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Título: MicroRNAs como reguladores do desenvolvimento em plantas e o papel regulatório em raízes de arroz (*Oryza sativa* L.) na resposta ao alumínio.

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SUMÁRIO

Lista de Abreviaturas

µg – micrograma

ng - nanograma

µL – microlitro

5`RACE – Rapid Amplification of cDNA Ends (Amplificação e ou mapeamento de cDNAs na região terminal 5')

AA – aminoácidos

ABA – hormônio ácido abscísico

ABRE – "ABA responsive element" (elemento responsivo ao hormônio ácido abscísico - ABA)

ABREBP – "ABA responsive element binding protein" (proteína de ligação ao elemento responsivo ao hormônio ácido abscísico - ABA)

ABC – "ATP Binding Cassete" (proteína de ligação e transporte de ATP)

AGO 1 – proteína Argonauta 1 da família das RNase do Tipo III.

AGL16 – "Agamous Like 16" (gene da família "MADS Box" envolvido no desenvolvimento floral)

APETALA 1- fator de transcrição do tipo "MADS Box" envolvido no desenvolvimento floral

ARF (ARF6, ARF8, ARF10, ARF16, ARF17) – "auxin response factors" (fatores de transcrição responsivos a auxina).

At – Arabidopsis thaliana

ATP – "Adenosine triphosphate" (adenosina trifosfato)

AtALMT1 – Aluminum Activated Malate Transporter 1 gene (transportador de malato ativado por alumínio)

AtALS1 – Aluminum Sensitive 1 gene (gene cuja proteína está envolvida na resposta ao Alumínio)

AtALS3 - Aluminum Sensitive 3 gene (gene cuja proteína está envolvida na resposta ao Alumínio)

AtNF-YA – gene que codifica o fator de trancrição do tipo NF-YA.

BLAST – "Basic Local Alignment Search Tool" – ferramenta de procura de sequências

cDNA – DNA complementar

CDS – “Coding Sequence” (Sequência Codificante)

CCCH – domínio protéico contendo resíduos de cisteína (C) e histidina (H)

Cg1 – “corn grass 1” (mutante de milho que superexpressa o miR156)

CUC (*CUC1, CUC2*)– “cup shaped cotyledon” proteins (cotilédone em forma de copo ou taça) – estas proteínas são FTs.

CSRDB – Cereal Small RNA Database (Banco de dados de pequenos RNAs de cereais)

Dicer like I – proteína da família das RNase do Tipo III cuja função é reconhecer RNA dupla fita

DNA – “Desoxiribonucleic Acid” (Ácido Desoxirribonucléico)

DRE – “dehydration responsive element” (elemento responsivo a seca)

DREB – “dehydration responsive element binding protein” (proteína de ligação a elemento responsivo a seca)

EFs – “Elongation Factors” (Fatores de Elongação)

ER – “Endoplasmatic Reticulum” (Retículo Endoplasmático)

EROs - (espécies reativas de oxigênio)

EST – “Expressed Sequence Tag” (fragmento de sequência expressa)

FT – fator(es) de transcrição(cionais)

GAMYB – Giberelic Acid MYB (fatores de transcriçção do tipo MYB dependentes de ácido giberélico)

GRF – “Growth Regulating Factors” (fator de regulação do crescimento)

GUS – gene repórter que codifica para a enzima B-Glucoronidase

HEN 1 – Hua Enhancer 1 (metiltransferase)

HD-ZIPIII – fator de transcrição do tipo “Zipper de Leucina”

Hv – Hordeum vulgare (cevada)

HvAACT1- Aluminum Activated Citrate Transporter 1 (gene de cevada cuja proteína transporta citrato)

HYL 1 – “Hyponastic Leaves 1” (proteína envolvida na estabilização dos pre-miRNAs, cuja planta mutante tem folhas hiponásticas)

IPS – RNA “Induced by Phosphate Starvation” (RNA não codificante que é induzido por deficiência de fosfato

Kg - quilograma

M – molar

MADS Box – a sigla origina-se de uma família bastante conservada de FT em diversos eucariotos. *M* significa "MCM1", *A* significa "Agamous", *D* significa "DEFICIENS" e S significa SRF (serum response factor – fator responsivo ao soro).

MAP Kinases – Mitogen activated Protein Kinases (Proteínas Quinases ativadas por mitógeno – substância elicitora de divisão celular)

miRBase – "MicroRNA Database" (base de dados de microRNAs)

mg - milgramas

mRNA – RNA mensageiro

miRNA – pequeno RNA de aproximadamente 21 nucleotídeos de comprimento

microRNA – sinônimo de miRNA

NAC – "no apical meristem" proteins (sem meristema apical) – estas proteínas são FTs.

NCBI – "National Center for Biotechnology Information" (Centro Nacional de Informação Biotecnológica)

NF-YA – "nuclear transcription factor Y subunit alpha" (subunidade alfa do fator de transcrição Y)

ºC – graus Celsius

Os – Oryza sativa

OsART1 – "Aluminum Resistance Transcription Factor 1 gene" (gene de arroz cuja proteína é um FT envolvido na resposta ao alumínio)

OsNRAT1 – "Nramp Aluminum Transporter 1 gene" (gene de arroz cuja proteína transporta alumínio)

OsSTAR1 – "Sensitive to Aluminum Ryzhotoxicity 1" (gene de arroz cuja proteína está envolvida na resposta ao alumínio)

OsSTAR2 – "Sensitive to Aluminum Ryzhotoxicity 2" (gene de arroz cuja proteína está envolvida na resposta ao alumínio)

PCR – "Polymerase Chain Reaction" (Reação em Cadeia de Polimerase)

PHO2 – proteína repressora de transportadores de fosfato

PHR1 – FT ativador do gene do miR399.

PMRD – "Plant MicroRNA Database" (Banco de dados de microRNA de plantas)

PmiRKB – Plant MicroRNA Knowledge Base (Base de Conhecimento de microRNAs de Plantas)

Pre-miRNA – " Precursor of microRNA" (RNA longo, precursor secundário do miRNA)

Pri-miRNA – "Primary precursor of microRNA" (RNA longo, precursor primário do miRNA)

QTL – "Quantitative trait Loci" (Locos envolvidos na herança e controle de características quantitavitas)

RISC – "RNA Interference Silencing Complex" (Complexo de silenciamento via RNA de interferência)

RDR6 – "RNA Dependent RNA Polimerase 6" (RNA polimerase dependente de RNA)

RNA – "Ribonucleic Acid" (Ácido Ribonucléico)

rRNA – RNA Ribossomal

RNA Pol II – RNA polimerase do Tipo II

RT-qPCR – Reverse Transcriptase quantitative Polimerase Chain Reaction (Reação da Polimerase em Cadeia para quantificação de DNA e RNA)

SbMATE1- gene de sorgo cuja proteína transporta citrato

SCR – "Scarecrow Like protein" (fator de transcrição do tipo "Scarecrow like")

SE – Serrate (proteína envolvida na estabilização dos pre-miRNAs, cuja planta mutante tem folhas serradas)

Self-reg – "Self regulation" (auto regulação)

SERK – "Somatic Embryogensis Receptor Kinase" (Proteína quinase receptora)

SGS3 – "Supressor of Gene Silencing 3" (Supressor de Silenciamento gênico – proteína envolvida na biogênese de pequenos RNAs)

SHR – "Short Root protein" (raiz curta – refere-se ao fenótipo do mutante) – esta proteína é um FT.

siRNAs – "small interfering RNAs" (pequenos RNAs de 24 nucleotídeos de comprimento)

SNF1 tipo quinase 2 –

SNP – Single Nucleotide Polymorphism (Polimorfismo de nucleotídeo único)

SOD1 e SOD2 – proteínas superóxido dismutases 1 e 2.

SPL – "Squamosa Promoter Binding like protein" (fator de transcrição do tipo "SBP Box")

Ta-siRNAs – "*Trans* acting small interefering RNAs" (pequenos RNAs que atuam em *trans*)

Ta – Triticum aestivum (trigo cultivado)

TaALMT1- gene de trigo cuja proteína transporta malato

TaMATE1- gene de trigo ortólogo do gene SbMATE 1 de sorgo cuja proteína transporta citrato

TAS – RNAs longos não codificantes envolvidos na formação de siRNAs.

TCPs – Teosinte Branched/ Cycloidea/ PCF – fatores de transcrição envolvidos na síntese de ácido jasmônico.

TIGR – "The Institute for Genomics Research" (Instituto para Pesquisa Genômica)

TOE 1 – Target of Eat 1 (fator de trancrição)

TOE 2 – Target of Eat 2 (fator de trancrição)

TZF1- Tandem Zinc Finger proteins (fator de transcrição do tipo "dedo de zinco")

UV-B - luz ultra violeta do tipo B

WRKY – fator de transcrição bastante conservado em plantas contendo o domínio WRKYGQK (W = Triptofano; R = Arginina; K = Lisina; Y = Tirosina; G = glicina; Q = Glutamina; K = Lisina).

Zm – Zea mays (milho)

ZmMATE1 – gene de milho ortólogo do gene *SbMATE 1* de sorgo cuja proteína transporta citrato

ZmTSH4 – "tassel sheath 4 gene" (gene de milho cuja proteína contém o domínio do tipo SBP – "Squamosa promoter binding like")

RESUMO

Diversos trabalhos demonstram que a resposta das plantas ao alumínio é complexa. Porém, nenhum trabalho caracterizou o envolvimento de microRNAs nesta resposta. Parte deste trabalho visou caracterizar o perfil de expressão de diferentes famílias de microRNAs em resposta ao alumínio comparando-se as raízes de plantas de arroz *japonica* e *indica*. De um total de dezesseis microRNAs diferencialmente expressos, treze microRNAs tiveram a expressão reduzida e outros seis tiveram a expressão aumentada nas raízes de plantas de arroz j*aponica* tratadas com 450 μM de $AlCl_3$ após 8h. Nas plantas de arroz *indica* tratadas nas mesmas condições, nove microRNAs foram detectados como diferencialmente expressos. Destes, quatro tiveram a expressão aumentada e os outros cinco tiveram a expressão reduzida. Por RT-qPCR foram confirmados dois alvos do miR528. Um dos alvos, o gene L-Ascorbato Oxidase está relacionado com a regulação da divisão celular. Sugere-se que a regulação pelo miR528 pode estar de acordo com a inibição do desenvolvimento das raízes em resposta ao alumínio em plantas sensíveis. Estes resultados demonstram que a resposta dos microRNAs ao tratamento com alumínio também é complexa, pois vários microRNAs, que provavelmente regulam alvos distintos tiveram sua expressão modulada após o tratamento das plantas.

Já foi demonstrado que o desenvolvimento de raízes laterais sofre regulação por microRNAs em Arabidopsis. A outra parte deste trabalho visou caracterizar funcionalmente o miR164 e a expressão espacial de diferentes membros da família miR164 em raízes de plantas de arroz. Plantas superexpressando o miR164 apresentaram as raízes laterais reduzidas em comparação com as plantas não transformadas. Interessantemente, a análise por RT-qPCR de dois alvos do miR164 revelaram resultados inversos. A análise das plantas contendo os promotores de três genes da família miR164 fusionados ao gene repórter GUS revelou uma sobreposição da expressão espacial no órgão. Os microRNAs miR164a e miR164d localizam-se nas raízes laterais, e o miR164f está localizado nas raízes laterais e também na raiz primária. Porém, cortes transversais das raízes demonstraram que os miR164a e o miR164f localizam-se na endoderme e no estelo, respectivamente. Uma análise *in silico* das seqüências dos promotores dos seis membros da família miR164 e de seus respectivos alvos revelou a presença de motivos de DNA provavelmente responsivos a fatores de transcrição envolvidos no desenvolvimento de

meristemas primários. Com base nestes resultados, sugere-se que os microRNAs miR164a e miR164f devem estar regulando seus alvos em diferentes tecidos da raiz. Este resultado está de acordo com o desenvolvimento inicial das raízes laterais, pois estas se originam do periciclo componente do estelo.

Os microRNAs têm função crucial ao longo do desenvolvimento e em resposta a estresses abióticos. O papel regulatório dos microRNAs reside na complexidade e diversidade das respostas a estresses abióticos e ao desenvolvimento, visto que diversos microRNAs, que provavelmente regulam distintos alvos, estão envolvidos em redes complexas na regulação da expressão gênica.

ABSTRACT

Previous works showed that the response to aluminum (Al) in plants is complex. However, at present, there is no data regarding microRNA expression in this response. Part of this work aimed to characterize the expression profile of different families of microRNAs in response to Al comparing roots of *japonica* and *indica* roots. From sixteen microRNAs differentially expressed, thirteen were down-regulated and six were upregulated in *japonica* rice roots treated with 450 μM of AlCl3 after 8h. For the *indica* rice roots under the same conditions, nine microRNAs were differentially expressed. From these microRNAs, four were up-regulated and five were down-regulated. Two miR528 targets were confirmed by RT-qPCR. One of the targets is the L-AScorbate oxidase gene, which regulate cell divisions. These results help explaining rice root inhibition under Al teatment in sensitive plants. Our results suggest that microRNA response to Al is also complex, because several microRNAs that had their expression modulated probably regulate distinct target genes.

It is known that lateral root development is regulated by microRNAs in Arabidopsis. The other part of this work was to characterize the miR164 function and the spatial expression of different members of the miR164 family in rice roots. Plantas overexpressing the miR164 had less lateral roots when compared to the non-transformed plants. Interestingly, the expression by RT-qPCR of two miR164 target was inverse in the miR164 overexpressing plants. The spatial expression analysis of different members of the miR164 family revealed overlapping domains. MicroRNAs miR164a and miR164d localized in the lateral roots, and miR164 is localized in lateral roots and also in primary roots. However, hand-sectioning of the miR164a and miR164f GUS fusion plants demonstrate that miR164a is expressed in the endodermis and miR164f is expressed in the stele. An *in silico* analysis of promoter sequences of all miR164 genes revealed the presence of DNA motifs probably responsive to transcription factors involved in the development of primary meristems. Base on these results, we suggest that different members of the miR164 family are regulating their targets in different tissues of rice roots. These results are in agreement with the initial development of lateral roots originating from the pericycle cells. The key of

the regulatory role of microRNAs in response to abiotic stresses and during development brought complexity to the regulatory networks of gene expression.

CAPÍTULO I: Introdução Geral

INTRODUÇÃO

A ESPÉCIE *ORYZA SATIVA* E O ARROZ CULTIVADO

O gênero *Oryza* está classificado na tribo Oryzeae, subfamília Oryzoideae, família Poaceae (Graminae). Este gênero possui duas espécies cultivadas, *O. sativa*, cultivada no mundo todo e *O. glaberrima*, cultivada em alguns países da África Ocidental, e mais de 20 espécies silvestres, distribuídas nas regiões tropical e subtropical. O arroz mais cultivado em todo mundo, domesticado e melhorado a partir da espécie *O. sativa*, tem origem no continente Asiático. Possui genoma diplóide do tipo AA totalmente seqüenciado e disponível à comunidade científica (IRGSP 2005). A espécie *Oryza sativa* é classificada em duas subespécies, *indica* e *japonica*. Estas subespécies foram domesticadas provavelmente na Índia e China, respectivamente (Khush 1997). É importante ressaltar que o arroz é parte da dieta básica de aproximadamente metade da população mundial. Isto significa um mercado consumidor gigantesco de aproximadamente 3 bilhões de pessoas (http://www.rice-trade.com/articles/rice-production.html). Portanto, a pesquisa científica se justifica pelo alto impacto econômico e social desta cultura.

O Brasil figura entre os dez países onde mais se produz arroz (Tabela 1), sendo a China e a Índia os maiores produtores.

Tabela 1. Os dez países que mais produzem de arroz.

País	Toneladas (milhões)
China	166.417.000
Índia	132.013.000
Indonésia	52.078.832
Bangladesh	38.060.000
Vietnã	34.518.600

Tabela 1. Continuação

País	Toneladas (milhões)
Tailândia	27.000.000
Mianmar	24.640.000
Filipinas	14.031.000
Brasil	10.198.900
Japão	9.740.000

Fonte: www.rice-trade.com

A escolha do arroz cultivado como modelo de estudos em genética e biologia molecular para as plantas monocotiledôneas reside nos seguintes fatores: menor tamanho de genoma quando comparado a outras espécies importantes como trigo, cana-de açúcar e milho; genoma totalmente seqüenciado; metodologias de transformação genética muito bem estabelecidas; boa capacidade de regeneração *in vitro*.

As subespécies *indica* e *japonica*, cuja importância econômica e social são fatos, apresentam características específicas quanto a anatomia. Isto torna relevante a condução de trabalhos que busquem diferenciar as duas subespécies quanto a aspectos morfológicos, anatômicos, e de biologia molecular envolvendo a fisiologia, bioquímica e genética. Apesar de não ser objeto desta tese, a literatura científica relata inúmeros trabalhos que tratam da caracterização e diferenciação destes dois tipos de arroz cultivado (Sang and Ge 2007; Chen et al., 2010; Li et al., 2010b; Jahn et al., 2011).

