of miRNA819b, for which the diacylglycerol kinase gene is a putative target. We suggest that miRNA819b and Al have a synergistic effect in the phosphatidic acid pathway, or cellular responses positively regulate miRNA819b, which then down-regulates the diacylglycerol kinase gene.

It was found earlier that plantacyanins respond to abiotic stress in chickpea plants and subsequently that miRNA408 targets plantacyanins (Abdel-Ghany and Pilon, 2008). Based in our results, plus those in the literature, down-regulation of miRNA408 could also be playing a role in the response to Al stress in rice roots (Figures 2 and 3).

Transcription factors

It was recently reported that the plant nuclear factor Y has a crucial role in response to

abiotic stress in maize, *Arabidopsis*, and rice (Zhao et al., 2007, 2009). Our analysis revealed that miRNA169g putatively targets genes from the nuclear factor Y family. Since miRNA169g is down-regulated in rice roots under Al treatment, this microRNA might have a role in increasing Y protein in rice roots under Al stress.

MiRNA156l and miRNA172a are down-regulated in rice roots under Al treatment (Figures 2 and 5). In *Arabidopsis*, miRNA172 was characterized as an APETALA repressor gene in floral development. More detailed studies revealed that miRNA172 and miRNA156 play critical roles during plant development (Zhu and Helliwell, 2011).
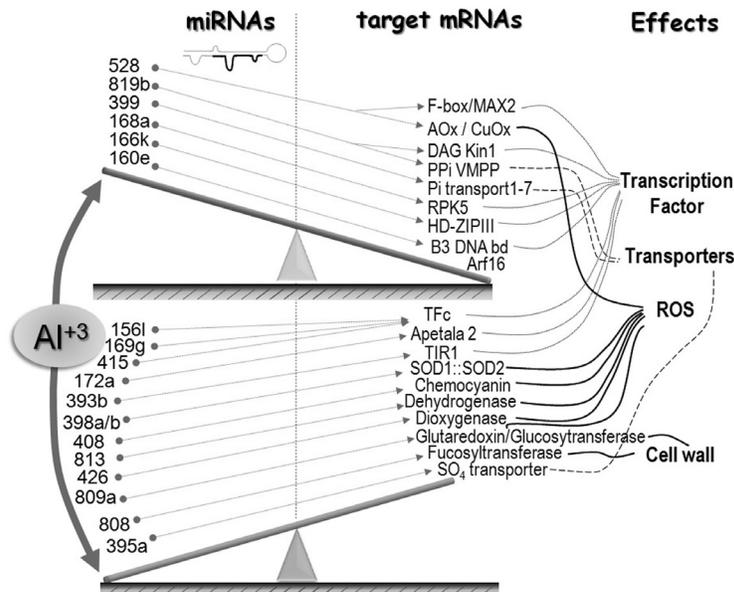

Figure 5. Diagram of the differentially expressed miRNAs in rice roots treated with aluminum (Al) and their putative targets. ROS = reactive oxygen species.

MiRNA responses to Al in Embrapa Taim

Our results showed that for the *indica* cultivar, only 5 miRNAs were down-regulated compared to 13 found to be down-regulated in the *japonica* cultivar. Only three miRNAs were significantly up-regulated in the *indica* cultivar comparing to the six up-regulated in the *japonica* cultivar. Among the seven miRNAs differentially regulated in response to Al in the *indica* cultivar (Figure 3), six have been characterized as important regulators of stress responses (Lu and Huang, 2008). MiRNA395, a sulfur homeostasis regulator, is up-regulated in maize roots of both tolerant and sensitive lines under salt stress (Ding et al., 2009). Our analysis showed that miRNA395 is down-regulated in both *indica* and *japonica* varieties (Figures 2 and 3). Also, miRNA168, miRNA528 and miRNA399 were up-regulated in both rice varieties; it is known that these miRNAs respond to abiotic stresses (Xue et al., 2009). This pattern of expression could be explained by activation of similar pathways in response to Al stress in rice plants from different genetic backgrounds. It is suggested that the slight

down-regulation of the miRNA528 target copper-ion gene, and especially of the L-ascorbate oxidase gene (Figure 4), results from the involvement of these proteins in the regulation of cell division (Xue et al., 2009). Since the primary symptom of sensitive plants treated with Al is root growth impairment, we suggest an initial arrest of cell divisions regulated by miRNA528 in rice roots. MiRNA408, which was also down-regulated in both rice varieties (Figure 2 and 3), is mediated by down-regulation of copper (Abdel-Ghany and Pilon, 2008). MiRNA393, which targets the gene that codes for the receptor of auxin TIR1, is undoubtedly important in the response to abiotic stresses in plants (Navarro et al., 2006). In the eudicot, *Medicago truncatula*, it was found that miRNA398 was down-regulated in response to Al, which is expected based on our results. MiRNA398 is known to be a regulator of genes that respond to abiotic stress in plants. A comparison between an Al-tolerant line and an Al-sensitive line in *M. truncatula* showed similar molecular responses related to genes involved in cell death, senescence, and cell wall degradation. Most Al-tolerant rice cultivars are derived from a *japonica* background, and Al-sensitive cultivars are derived from an *indica* background. Recently, it was found that several *japonica* cultivars with a different membrane lipid composition have a different tolerance response to toxic Al concentrations (Khan et al., 2009). This suggests that even in Al-tolerant rice cultivars, the responses are complex. Moreover, the different responses to Al among *indica* and *japonica* backgrounds are not yet well characterized.

Plant responses to abiotic stresses are quite complex and activate several mechanisms. Al is a special case in the study of plant-metal interaction, because Al toxicity in acidic soils worldwide can cause severe damage to important crops such as rice. On the other hand, the molecular mechanisms of plant-aluminum interaction are far from being fully elucidated. Our findings concerning miRNA differential expression in rice roots have revealed new components within the network of plant responses to abiotic stress caused by Al. In depth, sequencing of small RNA libraries from rice plants treated with Al will be useful to confirm and reveal new miRNAs and their targets in this complex plant-metal interaction.

ACKNOWLEDGMENTS

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