ESTRATÉGIAS DAS PLANTAS NA RESPOSTA A ESTRESSES ABIÓTICOS

Além das plantas serem sésseis e possuírem um programa genético que esta na base de seu funcionamento ao longo de seus ciclos biológicos, a interação com inúmeros fatores bióticos (homem, vírus, bactérias, fungos) e abióticos (metais, sal, variação de temperatura, disponibilidade de água) torna-se inevitável. Interessantemente, todos estes fatores em

conjunto com o programa genético de cada planta co-evoluem de forma a afetar a resposta das plantas e o sucesso de seu desenvolvimento. São sugeridas três estratégias que as plantas utilizam para lidar com a variedade de ambientes: (1) uma estratégia mais especialista, cuja função pode determinar o aparecimento de espécies mais endêmicas e ou localmente adaptadas; (2) plantas com respostas mais generalistas, o que poderia ser vantajoso na dispersão de espécies para novos ambientes; (3) plantas com alta plasticidade ambiental, respondendo de acordo com o ambiente onde estão inseridas (Des Marais and Juenger 2010). As espécies dentro do gênero *Oryza* podem ser consideradas dentro da primeira e terceira categorias, pois ocupam ambientes extremos, desde completamente alagados até solos com pouca água.

Uma miríade de sinais e cascatas regulatórias (Figura 1) torna complexa a compreensão de como as respostas são iniciadas e como a planta responde de forma sensível ou tolerante ao fator indutor (Hirayama and Shinozaki 2010). A disponibilidade de genomas completos como o de *Arabidopsis*, arroz e outros, e a geração de centenas de dados moleculares a respeito das respostas das plantas a partir das sequências de DNA disponíveis (genes, sequências promotoras, outras sequências regulatórias), tornou possível uma compreensão mais global de como plantas diferentes respondem aos mesmos estímulos (Hirayama and Shinozaki 2010). Há vários exemplos na literatura de genes, seqüências regulatórias e proteínas chave na determinação dos mecanismos de resposta a diferentes estresses. Proteínas SERK contendo domínios transmembrana funcionam como receptoras de moléculas sinalizadoras de estresses bióticos (Santos and Aragao 2009). Proteínas presentes nas cascatas intracelulares (quinases, fosfatases, inúmeros fatores transcricionais (FT)) são responsáveis pela indução da resposta (Shinozaki et al., 2003; Lumba et al., 2010). Proteínas do tipo MAP quinases são fundamentais na transdução de sinais nas redes de resposta a estresses em plantas (Bartels et al., 2010). Outro exemplo são as proteínas denominadas SNF1 – tipo quinase 2 que podem ser ativadas por estresse osmótico e ou ácido abscísico (Boudsocq et al., 2004).

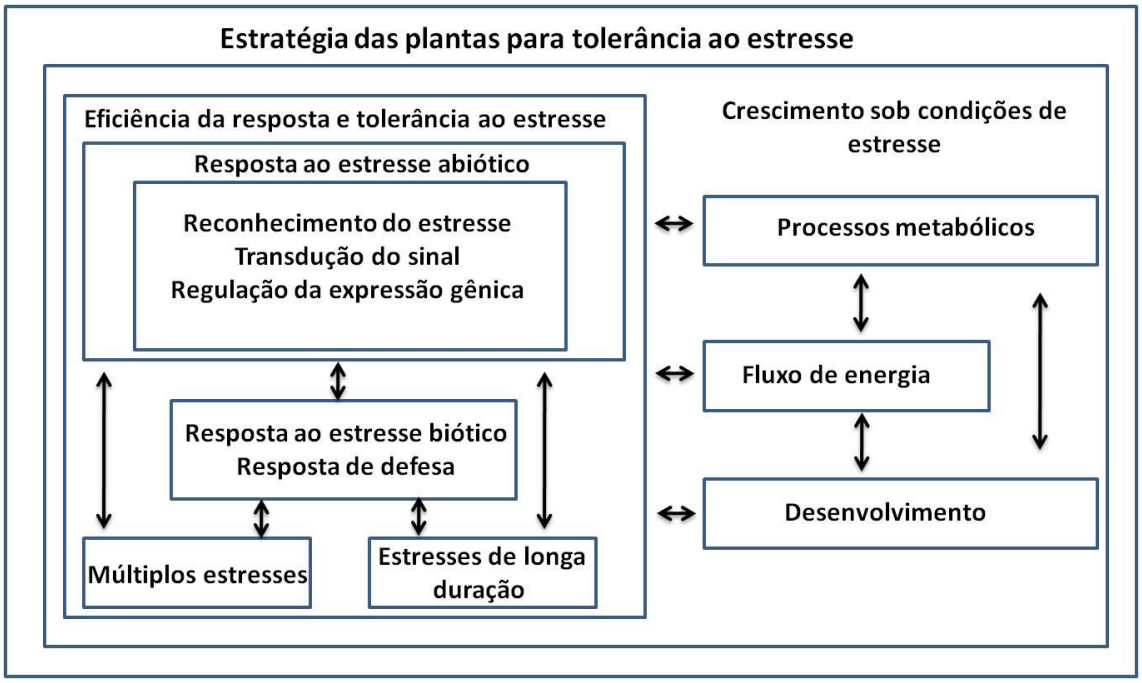

Figura 1. Estratégias das plantas nas respostas aos estresses. Primeiramente, a resposta aos estresses é percebida ou sinalizada na presença de um fator indutor, como variações de temperatura e íons presentes no solo. Estes sinais desencadeiam uma série de respostas, de curta e longa duração, que certamente afetam o crescimento e desenvolvimento ao longo do ciclo de vida das plantas. Modificado de Hirayama e Shinozaki (2010).

Motivos de DNA do tipo DRE (elemento responsivo `a desidratação) presentes nos promotores ocorrem em genes ativados por proteínas DREB (proteína de ligação a elemento responsivo a seca) em resposta a estresses por seca em plantas (Islam and Wang 2009; Zhang et al., 2009a; Zhang et al., 2009b). Sequências de DNA do tipo ABRE (elemento responsivo ao hormônio ácido abscísico - ABA) também são bastante comuns como elementos regulatórios em genes ativados por FT do tipo ABREBP (proteína de ligação ao elemento responsivo ao hormônio ácido abscísico - ABA) em resposta a estresses dependentes de ABA (Yamaguchi-Shinozaki and Shinozaki 2005; Hossain et al., 2010). Isto indica que existem respostas dependentes e independentes de ABA em plantas. Estudos recentes revelaram que a proteína TZF1, que possui domínios CCCH em série, deve desempenhar um papel de reguladora geral em resposta a hormônios e a estresses por seca e baixas temperaturas em plantas (Lin et al., 2011). O cálcio (Ca), importante

mensageiro celular, desempenha papel fundamental nas respostas diretas (enrijecimento da parede celular) e indiretas (transdução de sinal via proteínas quinases) em plantas (Boudsocq 2010). Superóxido dismutases, ascorbato peroxidases, glutationa peroxidases são proteínas com atividade na regulação, conversão e sequestro de EROs (espécies reativas de oxigênio) nas respostas a estresses oxidativos (Teixeira et al., 2006; Jagadeeswaran et al., 2009; Blokhina and Fagerstedt 2010). Além de ser ampla, genes da família dos FT WRKY são bastante estudados. Compreende-se que proteínas WRKY regulam, reprimindo ou ativando, diversos genes importantes em várias rotas de sinalização ao desenvolvimento e em resposta a estresses (Qiu et al., 2009; Rushton et al., 2010). Há sem dúvida um aparato complexo, porém organizado, que é ativado frente aos diversos estresses e também aos sinais ao longo do desenvolvimento das plantas.

A disponibilidade de íons no solo, especialmente íons metálicos (cádmio, ferro, cobre e alumínio), também é um fator de indução de diferentes respostas nas plantas. Em solos ácidos, o excesso de metais livres como o alumínio pode ocasionar prejuízos consideráveis à agricultura, pois plantas sensíveis têm seu crescimento inibido com conseqüências negativas a reprodução e produção de sementes. Uma revisão mais detalhada das respostas das plantas ao alumínio segue no item posterior.

AS RESPOSTAS DAS PLANTAS NA INTERAÇÃO COM ALUMÍNIO

O alumínio (Al) é o terceiro elemento químico mais abundante na face da terra, depois do oxigênio e do silício (Ma et al., 2001). Em solos ácidos, a solubilidade do Al aumenta e seu cátion trivalente (Al^{3+}) torna-se disponível às plantas. Um dos primeiros sintomas do contato do Al com as raízes é a inibição de seu crescimento. Porém, há também outras conseqüências para a planta na interação com alumínio, como inibir o desenvolvimento de raízes laterais, afetar o aporte de cátions importantes como Ca^{2+} e potássio (K^{+}), ocasionar estresses oxidativos, desorganizar o citoesqueleto e o transporte intercelular (Matsumoto 2000; Sivaguru et al., 2000; Yamamoto et al., 2002; Kochian et al., 2004). A síntese de calose (1-3-β glucana) é uma das consequências da ruptura na homeostase de Ca^{2+} nos tecidos das raízes (Delhaize and Ryan 1995). A deposição de

calose nos plasmodesmas interrompe a comunicação intercelular impedido o tráfego normal de moléculas (Panda et al., 2009). A formação de EROs danosas à célula têm origem na peroxidação lipídica de membranas pela interação com Al (Yamamoto et al., 2002). O citoesqueleto é base para os processos de expansão e divisão celular. Porém, a desestruturação do citoesqueleto pela interação do Al com a actina e os microtúbulos afeta a correta expansão e divisão celular (Panda et al., 2009). O dano ao DNA também já foi relatado como um dos fatores importantes na inibição do crescimento em plantas (Meriga et al., 2004). Vários trabalhos contendo dados de expressão gênica revelaram que genes importantes na biossíntese da parece celular, metabolismo oxidativo, FT e sinalização celular estão envolvidos nas respostas ao Al (Yang et al., 2007; Zhang et al., 2007; Zhao et al., 2009b; Duressa et al., 2010; Grisel et al., 2010).

Foram caracterizados dois tipos de mecanismos que as plantas utilizam para sobreviver em solos ácidos: (1) aqueles mecanismos mais relacionados `a resistência ao Al e (2) aqueles mecanismos relacionados `a tolerância do metal no meio intracelular (Ryan et al., 2011).

Os mecanismos em torno da liberação de ácidos orgânicos na rizosfera estão dentre os mais aceitos como sendo uma forma de resistência ao Al. Estes compostos se complexam com o Al livre impedindo danos à raiz (Delhaize et al., 2007). A clonagem do gene *TaALMT1* foi um dos primeiros indícios moleculares de que um transportador de malato codificado por este gene desempenha papel fundamental na resistência ao Al em trigo (Sasaki et al., 2004). Em *Arabidopsis* e centeio, as proteínas dos genes ortólogos do *TaALMT1* também desempenham função similar, transportando malato e formando complexos com Al não danosos à célula (Hoekenga et al., 2006; Collins et al., 2008). O citrato é outro tipo de ácido orgânico que têm papel na resistência ao Al em plantas. A proteína do gene *SbMATE1*, transportadora de citrato, é uma das principais fontes de resistência ao Al em sorgo (Magalhaes et al., 2007). O gene ortólogo *ZmMATE1* compõe um QTL envolvido na resposta de resistência da planta de milho ao Al (Maron et al., 2010). Interessantemente, em uma análise do transcriptoma de raízes de plantas de milho tolerante e sensível ao Al não foram encontrados genes de transporte de ácidos orgânicos diferencialmente expressos (Mattiello et al., 2010). A partir de evidências genéticas e fisiológicas, o gene *TaMATE1* de trigo é sugerido como parte de um segundo mecanismo

de resistência ao Al, envolvendo efluxo de citrato das raízes (Ryan et al., 2009). Em cevada, o gene *HvAACT1* que confere resistência ao Al é expresso constitutivamente nas raízes e sua proteína transporta especificamente citrato (Furukawa et al., 2007). Com base nestes estudos, sugere-se que proteínas transportadoras de malato e citrato sejam componentes cruciais na interação com Al, impedindo que sua forma livre provoque danos irreversíveis à planta. Porém, em plantas de arroz, estudos recentes demonstram que há outros mecanismos de resposta da planta na interação com Al.

Comparando-se plantas de arroz mais tolerantes com plantas mais sensíveis ao Al, sugeriu-se que a presença de metilações nos açúcares da parede celular são responsáveis por parte da resistência ao Al tóxico (Yang et al., 2008).Os genes *OsSTAR1* e *OsSTAR2* codificam uma proteína de ligação a nucleotídeo e uma proteína do tipo transportadora ABC, respectivamente. Como um mecanismo de resistência do arroz ao Al, foram sugeridas modificações de parede celular a partir da interação das proteínas STAR1 e STAR2 e conseqüente transporte de UDP-glicose (Huang et al., 2009). Dados de microarranjo demonstraram que a regulação dos genes *OsSTAR1* e *OsSTAR2* está sujeita à ativação do fator transcricional OsART1 em resposta ao Al em arroz. (Yamaji et al., 2009). O gene *OsART1* tem função crucial no mecanismo de tolerância ao Al em arroz, pois plantas mutantes são bastante sensíveis ao Al (Yamaji et al., 2009). Recentemente, foi identificado o gene *OsNRAT1,* regulado pela proteína ART1, que codifica uma proteína transportadora de Al envolvida no seu influxo para o vacúolo, o que sugere uma forma de acumular Al de modo seguro dentro da célula em plantas de arroz (Xia et al., 2010; Xia et al., 2011). A expressão do gene *AtSTAR1* de *Arabidopsis* não é modulada por Al. Porém, plantas *atstar1* transformadas com o gene *OsSTAR1* resgataram a tolerância ao Al, o que sugere uma função similar para o ortólogo *AtSTAR1* em plantas de *Arabidopsis* (Huang et al., 2010). Sugere-se que as proteínas do tipo transportadoras ABC em *Arabidopsis*, codificadas pelos genes *AtALS1* e *AtALS3* quelam Al e o redirecionam para o apoplasto e para o vácuolo, respectivamente (Larsen et al., 2005; Larsen et al., 2007). Uma análise do transcriptoma de raízes do mutante *stop1* em *Arabidopsis* revelou que este gene, que codifica um fator transcricional, regula outros genes em resposta ao Al como *AtALMT1* e *AtALS3* (Sawaki et al., 2009).

MIRNAS E REGULAÇÃO DA EXPRESSÃO GÊNICA

Os miRNAs são uma classe de pequenos RNAs de 21 nucleotídeos (nt) de comprimento que têm papel crucial na regulação da expressão gênica (Jones-Rhoades et al., 2006; Bartel 2007). Em estudos relacionados ao desenvolvimento, realizados com o nematóide modelo *Caenorhabditis elegans*, foi identificado o primeiro microRNA denominado *lin-4* (Lee et al., 1993).

Os diversos estudos envolvendo microRNAs tornaram evidente seu papel na regulação transcricional e pós-transcricional da expressão gênica. Na área humana e animal já se compreende que estes pequenos RNAs regulam genes importantes na proliferação celular, no desenvolvimento, na diferenciação, na plasticidade genotípica, nas respostas a estresses e na regulação de células tronco totipontentes (Jeanteur 2010; Leung and Sharp 2010; Shi et al., 2010; Yasuda 2010).

Postula-se que os genes dos miRNAs em plantas, evoluíram a partir da duplicação invertida de sequências dos alvos dos miRNAs. Outra hipótese discute que alguns genes mais recentes evolutivamente podem ter surgido ao acaso, pois não apresentaram similaridade alguma com outras sequências de seus genomas hospedeiro (Allen et al., 2004; Felippes et al., 2008).

Em plantas, a biogênese dos miRNAs (Figura 2) é um processo que inicia na transcrição dos genes de miRNAs por uma RNA polimerase Tipo II em uma molécula denominada pri-miRNA. Este RNA longo, que forma uma estrutura secundária, é reconhecido por uma RNase do Tipo III, Dicer Like I, que o processa em um RNA menor denominado pre-miRNA. Em conjunto com outras duas proteínas, HYL1 e SE, a Dicer Like I processa o pre-miRNA no "duplex" miRNA/miRNA*. Estes RNAs são reconhecidos pela proteína HEN1 e metilados. Uma das fitas do "duplex" é carregada pela proteína AGO1 juntamente com um complexo protéico denominado RISC (Complexo de silenciamento via RNA de interferência) que reconhecerá por complementação parcial do miRNA seus alvos regulatórios (Chen 2009). A regulação por microRNAs pode ocorrer pela clivagem e ou inibição da tradução dos RNAs alvos (Chen 2009).

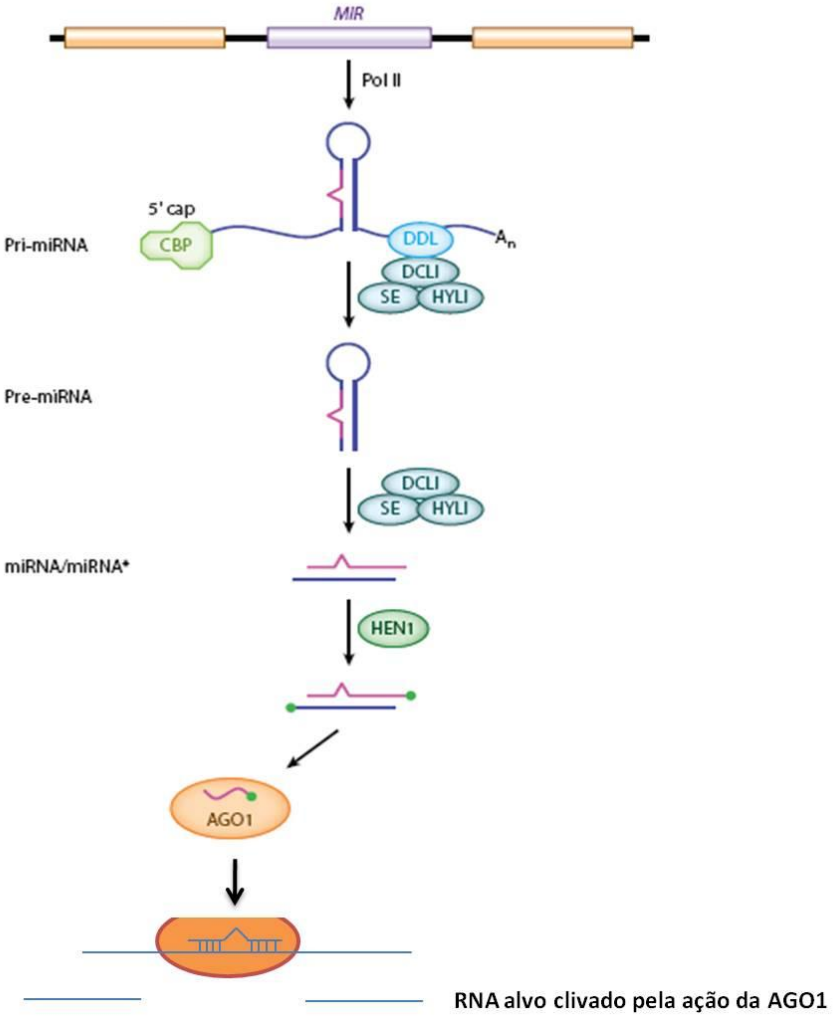

Figura 2. Biogênese dos miRNAs. O gene de miRNA é reconhecido e transcrito em um pri-miRNA pela RNA Pol II, poliadenilado e encapado. O pri-miRNA é processado em um pre-miRNA pela DCL1 (Dicer Like 1), que é novamente processado no "duplex" miRNA/miRNA* pela DCL1. O "duplex" é metilado pela ação da HEN1. Uma das fitas do "duplex" é reconhecida e selecionada pela AGO1 (Argonaute 1) e direcionada ao complexo RISC. Este complexo determina o reconhecimento e a ação regulatória do miRNA. (modificado de Chen, 2009).

MIRNAS E DESENVOLVIMENTO EM PLANTAS

Em plantas, mutações nos genes envolvidos na biogênese e no papel regulatório dos miRNAs afetam de forma expressiva o desenvolvimento. O que sugere uma importância crucial para os miRNAs ao longo do desenvolvimento (Ramachandran and Chen 2008; Chen 2009; Xie et al., 2010). Os genes *AGO*, principalmente *AGO1* regulados pelos miR168a e miR168b, desempenham papel fundamental na estabilização e ação regulatória de outros miRNAs (Vaucheret et al., 2004). Em *Arabidopsis*, mutações no gene MIR168a não afetam o desenvolvimento da planta sob condições normais de cultivo. Porém, sob condições de estresse, a planta apresentou menor vigor e florescimento precoce (Vaucheret 2009). Ainda são poucos os estudos que objetivam caracterizar a função dos diferentes membros das famílias de miRNAs. Apesar disto, há trabalhos que relatam que pode haver sobreposição de função e funções independentes para cada membro (Chen 2009).

Um dos exemplos melhor representados é o da família miR164 em *Arabidopsis thaliana*, que regula os FT NAC e CUC (Baker et al., 2005; Guo et al., 2005; Nikovics et al., 2006; Sieber et al., 2007). Provavelmente devido à redundância funcional dos diferentes membros, mutação de perda de função do gene *athMIR164b* não afetou o desenvolvimento floral. Porém, mutações no gene *athMIR164c* resultaram em plantas contendo flores com mais pétalas, o que sugere função específica para este membro da família miR164 (Baker et al., 2005). A geração de mutantes triplos *miR164abc* revelou que os genes *athMIR164a* e *athMIR164b* também sobrepõem parcialmente a função do athMIR164c no desenvolvimento floral, pois o fenótipo mutante ficou mais acentuado no triplo mutante (Sieber et al., 2007).

O gene *CUC2* em *A. thaliana*, regulado pelo miR164a, desempenha papel fundamental no correto estabelecimento da folha (Nikovics et al., 2006). O estabelecimento de meristemas axilares é regulado via redundância funcional dos genes *CUC1* e *CUC2* cujos mRNAs são clivados pela ação do miR164 (Raman et al., 2008). Mutantes individuais para os genes athMIR164a e athMIR164b apresentaram plantas com maior número de raízes (Guo et al., 2005). Interessantemente, a disponibilidade de nitrogênio afeta o desenvolvimento de raízes regulado pelo módulo regulador de auxina AFB3/miR393 em plantas de *Arabidopsis* (Vidal et al., 2010). Na determinação do correto desenvolvimento da raíz, as proteínas SHR e SCR, em ação conjunta, ativam os genes dos

miRNA165a e miR166b, que regulam negativamente os fatores transcricionais HD-ZipIII (Carlsbecker et al., 2010). Plantas de arroz mutantes para sensibilidade a auxina revelaram uma complexa rede de miRNAs e sinais regulatórios envolvidos no desenvolvimento da raiz (Meng et al., 2009).

Outro exemplo de regulação por miRNAs ao longo do desenvolvimento torna-se evidente na relação entre o miR156 e o miR172 na transição da fase vegetativa para a reprodutiva em plantas. Já se compreende que o miR156 regula fatores de transcrição do tipo SPL e que a superexpressão do miR156 resulta em plantas anãs ou semi-anãs, excesso de folhas e uma fase vegetativa mais prolongada (Xie et al., 2006a; Wang et al., 2008; Zhang et al., 2011c). A expressão espacial do gene *AthSPL3* em fusão com o gene GUS revelou uma correlação inversa ao longo do desenvolvimento, cuja expressão do miR156 diminui e a expressão do gene SPL3 aumenta (Wu and Poethig 2006). Em conjunto com a expressão do miR156 que diminui ao longo do desenvolvimento, está a expressão aumentada do miR172, que regula repressores de florescimento APETALA2, TOE1 e TOE2 (Aukerman and Sakai 2003; Wu et al., 2009; Zhu and Helliwell 2010). No desenvolvimento floral, também sugere-se que o miR172 atue tanto na regulação por clivagem do mRNA do gene APETALA, quanto na inibição da tradução (Chen 2004). Interessantemente, o mutante *Cg1* de milho que superexpressa o miR156 tem níveis reduzidos do miR172, o que sugere uma ação conjunta destes dois miRNAs na transição da fase vegetativa para a reprodutiva (Chuck et al., 2007). Sugere-se que sinais moleculares dependentes do miR156, que partem dos primórdios foliares determinam a mudança para a fase reprodutiva em plantas (Yang et al., 2011). Foi caracterizado, recentemente em milho, que a expressão espacial do miR156, que regula o gene *ZmTSH4*, é importante no correto estabelecimento de meristemas laterais (Chuck et al., 2010). A regulação do gene *OsSPL14* pelo miR156 define plantas de arroz com menos perfilhos e maior número de ramos na inflorescência (Jiao et al., 2010; Miura et al., 2010).

A sinalização por hormônios e a interação com a regulação da expressão gênica por miRNAs tem papel crucial no desenvolvimento em plantas (Liu and Chen 2009; Liu et al., 2009a). Em *Arabidopsis*, o miR159 tem como alvo os genes da família GAMYB (fatores de transcrição do tipo MYB dependentes de ácido giberélico) que atuam na germinação e formação de anteras (Reyes and Chua 2007). A superexpressão do miR159 e conseqüente inibição da expressão dos genes MYB ocasionou retardamento na floração e macho-

esterilidade (Millar and Gubler 2005). Plantas de *Arabidopsis miR159ab* tiveram o crescimento inibido pela redução na proliferação celular e morte celular programada (Alonso-Peral et al., 2010).

A biossíntese de ácido jasmônico é regulada pelas proteínas TCPs, que são FT. Estes FT que tem papel regulatório no desenvolvimento e na senescência foliar são regulados pelo miR319 (Schommer et al., 2008). Diversos genes que tem papel na via de sinalização por auxina são alvos de miRNAs. Plantas com formas resistentes dos genes *ARF10*, *ARF16* e *ARF17* (fatores responsivos a auxina) ao miR160 apresentaram efeitos pleiotrópicos, tanto na parte aérea quanto nas raízes (Mallory et al., 2005; Liu et al., 2007). A superexpressão do miR160 resultou em plantas com menor sensibilidade ao ácido giberélico na germinação (Liu et al., 2007). O miR167, que regula os genes *ARF6* e *ARF8*, tem papel no desenvolvimento de estames e do gineceu em flores (Wu et al., 2006). O desenvolvimento de meristemas axilares que dão origem as ramificações em plantas tem ação regulatória do miR171c sob os genes da família SCR (Wang et al., 2010). A proliferação celular em *Arabidopsis* é atenuada pelo aumento de expressão do miR396, que regula o gene GRF (fator de regulação do crescimento) envolvido na modulação dos genes do ciclo celular (Rodriguez et al., 2010). A presença de estômatos, importantes na transpiração da planta, depende, em parte, da regulação do miR824 sob o gene AGL16, que compõe a extensa família MADS Box (Kutter et al., 2007).

Existem miRNAs (miR173, miR390) que atuam (Figura 3) reconhecendo e clivando transcritos de locos no genoma denominados de TAS (RNAs longos não codificantes) e tem função ao longo desenvolvimento em plantas (Peragine et al., 2004). Os RNAs clivados são estabilizados pela proteína SGS3, servindo como molde para a proteína RDR6 responsável pela síntese de novos dsRNAs (fitas duplas de RNAs); estes dsRNAs são reconhecidos e clivados pela DCL4 gerando pequenos RNAs de 21 nt que são metilados pela proteína HEN1. Os ta-siRNAs reconhecidos pela AGO1 atuam regulando pos-transcricionalmente genes da família ARF (Peragine et al., 2004; Allen et al., 2005).

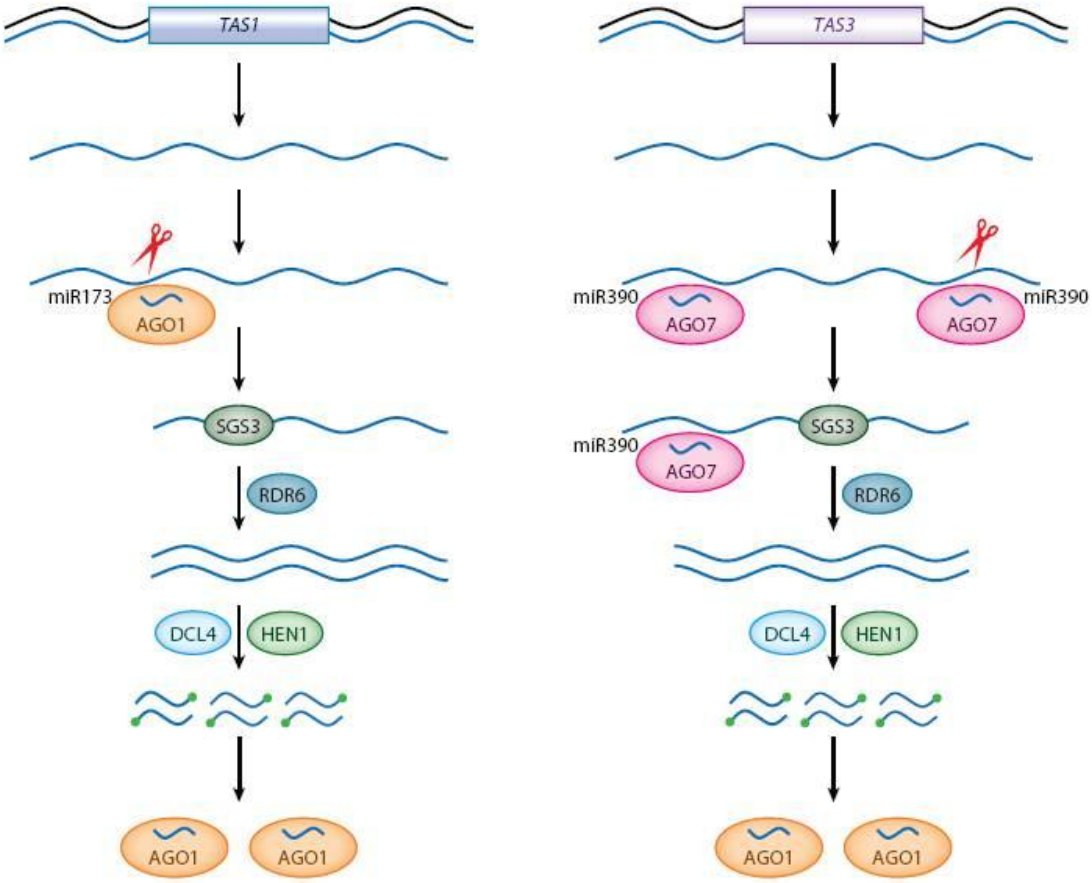

Figura 3. Biogênese dos ta-siRNAs. Transcritos gerados a partir dos locos TAS são reconhecidos e clivados pelos miR173 e miR390 por ação das AGO1 e AGO7. Os transcritos estabilizados pela proteína SGS3 são utilizados como molde pela proteína RDR6 para síntese de novas dsRNAs, que são reconhecidas pela DCL4 e clivadas em pequenos RNAs que atuam regulando outros genes via AGO1 (modificado de Chen, 2009).

Os tasiR-ARFs promovem a identidade de órgãos em meristemas foliares regulando os genes ARF (Chitwood et al., 2009). Mutantes de *Arabidopsis* para os genes de biogênese de ta-siRNAs não apresentam defeitos óbvios na polaridade de órgãos (Garcia et al., 2006). Já em milho, o mutante *lbl* resulta em inversão de polaridade do órgão ou abaxialização foliar (Nogueira et al., 2007). Em arroz, mutações nos genes de biogênese de ta-siRNAs SHL (*SHL1*, *SHL2* e *SHL4*), que são homólogos dos genes *DCL4*, *AGO7* e

RDR6 de *Arabidopsis*, afetam diretamente o desenvolvimento do meristema apical na parte aérea da planta (Nagasaki et al., 2007). A auxina afeta a ação regulatória do miR390 sobre o gene não codante TAS3, promovendo a correta formação de raízes laterais em *Arabidopsis* (Yoon et al., 2010). Estes mecanismos sugerem a importância da rota de formação de ta-siRNAs na manutenção da identidade e polaridade dos órgãos na planta.

MIRNAS EM RESPOSTA A ESTRESSES ABIÓTICOS

Além do papel dos miRNAs ao longo do desenvolvimento em plantas, eles regulam diversos genes em respostas a estresses abióticos (Reyes et al., 2010; Sunkar 2010). A compreensão de como os miRNAs atuam na regulação da expressão gênica e quais genes têm a expressão modulada pelos miRNAs em resposta a estresses como seca, sal, metais, baixas temperaturas e na homeostase de nutrientes, auxiliarão na geração de plantas mais tolerantes (Sunkar 2010).

A EXPRESSÃO DIFERENCIAL DOS MIRNAS E SEU PAPEL REGULATÓRIO EM RESPOSTA A SECA, SALINIDADE E BAIXAS TEMPERATURAS

Diferentes estresses abióticos modulam as respostas gênicas nas plantas. Os miRNAs também respondem aos estresses regulando os diversos genes nas complexas respostas das plantas. O miR393 deve ser um dos moduladores chave nas respostas a estresses, pois sua expressão está alterada em plantas de *Arabidopsis thaliana*, *Oryza sativa*, *Medicago truncatula* e *Phaseolus vulgaris* sob condições de seca, salinidade e baixas temperaturas (Sunkar and Zhu 2004; Zhao et al., 2007; Liu et al., 2008; Zhou et al., 2008b; Arenas-Huertero et al., 2009; Trindade et al., 2010). Porém, a evidência molecular de que o miR393 regula seus alvos sob diferentes condições de estresse ainda deve ser melhor considerada. Recentemente, plantas de *Arabidopsis*, superexpressando osaMIR393 tornaram-se mais sensíveis ao tratamento com excesso de sal, o que sugere sua importância na tolerância `a salinidade (Gao et al., 2011). Sabe-se que diferentes miRNAs da família

miR169 respondem diferencialmente a estresses por seca, salinidade e baixas temperaturas em plantas (Zhao et al., 2007; Liu et al., 2008; Zhou et al., 2008b; Zhao et al., 2009a). Em resposta a estresse por salinidade e seca em arroz, os fatores de transcrição da família NF-YA tem sua expressão modulada por membros da família miR169 (Zhao et al., 2009a). Foi demonstrado em *Arabidopsis,* que plantas mutantes para o gene *AthNF-YA* e plantas superexpressando o miR169 são mais sensíveis à seca (Li et al., 2008). Interessantemente, em tomateiro, a tolerância à seca pela superexpressão do miR169c se deve `a regulação negativa de um gene envolvido na abertura e fechamento de estômatos (Zhang et al., 2011a). Em *Populus trichocarpa*, a expressão dos miR530a, miR1445, miR1446a-e e miR1447 é reduzida em resposta `a seca e salinidade, diferentemente do miR1450 que teve sua expressão reduzida em resposta `a seca e aumentada em resposta a salinidade (Lu et al., 2008). No ancestral do trigo cultivado, *Triticum dicoccoides*, a expressão aumentada do miR1450 revela uma inversão na resposta `a seca em comparação com *Populus trichocarpa* (Kantar et al., 2011a). Apesar da presença do gene MIR1450 em genomas de monocotiledôneas e dicotiledôneas, os padrões de resposta sugerem diferenças regulatórias em resposta `a seca (Lu et al., 2008; Kantar et al., 2011a). A inversão da expressão dos miRNAs miR156, miR166, miR171, miR408 e seus alvos foram detectadas em resposta `a seca em plantas de cevada (Kantar et al., 2010). Em tecidos de diferentes estádios de desenvolvimento, em plantas de arroz sob condição de seca, os miRNAs miR156, miR171 e miR408 dentre outros foram detectados como diferencialmente expressos (Zhou et al., 2010). Em *Medicago truncatula*, o miR408 também atua como modulador em resposta `a seca regulando genes da família das plantacianinas (Trindade et al., 2010).

A EXPRESSÃO DIFERENCIAL DOS MIRNAS E SEU PAPEL REGULATÓRIO EM RESPOSTA A RADIAÇÃO UV-B, HIPOXIA E ESTRESSE OXIDATIVO

O estado “redox” no ambiente celular e a geração de EROs como conseqüência da exposição `a radiação UV-B e hipoxia podem reprogramar as respostas das plantas em função de eventuais danos irreversíveis (Blokhina and Fagerstedt 2010; De Gara et al., 2010). A indução dos miRNAs miR166, miR396, miR395 e miR528 na exposição `a hipoxia em plantas de milho, aponta para o papel dos miRNAs nas alterações morfológicas

e no metabolismo (Zhang et al., 2008). Em resposta a estresses, a presença de EROs nas plantas pode ser regulada pelas proteínas SOD1 e SOD2 (superóxido dismutases), cujos mRNAs são alvos do miR398 (Sunkar et al., 2006; Jagadeeswaran et al., 2009; Trindade et al., 2010; Kantar et al., 2011a). A repressão do miR395 e a indução do miR398 e a consequente inversão da expressão de seus alvos na resposta a UV-B em plantas de *Populus tremula*, sugere diferenças no ajuste metabólico quando comparado a *Arabidopsis* (Jia et al., 2009).

O PAPEL DOS MIRNAS NA HOMEOSTASE DE NUTRIENTES

A absorção de nutrientes é indispensável para as plantas. Do mesmo modo, a homeostase destes nutrientes é um fator preponderante na manutenção do crescimento e desenvolvimento (Giehl et al., 2009; Yang and Finnegan 2010). O enxofre transportado para dentro da célula na forma de sulfato tem função nas respostas da planta a estresses, pois constitui a estrutura de proteínas e outras moléculas (metabólitos secundários, glutationa) importantes nestas respostas (Rausch and Wachter 2005). Na deficiência de sulfato, o miR395 regula transportadores de sulfato e ATP sulfurilases, que têm papel no transporte e assimilação pela célula (Jones-Rhoades and Bartel 2004). Em raízes de *Arabidopsis*, ambas as expressões do transportador de sulfato AST68 e do miR395 foram induzidas. Em maior detalhe, foi sugerido que a expressão espacial do miR395 limita a expressão de seu alvo nas células do floema (Kawashima et al., 2009).

A homeostase de fosfato pela célula envolve a regulação pelo miR399. Baixos níveis de fosfato na célula ativam via FT PHR1 o miR399, que age reprimindo o gene *PHO2*, envolvido na degradação de transportadores de fosfato (Bari et al., 2006). Uma regulação alternativa na sinalização da homeostase de fosfato na célula ocorre pela expressão de um RNA não codante IPS que seqüestra o miR399 em condições ideais e impede que ele regule negativamente o gene PHO2 (Franco-Zorrilla et al., 2007). Também foram identificados, por seqüenciamento em massa, diversos outros miRNAs possivelmente envolvidos nas vias de sinalização de fosfato na célula (Hsieh et al., 2009; Gu et al., 2010).

Os metais cobre e ferro são micronutrientes essenciais à vida da planta. Os miRNAs miR398, 408 e 857 fazem parte da rede de sinais que regulam os níveis de cobre nas plantas. Em condições de deficiência de cobre, estes miRNAs são induzidos causando a repressão de seus alvos regulatórios (Yamasaki et al., 2007; Burkhead et al., 2009). Vários miRNAs foram induzidos em resposta à deficiência de ferro em *Arabidopsis* (Kong and Yang 2010). Interessantemente, um membro da família miR854, induzido em plantas com deficiência de ferro, está conservado em plantas e animais e seus alvos preditos podem ser regulados via repressão da tradução (Arteaga-Vazquez et al., 2006; Kong and Yang 2010).

PRINCIPAIS BANCOS DE DADOS DE MIRNAS DE PLANTAS

A identidade e a seqüência de milhares de miRNAs em plantas está disponível à comunidade científica em diferentes bancos de dados. Está descrito a seguir, uma breve discussão sobre a existência das seqüências de pequenos RNAs e ferramentas de análise nos principais bancos de dados de miRNAs.

O miRBase

O miRBase congrega não somente miRNAs de plantas, mas de diversos outros organismos (Griffiths-Jones 2004; Griffiths-Jones 2006; Griffiths-Jones et al., 2008). O dado do número de sequências do miRBase revela mais de 15000 miRNAs. Há sequências de miRNAs disponíveis para 29 plantas dicotiledôneas, 10 plantas monocotiledôneas, dentre outras como para a alga *Chlamydomonas reinhardtii*. O usuário pode identificar os miRNAs ou seus respectivos genes de vários modos:

- na utilização do código identificador da sequência do miRNA ou palavra-chave;

- através da sequência de um provável miRNA ou precursor proveniente de seqüenciamento de DNA, bibliotecas enriquecidas para pequenos RNAs ou integração dos dados de seqüenciamento em massa com a anotação do miRBase (Kozomara and Griffiths-Jones 2010);

- através da localização genômica do provável loco que contém o miRNA.

Além destas informações, o miRBase disponibiliza as seqüências e as estruturas dos precursores dos miRNAs, bem como a dos prováveis alvos para alguns dos organismos presentes.

O PMRD (Banco de dados de microRNA de plantas)

É um banco de dados de miRNAs de plantas somente. O PMRD conta com por exemplo, mais de 2600 miRNAs de arroz e mais de 1400 miRNAs de *Arabidopsis* identificados experimentalmente e por análise computacional (Zhang et al., 2010). Este banco possui similaridades em termos de ferramentas de busca e análise de miRNAs:

- uso de palavras chave, localização genômica e dados de expressão.

Um diferencial deste banco é que o usuário pode identificar miRNAs responsivos a diversos estresses e fases do desenvolvimento para cada espécie individualmente.

O PmiRKB

Um dos bancos mais importantes que surgiu recentemente é o PmiRKB, que possui novos dados que os anteriores não possuíam (Meng et al., 2010a). Este banco conta com dados para *Arabidopsis* e arroz até o momento. Porém, há quatro tipos de dados indispensáveis nos estudos de miRNAs disponíveis:

- os módulo "SNP", que disponibiliza os polimorfismos, ao nível de nucleotídeo, dentre os precursores de diferentes acessos de arroz (*Oryza sativa*) e *Arabidopsis thaliana*;

- o módulo "pri-miR", que possui dados de expressão dos precursores primários dos miRNAs;

- o módulo "Mir-Tar", que conta com os dados do "sequenciamento de degradomas" para confirmação dos alvos dos miRNAs;

- o módulo “Self-reg”, que disponibiliza dados para compreensão de mecanismos envolvendo a auto-regulação dos miRNAs.

O CSRDB (Banco de dados de pequenos RNAs de cereais)

É um banco de dados de pequenos RNA de arroz e milho (Johnson et al., 2007). Este banco utiliza uma interface similar ao banco de dados de arroz (TIGR - http://rice.plantbiology.msu.edu/) e o banco de dados de Arabidopsis (TAIR - http://www.arabidopsis.org/). Apesar da interface oferecida neste banco de dados não ser um tanto amigável, ela possui uma série de dados como de mapeamento dos microRNAs e outros pequenos RNAs (siRNAs), dados de expressão e predição de alvos dos microRNAs.

A integração de todos esses bancos torna prontamente acessível diversos tipos de informações referentes aos miRNAs. Desde a posição do gene do miRNA até a identificação e confirmação de seus alvos por 5`RACE e o seqüenciamento do degradoma (German et al., 2008). Os bancos de dados têm papel fundamental no auxílio e compreensão da função que os miRNAs desempenham. Principalmente, porque as tecnologias de identificação e caracterização da expressão dos miRNAs geram um número crescente de dados. De fato, seria muito difícil trabalhar experimentalmente na bancada, sem a correta sistematização das seqüências dos miRNAs e suas funções.

FERRAMENTAS DE BIOINFORMÁTICA PARA A ANÁLISE DE MIRNAS E SEUS POSSÍVEIS ALVOS

Diversas ferramentas de bioinformática para a análise de microRNAs estão disponíveis na “internet” e sem custo algum para o usuário. Existem ferramentas que são específicas quanto a análise dos microRNAs, se de humanos e animais e ou de plantas. É interessante notar que estas ferramentas são bastante recentes, o que sugere que a área da bioinformática ligada `a biologia e genética molecular dos microRNAs ainda avançará

muito. Na Tabela 2 são citadas somente algumas das principais ferramentas disponíveis e suas funções.

Tabela 2. Ferramentas de bioinformática para análise de microRNAs.

Categoria	Nome da ferramenta	Endereço eletrônico	Detalhes das funções	Referências
Predição de microRNAs e mapeamento *de novo*	UEA sRNA toolkit	http://srna-tools.cmp.uea.ac.uk./	Mapeamento de microRNAs de plantas e animais, determinação de estrutura secundária, predição de alvos, determinação de perfis de expressão, contagem da freqüência de microRNAs a partir dos dados de seqüenciamento em massa.	(Moxon et al. 2008)
	PlantMiRNAPred	http://nclab.hit.edu.cn/PlantMiRNAPred/	Predição de pre-microRNAs de plantas.	(Xuan et al. 2011)
	mirAnalyser	http://web.bioinformatics.cicbiogune.es/microRNA/.	Predição de microRNAs de humanos e de animais.	(Hackenberg et al. 2009)
	MapMi	http://www.ebi.ac.uk/enright-srv/MapMi/	Mapeamento e análise de microRNAs humanos e animais	(Guerra-Assuncao and Enright 2010)
Predição de alvos	psRNAtarget	http://plantgrn.noble.org/psRNATarget/	Predição de alvos de microRNAs de plantas	
	Target-align	http://www.leonxie.com/targetAlign.php	Predição de alvos de microRNAs de plantas	(Xie and Zhang 2010)

OBJETIVOS

Parte desta tese de doutorado visou caracterizar o perfil de expressão de diferentes famílias de microRNAs em raízes de plantas de arroz *japonica* e *indica* tratadas com alumínio e determinar os prováveis alvos e papéis regulatórios destes microRNAs na resposta ao alumínio.

O trabalho desenvolvido no CSIRO Plant Industry na Austrália, visou a caracterização funcional e da expressão espacial do miR164 em plantas de arroz e a discussão do seu papel regulatório no desenvolvimento de raízes laterais.

O capítulo desta tese que tratou do artigo de revisão visou relatar o estado da arte do papel regulatório dos microRNAs em diferentes condições biológicas e do crescente número de novos microRNAs identificados pelas novas tecnologias de seqüenciamento em massa.

CAPÍTULO II: Aluminum triggers broad changes in microRNA expression in rice roots.

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Aluminum triggers broad changes in microRNA expression in rice roots.

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Running title: Aluminum and miRNA

ABSTRACT.

Micro RNAs 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 four 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 (Sunkar et al., 2005; 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 et al., 2010b) and by in-depth sequencing of small RNA libraries (Reyes et al., 2010; 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, 2010). 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 (Gao et al., 2010). *Arabidopsis* cultivated under cold, high salt concentrations or with the plant hormone, abscisic acid, showed differential expression of several miRNAs (Sunkar et al., 2006; Jung and Kang, 2007). Increasing concentrations of sulphate repress the expression of miRNA395 and up-regulated the expression of its target ATP sulphurylase-1. *Arabidopsis* plants overexpressing miRNA395 result in strong down-regulation of ATP sulphurylases and sulphate transporters (Chiou, 2007; 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 (Chiou, 2007). *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)(Sunkar et al., 2006). 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 (Kochian et al., 2004). 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 reactive oxygen species (ROS) (Kochian et al., 2004). A major gene conferring tolerance to Al, *ALMT1*, was cloned from wheat and also conferred Al tolerance in transgenic rice and tobacco cells (Sasaki et al., 2004). 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 (Huang et al., 2009). Also, in *Arabidopsis*, the ortholog of the *Os*STAR2 gene is involved in Al detoxification, indicating a similar functional mechanism (Huang et al., 2010a). 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 (Huang et al., 2009). 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 (Yang et al., 2008; 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 (*Oryza sativa ssp japonica*) and cv. Embrapa Taim (*Oryza sativa ssp indica*) were used in this study. Seeds were sown in Petri-dishes and germinated during four days (48 h dark/48 h light). 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 $MgCl_2$. 6 H_2O; 40 µM NH_4NO_3; 650 µM KNO_3. For the aluminum treatment, plants were cultivated with 450 µM of $AlCl_3$ during 4 h 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 the instructions of the manufacturer (Invitrogen). A stem loop primer was used to synthesize the miRNA cDNAs (5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACNNNNNN3'), 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 according to the following: 0.1 to 2 µg of total RNA; 100 units of MMLV reverse transcriptase (Promega); 0.05 µM of stem loop primer; 1X enzyme buffer; 0.33 mM of dNTP mix in a final vol. of 30 µl. cDNA synthesis conditions: 16ºC for 30 min, 42ºC for 30 min, 85ºC for 5 min. RT-qPCR mix: 10 µl of cDNA (1:2); 0.4 µl of 5 mM dNTPs; 0.2 µl of each forward (10 µM) and universal (10 µM) primers; 2 µl of 10 X PCR buffer; 1.2 µl of $MgCl_2$; 2 µl of 1 X SYBR Green (Molecular Probe); 0.1 µl of ROX (1:100 in water) (Invitrogen); 0.05 µl of Taq Platinum (Invitrogen) to a final volume of 20 µl. A forward primer (Table 1) and a universal reverse primer (5'GTGCAGGGTCCGAGGT3') were used in the quantitative PCR essays. The primers *Os18S*R (5'ACACTTCACCGGACCATTCAA), *Os18S*F (5'CTACGTCCCTGCCCTTTGTACA); *OsS27a*R (5'ACGCCTAAGCCTGCTGGTT), *OsS27a*F (5'ACCACTTCGACCGCCACTACT), *OseFα1*R (5'GACTTCCTTCACGATTTCATCGTAA), *OseFα1*F (5'TTTCACTCTTGGTGTGAAGCAGAT); *OsFDH* R (5'TTCCAATGCATTCAAAGCTG), *OsFDH*F (5'CAAAATCAGCTGGTGCTTCTC); *OssnU6*R (5'AGGGGCCATGCTAATCTTCT), *OssnU6*F (5'GGGGACATCCGATAAAATTG) were used as internal reference genes to normalize the expression of the miRNAs. RT-qPCR conditions: 94ºC for 5 min followed by 40 cycles at 95ºC for 15 s, 60ºC for 10 s, 72ºC for 15 s. Then the samples were heated from 55 to 99ºC with an increase of 0.1ºC/ 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 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. 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 of 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 8h 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 of 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 expresson 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 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 Al for 4 h, and 8 h. 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 Al. 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 down-regulated after 8 h of Al treatment (Figure 3). The expression of these miRNAs was reduced approximately 20 to 40 %. Only miRNA168a, miRNA399d, miRNA528 and miR808 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 a RT-qPCR for two miRNA528 targets; a slight down regulation was observed for one of the targets (Figure 4 and Tables 2 and 3).

Identification of putative targets for rice miRNAs differentially expressed in response to Al treatment may contribute to our understanding 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 of 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 we identified as putative ones in this report (Li et al., 2010).

Figure 3. Comparative expression levels of miRNAs down- and up-regulated in Embrapa Taim rice roots after 8 h treatment with Al. 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.

Figure 4. Comparative expression levels of miRNA528 targets in Embrapa Taim rice roots after 8 h treatment with Al. 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.

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 been also 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 a 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 (Hawker and Bowman, 2004). It was also shown that miRNA166 is involved in the regulation of class III HD-ZIP genes in shoot apical meristem formation in *Arabidopsis* (Williams et al., 2005) 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 reactive oxygen species, causing oxidized proteins to be degraded (Smalle and Vierstra, 2004). 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 (Yang et al., 2008). 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 (Chiou, 2007; Liang et al., 2010). MiRNA399d was up-regulated in our experiments. It regulates the PHO2 protein (UBC - ubiquitin conjugating enzyme) 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 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 sulphurylases, key proteins in sulphur homeostasis (Chiou, 2007; Liang et al., 2010). In roots of rice seedlings, expression of genes related to sulphur metabolism, including ATP sulphurylases, 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 8h of Al treatment (Figures 2 and 3).

Reactive oxygen species (ROS) 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 are in contrast to the down-regulation of miRNA398 (Sunkar et al., 2006). 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, miRNAs 398a 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 (Ramos-Diaz et al., 2007). 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 (Romo et al., 2001) and subsequently that miRNA408 targets plantacyanins (Sunkar et al., 2005; 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).

T*ranscription 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.

MiRNAs 156l and 172a 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 (Wu et al., 2009; Zhu and Helliwell, 2010).

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 four miRNAs were significantly up-regulated in the *indica* cultivar comparing to the six up-regulated in the *japonica* cultivar. Among the nine 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 sulphur homeostasis regulator, is up-regulated in maize roots of both tolerant and sensitive lines under salt stress (Chiou, 2007; Ding et al., 2009). Our analysis showed that miRNA395 is down-regulated in both *indica* and *japonica* varieties (Figures 2 and 3). Also, miRNAs 168, miRNA528 and 399 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 down-regulation of the miRNA528 target copper-ion gene, and especially the L-ascorbate oxidase gene (Figure 4), results from the involvement of these genes 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 (Zhou et al., 2008), which is expected based on our results. MiRNA398 is known to be a regulator of genes that respond to abiotic stress in plants (Sunkar et al., 2006). A comparison between an Al tolerant line and an Al sensitive line in *Medicago truncatula* showed similar molecular responses related to genes involved in cell death, senescence, and cell wall degradation (Chandran et al., 2008). Most Al tolerant rice cultivars are derived from a *japonica* background, and Al sensitive cultivars are derived from an *indica* background (Kochian et al., 2004; Yang et al., 2008). 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 (Chinnusamy et al., 2004). 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 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.

Figure 5. Diagram of the differentially expressed miRNAs in rice roots treated with Al and their putative targets.

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REFERENCES

Abdel-Ghany SE and Pilon M (2008). MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in Arabidopsis. *J. Biol. Chem.* 283: 15932-15945.

Baier AC, Somers DJ and Gustafson JP (1995). Aluminum Tolerance in Wheat - Correlating Hydroponic Evaluations with Field and Soil Performances. *Plant Breed.* 114: 291-296.

Bari R, Datt Pant B, Stitt M and Scheible WR (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* 141: 988-999.

Brennecke J, Stark A, Russell RB and Cohen SM (2005). Principles of microRNA-target recognition. *PLoS Biol.* 3: e85.

Chandran D, Sharopova N, VandenBosch KA, Garvin DF, et al. (2008). Physiological and molecular characterization of aluminum resistance in Medicago truncatula. *BMC Plant Biol* 8: -.

Chen C, Ridzon DA, Broomer AJ, Zhou Z, et al. (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 33: e179.

Chen ZY, Zhang JJ, Kong J, Li SQ, et al. (2006). Diversity of endogenous small non-coding RNAs in *Oryza sativa*. *Genetica* 128: 21-31.

Chinnusamy V, Schumaker K and Zhu JK (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J. Exp. Bot.* 55: 225-236.

Chiou TJ (2007). The role of microRNAs in sensing nutrient stress. *Plant Cell Environ.* 30: 323-332.

Ding D, Zhang L, Wang H, Liu Z, et al. (2009). Differential expression of miRNAs in response to salt stress in maize roots. *Ann. Bot. (Lond)* 103: 29-38.

Fukuda T, Saito A, Wasaki J, Shinano T, et al. (2007). Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. *Plant Sci.* 172: 1157-1165.

Gao P, Bai X, Yang L, Lv D, et al. (2010). osa-MIR393: a salinity- and alkaline stress-related microRNA gene. *Mol. Biol. Rep.* 38: 237-242.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, et al. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* 34: D140-144.

Grotz N and Guerinot ML (2002). Limiting nutrients: an old problem with new solutions? *Curr. Opin. Plant Biol.* 5: 158-163.

Hawker NP and Bowman JL (2004). Roles for Class III HD-Zip and KANADI genes in Arabidopsis root development. *Plant Physiol.* 135: 2261-2270.

Huang CF, Yamaji N and Ma JF (2010a). Knockout of a bacterial-type ATP-binding cassette transporter gene, AtSTAR1, results in increased aluminum sensitivity in Arabidopsis. *Plant Physiol.* 153: 1669-1677.

Huang CF, Yamaji N, Mitani N, Yano M, et al. (2009). A bacterial-type ABC transporter is involved in aluminum tolerance in rice. *Plant Cell* 21: 655-667.

Huang SQ, Xiang AL, Che LL, Chen S, et al. (2010b). A set of miRNAs from Brassica napus in response to sulphate deficiency and cadmium stress. *Plant Biotechnol. J.* 8: 887-899.

Jung HJ and Kang H (2007). Expression and functional analyses of microRNA417 in Arabidopsis thaliana under stress conditions. *Plant Physiol. Biochem.* 45: 805-811.

Khan MS, Tawaraya K, Sekimoto H, Koyama H, et al. (2009). Relative abundance of Delta(5)-sterols in plasma membrane lipids of root-tip cells correlates with aluminum tolerance of rice. *Physiol. Plant.* 135: 73-83.

Kikui S, Sasaki T, Maekawa M, Miyao A, et al. (2005). Physiological and genetic analyses of aluminum tolerance in rice, focusing on root growth during germination. *J. Inorg. Biochem.* 99: 1837-1844.

Kochian LV, Hoekenga OA and Pineros MA (2004). How do crop plants tolerate acid soils? - Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu. Rev. Plant Biol.* 55: 459-493.

Li YF, Zheng Y, Addo-Quaye C, Zhang L, et al. (2010). Transcriptome-wide identification of microRNA targets in rice. *Plant J.* 62: 742-759.

Liang G, Yang F and Yu D (2010). MicroRNA395 mediates regulation of sulfate accumulation and allocation in Arabidopsis thaliana. *Plant J.* 62: 1046-1057.

Lindow M, Jacobsen A, Nygaard S,Mang Y, et al. (2007). Intragenomic matching reveals a huge potential for miRNA-mediated regulation in plants. *PLoS Compu.t Biol.* 3: e238.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.

Lu XY and Huang XL (2008). Plant miRNAs and abiotic stress responses. *Biochem. Biophys. Res Commun.* 368: 458-462.

Maron LG, Kirst M, Mao C, Milner MJ, et al. (2008). Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots. *New Phytol.* 179: 116-128.

Millar AA and Gubler F (2005). The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are MicroRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* 17: 705-721.

Nagasaki H, Itoh J, Hayashi K, Hibara K, et al. (2007). The small interfering RNA production pathway is required for shoot meristem initiation in rice. *Proc. Natl. Acad Sci. U S A* 104: 14867-14871.

Navarro L, Dunoyer P, Jay F, Arnold B, et al. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312: 436-439.

Ramos-Diaz A, Brito-Argaez L, Munnik T and Hernandez-Sotomayor SM (2007). Aluminum inhibits phosphatidic acid formation by blocking the phospholipase C pathway. *Planta* 225: 393-401.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, et al. (2002). MicroRNAs in plants. *Genes Dev.* 16: 1616-1626.

Reyes JL, Arenas-Huertero C and Sunkar R (2010). Cloning of stress-responsive microRNAs and other small RNAs from plants. *Methods Mol. Biol.* 639: 239-251.

Romo S, Labrador E and Dopico B (2001). Water stress-regulated gene expression in Cicer arietinum seedlings and plants. *Plant Physiol. Biochem.* 39: 1017-1026.

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, et al. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37: 645-653.

Sharma P and Dubey RS (2007). Involvement of oxidative stress and role of antioxidative defense system in growing rice seedlings exposed to toxic concentrations of aluminum. *Plant Cell Rep.* 26: 2027-2038.

Smalle J and Vierstra RD (2004). The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* 55: 555-590.

Stirnberg P, Furner IJ and Ottoline Leyser HM (2007). MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. *Plant J.* 50: 80-94.

Sunkar R (2010). MicroRNAs with macro-effects on plant stress responses. *Semin. Cell Dev. Biol* 21: 805-811.

Sunkar R, Girke T, Jain PK and Zhu JK (2005). Cloning and characterization of microRNAs from rice. *Plant Cell* 17: 1397-1411.

Sunkar R, Kapoor A and Zhu JK (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18: 2051-2065.

Vanzin GF, Madson M, Carpita NC, Raikhel NV, et al. (2002). The mur2 mutant of Arabidopsis thaliana lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proc. Natl. Acad. Sci. USA* 99: 3340-3345.

Williams L, Grigg SP, Xie MT, Christensen S, et al. (2005). Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. *Development* 132: 3657-3668.

Wu G, Park MY, Conway SR, Wang JW, et al. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* 138: 750-759.

Xie Q, Frugis G, Colgan D and Chua NH (2000). Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev* 14: 3024-3036.

Xie Z, Khanna K and Ruan S (2010). Expression of microRNAs and its regulation in plants. *Semin. Cell Dev. Biol.* 21: 790-797.

Xue LJ, Zhang JJ and Xue HW (2009). Characterization and expression profiles of miRNAs in rice seeds. *Nucleic Acids Res.* 37: 916-930.

Yamaji N, Huang CF, Nagao S, Yano M, et al. (2009). A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *Plant Cell* 21: 3339-3349.

Yang JL, Li YY, Zhang YJ, Zhang SS, et al. (2008). Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. *Plant Physiol.* 146: 602-611.

Yang QS, Wang YQ, Zhang JJ, Shi WP, et al. (2007). Identification of aluminum-responsive proteins in rice roots by a proteomic approach: Cysteine synthase as a key player in Al response. *Proteomics* 7: 737-749.

Zhang Y (2005). miRU: an automated plant miRNA target prediction server. *Nucleic Acids Res* .33: W701-704.

Zhang ZH, Yu JY, Li DF, Zhang ZY, et al. (2010). PMRD: plant microRNA database. *Nucleic Acids Res.* 38: D806-D813.

Zhao B, Ge L, Liang R, Li W, et al. (2009). Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Mol. Biol.* 10: 29.

Zhao BT, Liang RQ, Ge LF, Li W, et al. (2007). Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Commun.* 354: 585-590.

Zhou ZS, Huang SQ and Yang ZM (2008). Bioinformatic identification and expression analysis of new microRNAs from Medicago truncatula. *Biochem. Biophys. Res. Commun.* 374: 538-542.

Zhu QH and Helliwell CA (2010). Regulation of flowering time and floral patterning by miR172. *J. Exp. Bot.* 62: 487-495.

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

Table 1. Continued

miRNA	Forward primer 5’ – 3’
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

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

Locus	Forward	Reverse
Os07g38290	GCCACCACTACTTCCTCTGC	GACGGAGAGCACGGACAC
Os09g20090	TAACAAGCTCTGCAGCCTCA	CGACGTGAACTGGTAGCTGA

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, sb, zma, ptc, mtr
		Os10g08022	2.5	5	Fructose-bisphosphate aldolase	

Table 3. Continued.

miRNA	miRNA seq 5'-3'	Target loci	Score	Mm	Target function	orthologs
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	UCAGGUUAUAAGACUUUCUAGC	Os03g31180	0	0	Diacylglycerol kinase 1	NI
h,i,j,k**		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,* sof = ; *Saccarum 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 they have the same mature sequence and one letter indicate 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,
		Os03g44540	1.5	3	Nuclear transcription factor Y subunit A-10	ptc,bna,vvi
miRNA172a,d**	AGAAUCUUGAUGAUGCUGCAU	Os03g60430	0.5	2	Floral homeotic protein	ath,zma,sbi
		Os05g03040	0.5	2	Floral homeotic protein APETALA2	gma,ptc,vvi
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,
		Os03g09940	1	2	Low affinity sulphate transporter 3	zma,ptc,vvi
		Os03g09930	2	3	Sulfate transporter 2.1	
miRNA398a**	UGUGUUCUCAGGUCACCCCUU	Os07g46990	1.5	3	Superoxide dismutase 2	ath, gma, ptc,vvi

Table 4. Continued.

mRNA	miRNA seq 5' – 3'	Target loci	Score	Mn	Target function	orthologs
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-fucosyltransferase A	NI
miRNA809a,b,d,	UGAAUGUGAGAAAUGUUAGAAU	Os09g34250	0	1	Indole-3-acetate beta-glucosyltransferase	NI
e,f,g,h**		Os01g34620	0.5	1	OsGrx_S15.1 - glutaredoxin subgroup II	
miRNA813*	GGGUUAUGGAAUGGGUUUUACC	Os02g49720	2.5	4	Aldehyde dehydrogenase,	NI

ath =; *Arabidopsis thaliana,* zma = ; *Zea mays,* sbi = ; *Sorgum bicolor,* ppt =; *Populus trichocarpa,*ptc = ; *Phycomitrella patens,* vvi = ; *Vitis vinifera,* sof = ; *Saccarum 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 they have the same mature sequence and one letter indicate the miRNA mature sequence is different from the other members of the family

Fig. 1

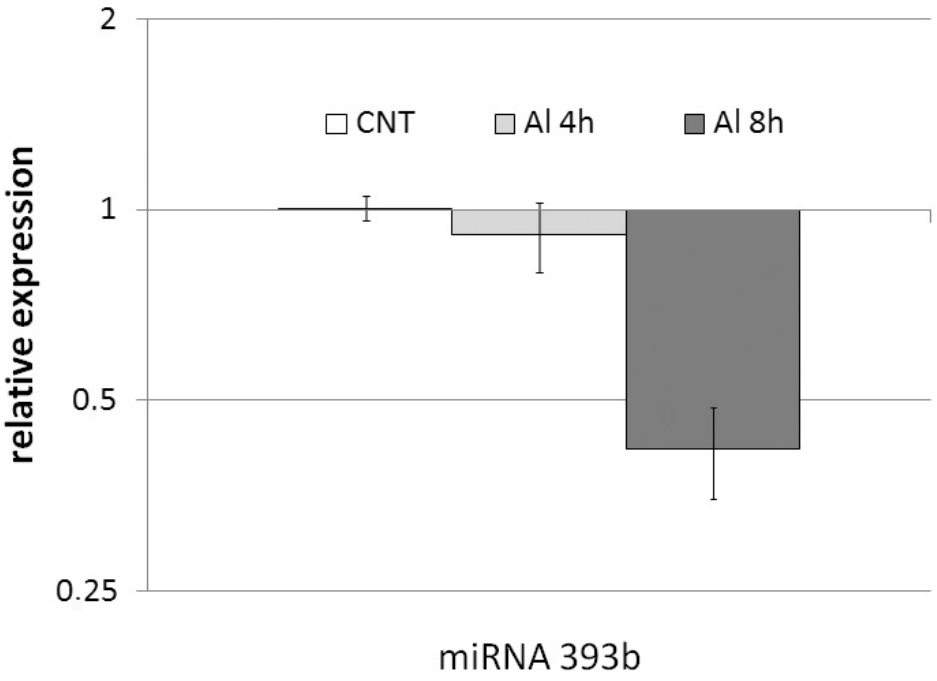

2
1
0.5
0.25
relative expression
CNT
Al 4h
Al 8h
miRNA 393b

Fig. 2

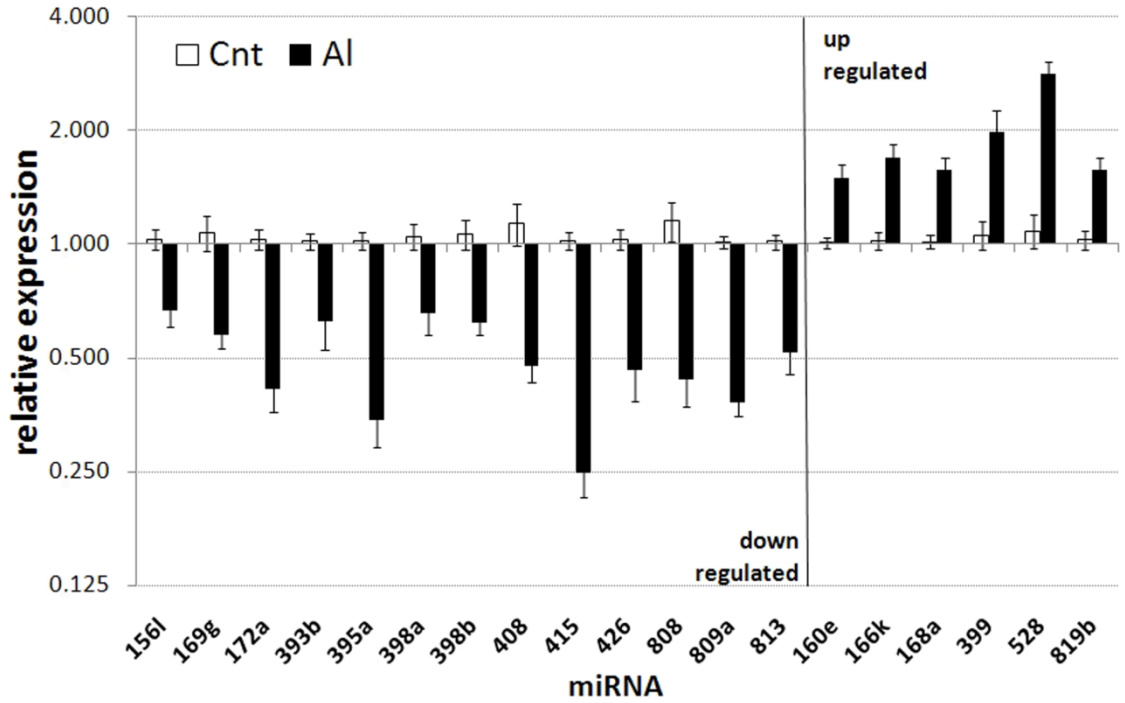

4.000
2.000
1.000
0.500
0.250
0.125
relative expression
Cnt
Al
up regulated
down regulated
156l
169g
172a
393b
395a
398a
398b
408
415
426
808
809a
813
160e
166k
168a
399
528
819b
miRNA

Fig 3.

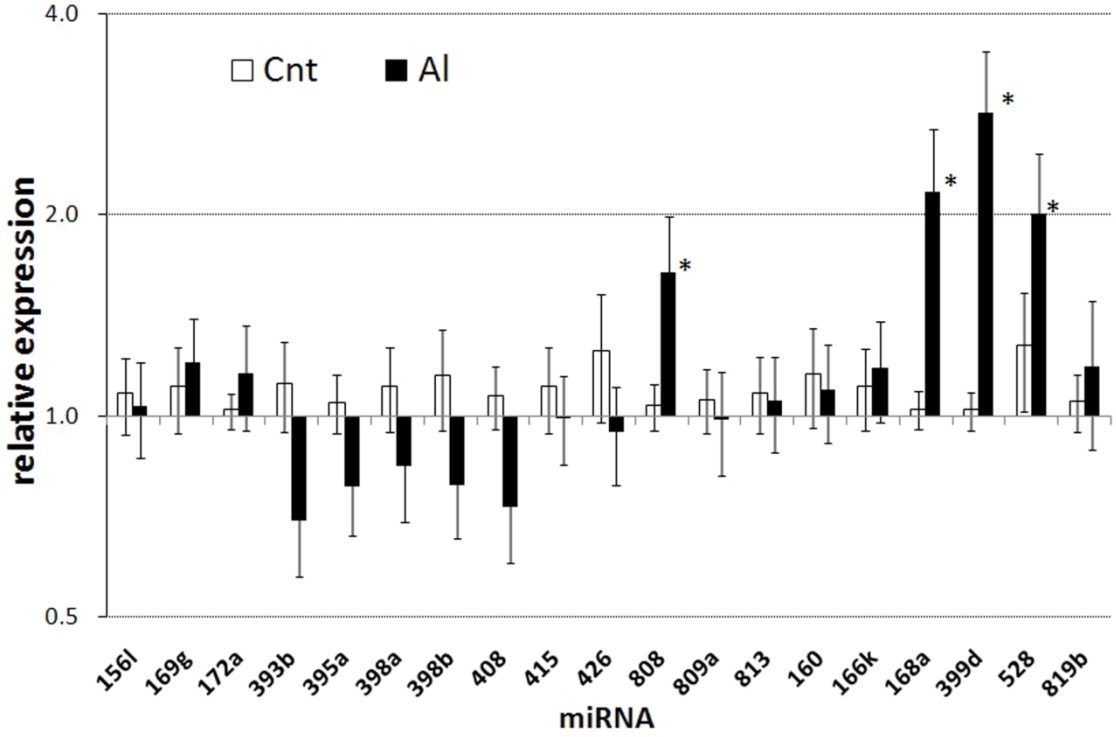

4.0
2.0
1.0
0.5
relative expression
Cnt
Al
*
*
*
*
156l
169g
172a
393b
395a
398a
398b
408
415
426
808
809a
813
160
166k
168a
399d
528
819b
miRNA

Fig. 4

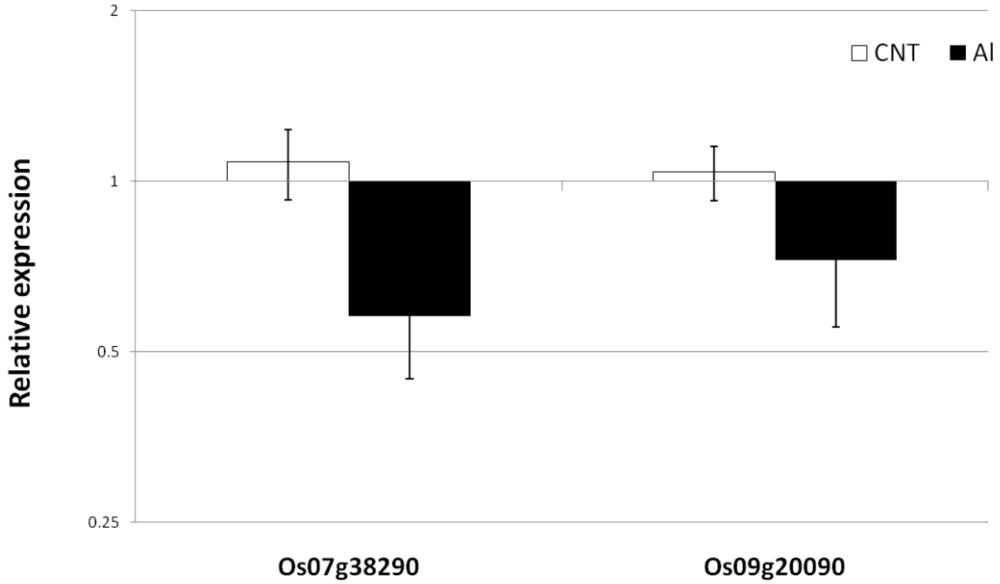
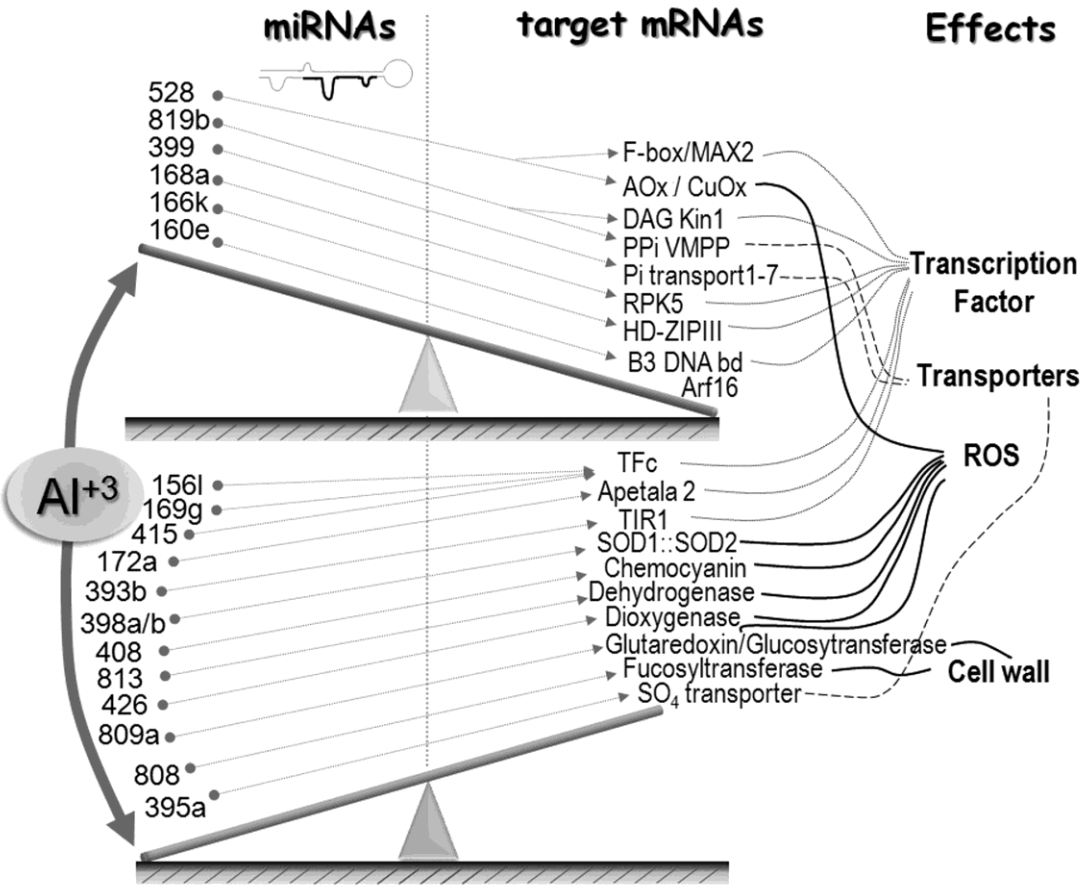

2
1
0.5
0.25
Relative expression
CNT
Al
Os07g38290
Os09g20090

Fig. 5


miRNAs
target mRNAs
Effects
528
819b
399
168a
166k
160e
F-box/MAX2
AOx / CuOx
DAG Kin1
PPi VMPP
Pi transport1-7
RPK5
HD-ZIPIII
B3 DNA bd
Arf16
Transcription Factor
Transporters
ROS
Cell wall
Al+3
156l
169g
415
172a
393b
398a/b
408
813
426
809a
808
395a
TFc
Apetala 2
TIR1
SOD1::SOD2
Chemocyanin
Dehydrogenase
Dioxygenase
Glutaredoxin/Glucosytransferase
Fucosyltransferase
SO4 transporter

CAPÍTULO III: Lateral root development is dependent on miR164 regulatory action in rice

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Artigo *em preparação*

Title: Lateral root development is dependent on miR164 regulatory action in rice

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Key-words: lateral root, miR164, meristem, *Oryza sativa*.

Abstract: Lateral roots play a key role in the uptake of nutrients during plant development. Moreover, microRNAs are regulatory molecules with a deterministic role in plant development. Here we demonstrate that miRNA164 regulating NAC transcription factors have a key role in the development of lateral roots in rice plants. The analyses of DNA motifs in the promoters of the six members of the miR164 family and their target genes revealed the co-occurrence of important regulatory sequences involved in meristem development. The analyses of promoter GUS fusion lines have shown overlapping and specific domains for the spatial expression of different members of miRNA164 in rice roots. MiRNA164a and miRNA164d are more expressed in lateral roots. MiRNA164f is expressed in both lateral roots and in the primary root. Also, miRNA164f is expressed in the stele and miR164a is expressed in the endodermis. Plants overexpressing miRNA164 have less lateral roots, which is in agreement with *Arabidopsis* plants overexpressing miRNA164 (Guo et al., 2005). These results suggest that the miRNA164 family has a crucial conserved role during the proper development of roots.

Introduction

Important cereals like rice (*Oryza sativa*) will have to face severe conditions to grow healthily and have high yields in the changing environment worldwide. Plants with a very efficient root system in such environments could have several developmental advantages over others. Several functional genomic strategies for understanding root architecture have been applied widely in the model plant *Arabidopsis* (Iyer-Pascuzzi et al., 2009; Iyer-Pascuzzi and Benfey 2009). However, there are important differences between *Arabidopsis* and cereals which have a more complex branched system (Coudert et al., 2010). Lateral roots have an important role in the formation of this branched system and are highly dependent on the initial local activation of pericycle cells (Fukaki et al., 2007). In rice, different mutants for root development have been characterized (Inukai et al., 2005; Liu et al., 2005; Han et al., 2008; Liu et al., 2009b). Extensive studies on the identification of QTLs controlling root development have been carried out (Zheng et al., 2003; Zheng et al., 2006; Suryapriya et al., 2009). Moreover, important molecular mechanisms in the development of rice roots reveal a complex molecular interaction (Coudert et al., 2010). However, there is still a lack of information regarding the molecular basis of lateral root development.

It has been shown that miRNAs are required in several developmental processes, including lateral root development (Chen 2005; Mallory and Vaucheret 2006). Attempts to understand root development based on the regulatory action of miRNAs have been described (Wang et al., 2005; Boualem et al., 2008; Gutierrez et al., 2009; Carlsbecker et al., 2010; Meng et al., 2010b). It was found that redundancy and specialization among different miRNA164 members contribute to proper meristem identity (Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007). In *Arabidopsis*, miRNA164 targeting the NAC1 transcription factor has a deterministic role in the development of lateral roots. Plants overexpressing NAC1 have more lateral roots and plants overexpressing miR164 have less lateral roots (Guo et al., 2005).

In rice, an auxin resistant mutant revealed a complex miRNA dependent regulatory network in root development involving miRNA164 (Meng et al., 2009). According to

bioinformatic analysis, rice has six different miR164 genes (Griffiths-Jones et al., 2008; Zhang et al., 2010). However, there are no reports in the literature regarding functional characterization of the different members of the miR164 family in rice. Although the analysis of different members of a miRNA family is a challenging task, strategies like identification and characterization of insertional mutants and transgenic lines carrying reporter genes are useful tools in the functional characterization of individual members of the same family (Baker et al., 2005; Guo et al.et al., 2005; Navarro et al., 2006; Sieber et al., 2007; Martinez et al., 2008; Parry et al., 2009). Here we report results of determining the spatial expression patterns of three members of this family, miR164a, miR164d, and miR164f. Also, rice plants overexpressing miR164 have a similar phenotype to *Arabidopsis* traits overexpressing this miRNA.

Material and methods

Plant material

All rice plants for the experimental procedures were derived from the *japonica* background (*Oryza sativa* ssp. *japonica cv* Nipponbare). Transgenic plants were obtained via tissue culture based on the method previously described (Upadhyaya et al.,, 2000). Plants with four fully expanded leaves were used for the GUS assay. MiRNA164 overexpressing plants with two fully expanded leaves were used for the phenotypic and molecular analyses.

Growth conditions

Rice plants at the developmental stage mentioned above were grown in a hydroponic system at pH 5.7 $\pm$ 0.1 adjusted with NaOH 5M. The components of the hydroponic liquid medium were: 2mM $(NH_4)_2SO_4$; 0,3 mM KNO_3; 2mM KH_2PO_4; 2,5 mM NH_4NO_3; 0,67 mM $Ca(NO_3).2H_2O$; 0,4 mM $MgSO_4.7H_2O$; 10mg/L FeEDTA and 5

mL of 10X micronutrient solution. 96 PCR plates covered with aluminium foil had the bottom removed to serve as a floating base for the growth of germinating seeds. The hydroponic solution was replaced every two days.

DNA constructs

For the promoter GUS fusion transgenic lines, up to 1.5 Kb DNA sequence upstream of pre-miRNA of miR164a, miR164d, and miR164f was fused to the GUS reporter gene and cloned into the pWBvec vector. For the overexpression lines, a pre-miRNA164b was placed under the control of the ubiquitin promoter from maize within the T-DNA construct.

Histochemical analysis and GUS assay

Histochemical GUS analysis was performed as described (Sheldon et al., 2002). Roots were cut at the base from the plants and were immersed in an X-Gluc solution containing: 1g X-GLUC; 10 mM EDTA; 100 mM phosphate buffer pH 7.2; 0,2 % TRITON X-100; 0,5 mM $K_3Fe(CN)_6$; 0,5 mM $K_4Fe(CN)_6.3H_2O$; 5 ml of methanol to a final volume of 667 ml. After 20h at 37° C, roots were incubated consecutively for 1h at room temperature with 30 %, 50 %, 70 %, 85 % and 100 % ethanol, respectively. Roots in fresh 100 % ethanol were kept at 4° C overnight and were incubated consecutively for 1h in 85 % and 70 % ethanol at room temperature. GUS stained roots were visualized in a ZEISS microscope. Cross sections were done using hand-sectioning.

Gene expression analysis of target genes in the miRNA164 overexpression lines

To confirm the presence of T-DNAs in the transgenic rice plants, DNA was extracted using a very simple method: a small piece of leaf was cut from rice seedlings, ground with a pestle in a 1.5 ml microtube in 1% SDS, and incubated in a water bath at 65° C for 45 min. Five microliters (1:25 diluted) was used for the PCR analysis. PCR was run

using: 1μl of 10X buffer, 07 μl of 50mM $MgCl_2$, 0.4 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer F and 0.4 μl of primer R, 0.5 μl SYBER GREEN (1:10000), 0.05 μl of 5 U/ μl and water to 10 μl. PCR products were visualized in an 2% agarose gel stained with ethidium bromide. Total RNA was extracted using RNeasy Plant Mini kit. cDNA was synthesized with Superscript III from Invitrogen using 1 μl of enzyme per μg of total RNA. qRTPCR was run in a ABI 7900 HT Fast Real time PCR System. For each PCR 5 μl of 1:50 diluted cDNA sample was mixed with 1 μl of 10X PCR buffer, 0.7 of 50 mM $MgCl_2$, 0.4 of 5 mM dNTP, 0.4 of each 10 μM forward and reverse primers, 0.5 μl of 1:10000 Sybr Green, 0.1 μl of Platinum Taq DNA polymerase, and 1.5 μl of autoclaved deionised water to a final volume of 10 μl. The quantification of cDNA amounts was based on a mathematical model already described (Pfaffl 2001).

Identification of *cis*-elements in the promoters of the osa-MIR164 genes and in their target genes

All promoter sequences were retrieved from the Gramene (http://www.gramene.org/) and JGI Genome Portal databases (http://www.gramene.org/; http://genome.jgi-psf.org/). The putative promoter for all sequences were retrieved based on the methodology already described (Zhou et al., 2007). For the two analyses, up to 1.5kb upstream of the pre-miRNA were used. To identify *cis*-elements among all sequences, PLACE (http://www.dna.affrc.go.jp/PLACE/index.html) and PlantPAN (http://plantpan.mbc.nctu.edu.tw/gene_group/index.php) were used. The plant species used in this analysis were: *Arabidopsis thaliana, Populus trichocarpa*, *Vitis vinifera, Sorghum bicolor* and *Zea maiz*.

Results and Discussion

Co-occurrence of DNA motifs in the promoter sequences of MIRNA164 genes in different plant species and in rice target genes

An *in silico* analysis using PlantPAN to identify putative *cis*-elements in the miRNA164 promoter sequences of all species analysed and in the rice target genes,

identified co-occurring DNA motifs (Table 1 and Table 2). Based on our analysis, DOF DNA motifs were the most frequent within the promoter consensus sequences. These motifs are binding sites for DOF transcriptional factors, which are found exclusively in plants and play a variety of regulatory roles including meristem development in different organs (Kushwaha et al., 2010). The second most frequent consensus sequence revealed binding sites for RAV transcriptional factors that regulate growth negatively (Ikeda and Ohme-Takagi 2009). According to our analysis, RAV binding sites were found both in the miR164 promoters and in the promoters of miR164 target genes (Table 1 and Table 2). Although promoter sequences containing CTCTT have been identified to be active in infected roots in mycorrhizal symbioses (Vieweg et al., 2004), they may be playing regulatory roles in miR164 genes regarding lateral root development. It is well documented that ARR1 transcriptional factors regulate meristem development in roots (Sakai et al., 2001; Ross et al., 2004; Dello Ioio et al., 2008). The occurrence of ARR1 binding sites in both promoters of miR164 and miR164 target genes, point to a possible regulatory feedback loop (Table 1 and Table 2). The other occurring and co-occurring cis-elements as GATA-Box, and Core are general DNA motifs found in the majority of promoters.

DNA regulatory elements have key roles in promoter activity. The computational identification of regulatory elements for the miR164 family in this report is a first step for a further biological confirmation.

Localization of different members of the osamiRNA164 family in rice roots.

To investigate the spatial expression patterns of different miRNA164 family members, we analysed T1 promoter GUS fusion lines from miRNA164a, miRNA164d, and miRNA164f. Based on the GUS activity, the spatial expression of miRNA164a and miRNA164d were more pronounced in lateral roots coming from mature and young regions (Fig 1a, b). Also, miRNA164a was expressed in the apex of primary roots (Fig 1a). MiRNA164f was expressed in the primary roots, and also in lateral roots (Fig 1c). Cross sections have shown that miRNA164a was expressed in the endodermis (Fig 1d) and miRNA164f was expressed in the stele (Fig 1e). The stele is composed of pericycle, fibers, metaxylem, and floem (Coudert et al.,2010). It is well established that lateral roots originate from the pericyle cell layers, being repressed by cytokinin and promoted by auxin

(Laplaze et al., 2007; Parizot et al., 2008). Our results based on the histochemical analysis suggest that miRNA164f may have a key role in the lateral root specification during its initial development (Fig 1b). Interestingly miRNA164a was expressed in the apex of primary roots (Fig 1a). It is already reported that miRNA164 plays an important regulatory role in the expansion of meristematic regions in plants (Laufs et al., 2004; Mallory et al., 2004). Based on our results, we suggested that miRNA164a has a role regulating its targets during cell division in meristematic regions in the root apex. Moreover, miR164a is a TATA less promoter (Cui et al., 2009). Such type of promoters can be grouped into two classes of genes: (1) housekeeping genes, and (2) homeotic like genes important for proper developmental (Smale 2001). It is suggested that this kind of promoter regulates genes involved in developmental processes such as cell divisions during root development.

MiRNA164 overexpression has a deterministic role in an intricate molecular response in root development

Arabidopsis plants overexpressing miRNA164 have shown a reduction in the number of lateral roots (Guo et al., 2005). Here we demonstrate that transgenic rice lines overexpressing miRNA164 have smaller crown roots, and have fewer lateral roots (Fig 2b, e). It was difficult to obtain several transgenic lines with viable seeds because high sterility is another consequence of miRNA164 overexpression. Interestingly, one of the transgenic lines has a very strong dwarf phenotype (Fig 2c). This phenotype suggests a conserved regulatory role of miRNA164 in *Arabidopsis* and rice.

Previous publications regarding root development suggest a complex regulatory network. Auxin response factor transcripts (ARF) are dependent on the regulatory action of miRNA160 and miRNA167 in a feedback manner in *Arabidopsis* (Gutierrez et al., 2009). ARF10 and ARF16 targeted by miRNA160 control root cap development (Wang et al., 2005). Critical receptors in auxin signalling, TIR1/AFB, targeted by miRNA393 suggests a more complex gene regulatory action on root development in plants (Parry et al., 2009). Recently, it was published that miRNA165 and miRNA166 cleaved class III homeodomain-leucine zipper transcription factors are involved in the regulation of positional information and proper root development in *Arabidopsis* (Carlsbecker et al., 2010). The development of lateral roots is highly suppressed in the mutant *crl1*, which is

suggested to have *ARF* genes as targets (Inukai et al., 2005; Liu et al., 2005). Moreover, *WOX11* is a hormone dependent gene involved in crown root formation. Its loss of function mutation causes abnormal crown roots (Zhao et al., 2009c). The *IAA* gene is known to be a negative regulator dependent on auxin. It was found in rice that overexpression of the IAA3 protein produced auxin insensitive plants with abnormal leaves and crown root defects (Nakamura et al., 2006). Although a conserved mechanism is suggested between monocots and dicots for proper root development (McSteen 2010), the characterization of all the upstream and downstream gene factors in this complex gene regulatory network needs further investigation.

Lateral roots play crucial roles in the efficiency of the uptake of nutrients to the plant (Peret et al., 2009; Faiyue et al., 2010a; Faiyue et al., 2010b). Previous reports have shown that overexpression of miRNA164 can cause developmental defects (Laufs et al., 2004; Guo et al., 2005). Also, according to the results showed in this report, the development of lateral roots dependent on miRNA164 regulatory action could be a key point in the understanding of the complex root branching system in rice.

Rice roots overexpressing miRNA164 target NAC genes.

It is already shown that miRNA164 targets NAC genes downregulating their expression (Guo et al., 2005; Li et al., 2010c). To confirm the regulatory action of miRNA164 in rice roots, we analysed the expression of its target genes belonging to the NAC transcription factor family in the miRNA164 overexpression lines (Fig. 3). Our expression analysis revealed contradictory results. As expected, one the of the target genes, Os06g46270 was down regulated in plants overexpressing miR164. However, the other miR164 target gene, Os08g10080, was up regulated in some of the plants analysed. Both results still need confirmation.

References

Baker, CC, Sieber, P, Wellmer, F, Meyerowitz, EM. (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. *Curr Biol* 15: 303-315.

254 Boualem, A, Laporte, P, Jovanovic, M, Laffont, C, Plet, J, Combier, JP, Niebel, A, Crespi,
255 M, Frugier, F. (2008) MicroRNA166 controls root and nodule development in
256 *Medicago truncatula*. *Plant J* 54: 876-887.
257 Carlsbecker, A, Lee, JY, Roberts, CJ, Dettmer, J, Lehesranta, S, Zhou, J, Lindgren, O,
258 Moreno-Risueno, MA, Vaten, A, Thitamadee, S, Campilho, A, Sebastian, J,
259 Bowman, JL, Helariutta, Y, Benfey, PN. (2010) Cell signalling by microRNA165/6
260 directs gene dose-dependent root cell fate. *Nature* 465: 316-321.
261 Chen, X. (2005) MicroRNA biogenesis and function in plants. *FEBS Lett* 579: 5923-5931.
262 Coudert, Y, Perin, C, Courtois, B, Khong, NG, Gantet, P. Genetic control of root
263 development in rice, the model cereal. *Trends Plant Sci* 15: 219-226.
264 Coudert, Y, Perin, C, Courtois, B, Khong, NG, Gantet, P. (2010) Genetic control of root
265 development in rice, the model cereal. *Trends Plant Sci* 15: 219-226.
266 Cui, X, Xu, SM, Mu, DS, Yang, ZM. (2009) Genomic analysis of rice microRNA
267 promoters and clusters. *Gene* 431: 61-66.
268 Dello Ioio, R, Nakamura, K, Moubayidin, L, Perilli, S, Taniguchi, M, Morita, MT,
269 Aoyama, T, Costantino, P, Sabatini, S. (2008) A genetic framework for the control
270 of cell division and differentiation in the root meristem. *Science* 322: 1380-1384.
271 Faiyue, B, Al-Azzawi, MJ, Flowers, TJ. (2010a) The role of lateral roots in bypass flow in
272 rice (*Oryza sativa* L.). *Plant Cell Environ* 33: 702-716.
273 Faiyue, B, Vijayalakshmi, C, Nawaz, S, Nagato, Y, Taketa, S, Ichii, M, Al-Azzawi, MJ,
274 Flowers, TJ. (2010b) Studies on sodium bypass flow in lateral rootless mutants lrt1
275 and lrt2, and crown rootless mutant crl1 of rice (*Oryza sativa* L.). *Plant Cell*
276 *Environ* 33: 687-701.
277 Fukaki, H, Okushima, Y, Tasaka, M. (2007) Auxin-mediated lateral root formation in
278 higher plants. *Int Rev Cytol*256: 111-137.
279 Griffiths-Jones, S, Saini, HK, van Dongen, S, Enright, AJ. (2008) miRBase: tools for
280 microRNA genomics. *Nucleic Acids Res* 36: D154-158.
281 Guo, HS, Xie, Q, Fei, JF, Chua, NH. (2005) MicroRNA directs mRNA cleavage of the
282 transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root
283 development. *Plant Cell* 17: 1376-1386.
284 Gutierrez, L, Bussell, JD, Pacurar, DI, Schwambach, J, Pacurar, M, Bellini, C. (2009)
285 Phenotypic plasticity of adventitious rooting in Arabidopsis is controlled by
286 complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA
287 abundance. *Plant Cell* 21: 3119-3132.
288 Han, Y, Cao, H, Jiang, J, Xu, Y, Du, J, Wang, X, Yuan, M, Wang, Z, Xu, Z, Chong, K.
289 (2008) Rice ROOT ARCHITECTURE ASSOCIATED1 binds the proteasome
290 subunit RPT4 and is degraded in a D-box and proteasome-dependent manner. *Plant*
291 *Physiol* 148: 843-855.
292 Ikeda, M and Ohme-Takagi, M. (2009) A novel group of transcriptional repressors in
293 Arabidopsis. *Plant Cell Physiol* 50: 970-975.
294 Inukai, Y, Sakamoto, T, Ueguchi-Tanaka, M, Shibata, Y, Gomi, K, Umemura, I,
295 Hasegawa, Y, Ashikari, M, Kitano, H, Matsuoka, M. (2005) Crown rootless1,

296 which is essential for crown root formation in rice, is a target of an AUXIN
297 RESPONSE FACTOR in auxin signaling. *Plant Cell* 17: 1387-1396.
298 Iyer-Pascuzzi, A, Simpson, J, Herrera-Estrella, L, Benfey, PN. (2009) Functional genomics
299 of root growth and development in Arabidopsis. *Curr Opin Plant Biol* 12: 165-171.
300 Iyer-Pascuzzi, AS and Benfey, PN. (2009) Transcriptional networks in root cell fate
301 specification. *Biochim Biophys Acta* 1789: 315-325.
302 Kushwaha, H, Gupta, S, Singh, VK, Rastogi, S, Yadav, D. (2010) Genome wide
303 identification of Dof transcription factor gene family in sorghum and its
304 comparative phylogenetic analysis with rice and Arabidopsis. *Mol Biol Rep*
305 Laplaze, L, Benkova, E, Casimiro, I, Maes, L, Vanneste, S, Swarup, R, Weijers, D, Calvo,
306 V, Parizot, B, Herrera-Rodriguez, MB, Offringa, R, Graham, N, Doumas, P, Friml,
307 J, Bogusz, D, Beeckman, T, Bennett, M. (2007) Cytokinins act directly on lateral
308 root founder cells to inhibit root initiation. *Plant Cell* 19: 3889-3900.
309 Laufs, P, Peaucelle, A, Morin, H, Traas, J. (2004) MicroRNA regulation of the CUC genes
310 is required for boundary size control in Arabidopsis meristems. *Development* 131:
311 4311-4322.
312 Li, YF, Zheng, Y, Addo-Quaye, C, Zhang, L, Saini, A, Jagadeeswaran, G, Axtell, MJ,
313 Zhang, W, Sunkar, R. (2010) Transcriptome-wide identification of microRNA
314 targets in rice. *Plant J* 62: 742-759.
315 Liu, H, Wang, S, Yu, X, Yu, J, He, X, Zhang, S, Shou, H, Wu, P. (2005) ARL1, a LOB-
316 domain protein required for adventitious root formation in rice. *Plant J* 43: 47-56.
317 Liu, S, Wang, J, Wang, L, Wang, X, Xue, Y, Wu, P, Shou, H. (2009) Adventitious root
318 formation in rice requires OsGNOM1 and is mediated by the OsPINs family. *Cell*
319 *Res* 19: 1110-1119.
320 Mallory, AC, Dugas, DV, Bartel, DP, Bartel, B. (2004) MicroRNA regulation of NAC-
321 domain targets is required for proper formation and separation of adjacent
322 embryonic, vegetative, and floral organs. *Curr Biol*14: 1035-1046.
323 Mallory, AC and Vaucheret, H. (2006) Functions of microRNAs and related small RNAs
324 in plants. *Nat Genet* 38 Suppl: S31-36.
325 Martinez, NJ, Ow, MC, Reece-Hoyes, JS, Barrasa, MI, Ambros, VR, Walhout, AJ. (2008)
326 Genome-scale spatiotemporal analysis of *Caenorhabditis elegans* microRNA
327 promoter activity. *Genome Res* 18: 2005-2015.
328 McSteen, P. (2010) Auxin and monocot development. *Cold Spring Harb Perspect Biol* 2:
329 a001479.
330 Meng, Y, Huang, F, Shi, Q, Cao, J, Chen, D, Zhang, J, Ni, J, Wu, P, Chen, M. (2009)
331 Genome-wide survey of rice microRNAs and microRNA-target pairs in the root of
332 a novel auxin-resistant mutant. *Planta* 230: 883-898.
333 Meng, Y, Ma, X, Chen, D, Wu, P, Chen, M. (2010) MicroRNA-mediated signaling
334 involved in plant root development. *Biochem Biophys Res Commun* 393: 345-349.
335 Nakamura, A, Umemura, I, Gomi, K, Hasegawa, Y, Kitano, H, Sazuka, T, Matsuoka, M.
336 (2006) Production and characterization of auxin-insensitive rice by overexpression
337 of a mutagenized rice IAA protein. *Plant J* 46: 297-306.

Navarro, L, Dunoyer, P, Jay, F, Arnold, B, Dharmasiri, N, Estelle, M, Voinnet, O, Jones, JD. (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312: 436-439.

Nikovics, K, Blein, T, Peaucelle, A, Ishida, T, Morin, H, Aida, M, Laufs, P. (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. *Plant Cell* 18: 2929-2945.

Parizot, B, Laplaze, L, Ricaud, L, Boucheron-Dubuisson, E, Bayle, V, Bonke, M, De Smet, I, Poethig, SR, Helariutta, Y, Haseloff, J, Chriqui, D, Beeckman, T, Nussaume, L. (2008) Diarch symmetry of the vascular bundle in Arabidopsis root encompasses the pericycle and is reflected in distich lateral root initiation. *Plant Physiol* 146: 140-148.

Parry, G, Calderon-Villalobos, LI, Prigge, M, Peret, B, Dharmasiri, S, Itoh, H, Lechner, E, Gray, WM, Bennett, M, Estelle, M. (2009) Complex regulation of the TIR1/AFB family of auxin receptors. *Proc Natl Acad Sci U S A* 106: 22540-22545.

Peret, B, De Rybel, B, Casimiro, I, Benkova, E, Swarup, R, Laplaze, L, Beeckman, T, Bennett, MJ. (2009) Arabidopsis lateral root development: an emerging story. *Trends Plant Sci* 14: 399-408.

Pfaffl, MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.

Ross, EJ, Stone, JM, Elowsky, CG, Arredondo-Peter, R, Klucas, RV, Sarath, G. (2004) Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. *J Exp Bot* 55: 1721-1731.

Sakai, H, Honma, T, Aoyama, T, Sato, S, Kato, T, Tabata, S, Oka, A. (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* 294: 1519-1521.

Sheldon, CC, Conn, AB, Dennis, ES, Peacock, WJ. (2002) Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. *Plant Cell* 14: 2527-2537.

Sieber, P, Wellmer, F, Gheyselinck, J, Riechmann, JL, Meyerowitz, EM. (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* 134: 1051-1060.

Smale, ST. (2001) Core promoters: active contributors to combinatorial gene regulation. *Genes and Dev* 15: 2503-2508.

Suryapriya, P, Snehalatha, A, Kayalvili, U, Krishna, R, Singh, S, Ulaganathan, K. (2009) Genome-wide analyses of rice root development QTLs and development of an online resource, Rootbrowse. *Bioinformation* 3: 279-281.

Upadhyaya NM, Surin B, Ramm K, Gaudron J, Schünmann PHD, Taylor W, Waterhouse PM, Wang M-B. (2000) *Agrobacterium*-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. *Aust J Plant Physiol* 27:201–210.

Vieweg, MF, Fruhling, M, Quandt, HJ, Heim, U, Baumlein, H, Puhler, A, Kuster, H, Andreas, MP. (2004) The promoter of the *Vicia faba* L. leghemoglobin gene

VfLb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. *Mol Plant Microbe Interact* 17: 62-69.

Wang, JW, Wang, LJ, Mao, YB, Cai, WJ, Xue, HW, Chen, XY. (2005) Control of root cap formation by MicroRNA-targeted auxin response factors in Arabidopsis. *Plant Cell* 17: 2204-2216.

Zhang, ZH, Yu, JY, Li, DF, Zhang, ZY, Liu, FX, Zhou, X, Wang, T, Ling, Y, Su, Z. (2010) PMRD: plant microRNA database. *Nucleic Acids Res* 38: D806-D813.

Zhao, Y, Hu, Y, Dai, M, Huang, L, Zhou, DX. (2009) The WUSCHEL-related homeobox gene WOX11 is required to activate shoot-borne crown root development in rice. *Plant Cell* 21: 736-748.

Zheng, BS, Yang, L, Mao, CZ, Zhang, WP, Wu, P. (2006) QTLs and candidate genes for rice root growth under flooding and upland conditions. *Yi Chuan Xue Bao* 33: 141-151.

Zheng, BS, Yang, L, Zhang, WP, Mao, CZ, Wu, YR, Yi, KK, Liu, FY, Wu, P. (2003) Mapping QTLs and candidate genes for rice root traits under different water-supply conditions and comparative analysis across three populations. *Theor Appl Genet* 107: 1505-1515.

Zhou, X, Ruan, J, Wang, G, Zhang, W. (2007) Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol* 3: e37.

Table 1. Co-occurrence of *cis*-elements in all miR164 promoter sequences analysed

DNA motif	Consensus Sequence*
NODCON2GM	CTCTT
OSE2ROOTNODULE	CTCTT
PBF_01	NWNWAAAGNGN
DOF2_01	NNNWAAAGCNN
DOF3_01	NMNNAAAGNNN
Dof2	AAAGCN
WRKY71OS	TGAC
MYCCONSENSUSAT	CANNTG
Dof3	AAAGYV
POLLEN1LELAT52	AGAAA
RAV1AAT	CAACA
GATABOX	GATA
RAV1_01	NNGCAACAKAWN
RAV1-A	CAACA
GT1CONSENSUS	GRWAAW
ARR1AT	NGATT
Core	ATTA
TEIL_01	ATGWAYCT
DOFCOREZM	AAAG
MNB1A	AAAGH

* These DNA cis-elements found within the promoter sequences of miR164 genes of all species were analysed using Plant Pan (http://plantpan.mbc.nctu.edu.tw/gene_group/index.php). The variable nucleotides in the consensus sequences are N (A, G, C,and or T); W (A and or T); Y (T and or C); H (A, C, and or T); K (G and or T); V (G, A, and or C); R (A and or G).

Table 2. Common *cis*-elements in the promoter of all miR164 genes and their target genes .

DNA motif	Consensus Sequence*
MYCCONSENSUSAT	CANNTG
GT1CONSENSUS	GRWAAW
GATABOX	GATA
ARR1AT	NGATT
Core	ATTA
RAV1-A	CAACA
RAV1_01	NNGCAACAKAWN
RAV1AAT	CAACA

* These DNA cis-elements found within the promoter sequences of osaMIR164 target genes were analysed using Plant Pan (http://plantpan.mbc.nctu.edu.tw/gene_group/index.php). The variable nucleotides in the consensus sequences are N (A, G, C,and or T); W (A and or T); Y (T and or C); H (A, C, and or T); K (G and or T); V (G, A, and or C); R (A and or G).

Figure 1. Localization of different members of miR164 family in rice roots. A, spatial expression patterns of miR164a in lateral roots of the seminal root (a1: mature regions; a2: younger regions; a3: root apex. B, spatial expression patterns of miR164d in lateral roots. C, spatial expression patterns of miR164f in the main roots and in lateral roots (c1: seminal root; c2: crown roots). D, cross section of a crown root showing miR164a expression in the endodermis. E, cross section of a crown root showing miR164f expression in the stele.

Figure 2. Phenotype of miR164 overexpression lines. A, wild type. B, overexpression line L7. C, overexpression line L1. D, magnification of wild type roots. E, magnification of roots of overexpression line L7. SR: seminal roots; LR: lateral roots.

Figure 3. Real time analysis of miR164 target genes in the miR164 overexpression lines. Two miR164 target genes, Os06g46270 and Os08g10080 were analysed by RT-qPCR using OsUbi5 as a reference gene. Each bar represents a mean of four technical replicates.

Fig. 1.

Fig. 1. Continued

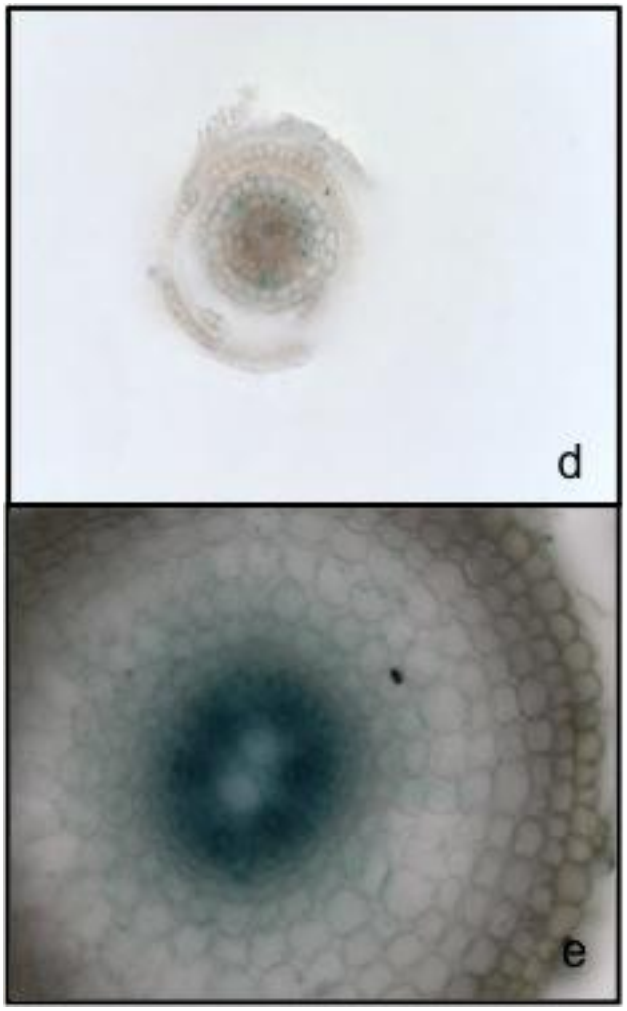


Fig.2.

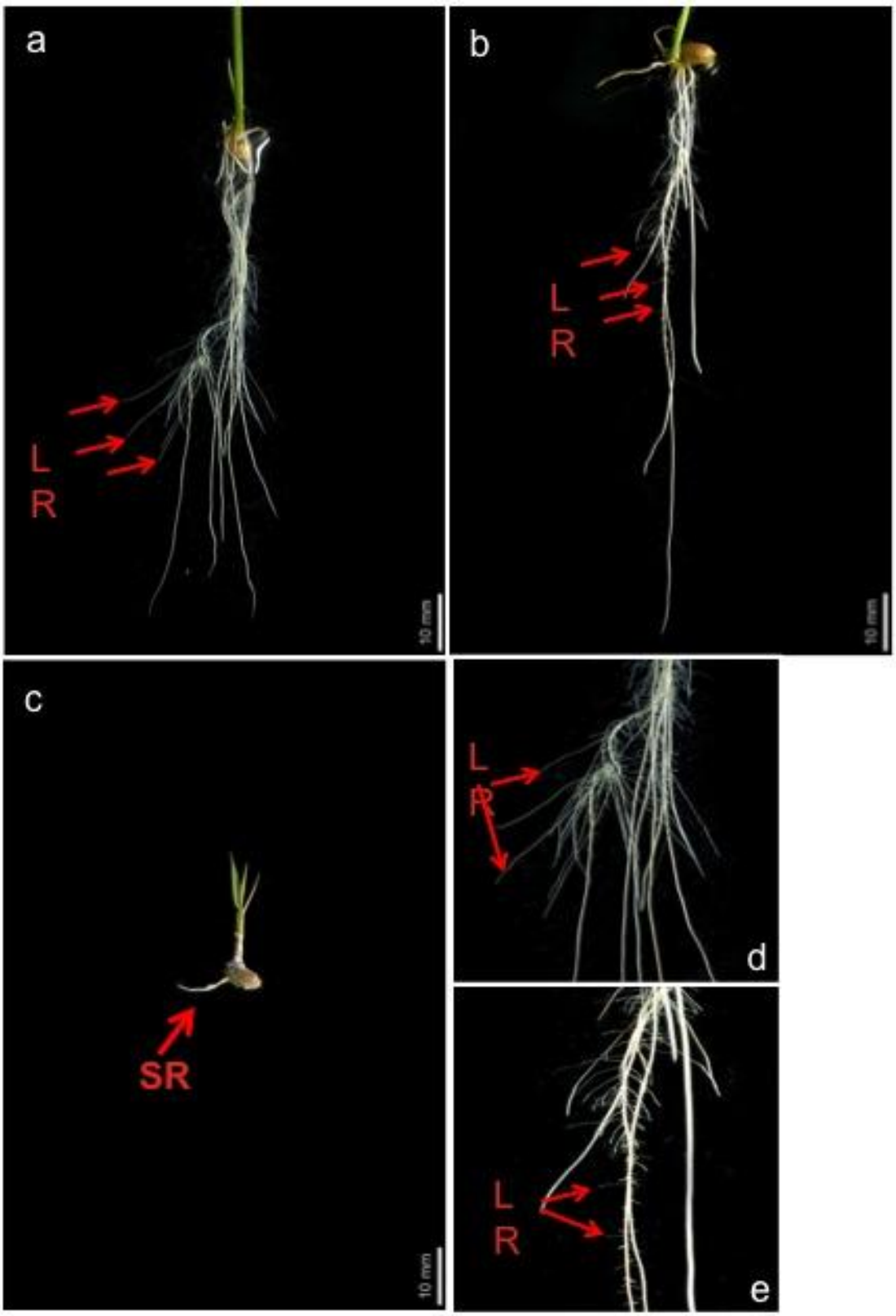
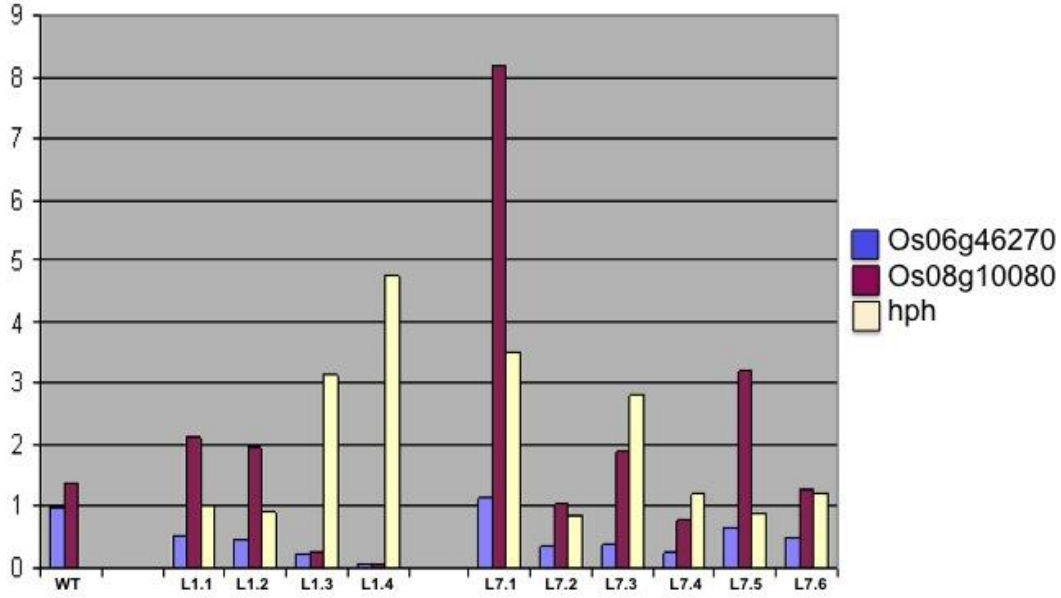


Fig. 3.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Os08g10080
hph

CAPÍTULO IV: Plant microRNAs as central regulators during development and abiotic stress responses.

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Title: Plant microRNAs as central regulators during development and abiotic stress responses.

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Running title: MicroRNAs and plants

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Abbreviations: TF – transcription factors; ROS – reactive oxygen species; ARFs – auxin response factors; GRF – growth-regulating factors; CBP – copper ion binding protein; RNA Pol II – RNA Polimerase II; AGO – Argonaute protein; NAC – no apical meristem (TF); CUC – cup-shaped cotyledon (TF); SCR – scarecrow like (TF); SHR – short root (TF); HD-Zip III – homeodomain of Class III TF; SPL – squamosa promoter binding like protein (TF); TOE1 and TOE2 – target of eat (TF); *cg1* – corn grass mutant gene; *ZmTSH4* – gene tassel sheath 4 of maize that codes for an SBP- box (TF); GAMYB – TF containing a giberelic acid R2R3 MYB domain; TCP - teosinte branched/Cycloidea/PCF (TF); SOD1 and SOD2 – superoxide dismutase proteins.

Abstract: MiRNAs have been identified as key molecules in regulatory networks. The fine-tuning role of miRNAs in addition to the regulatory role of transcription factors has shown that molecular events during development are tight regulated. In addition, several miRNAs play crucial roles in the response to abiotic stress induced by drought, salinity, low temperatures, and metals such as aluminium. Interestingly, several miRNAs have overlapping roles with regard to developmental, stress responses, and nutrient homeostasis. Moreover, in response to the same abiotic stresses, different expression patterns for some conserved miRNA families among different plant species revealed different metabolic adjustments. The use of deep sequencing technologies for the characterisation of miRNA frequency and the identification of new miRNAs adds complexity to regulatory networks in plants. In this review, we consider the regulatory role of miRNAs in plant development and abiotic stresses, as well as the impact of deep sequencing technologies on the generation of miRNA data.

INTRODUCTION

Gene transcription is a key mechanism regulated by transcription factors and also by distinct small RNAs of 21 to 24 nucleotide of length that can act at the transcriptional and post-transcriptional levels (Jamalkandi and Masoudi-Nejad 2009; Voinnet 2009). Distinct biochemical pathways generate three major classes of small RNAs: short interfering RNAs (siRNAs), piwi-interacting RNAs occurring exclusively in animals (piRNAs), and microRNAs (miRNAs) (Ramachandran and Chen 2008; Chen 2009; Jamalkandi and Masoudi-Nejad 2009; Liu and Paroo 2010). In plants, the regulation of gene expression mediated by small RNAs initiates after the generation of double stranded RNAs in the cells, which are recognized by RNase III-like enzymes called Dicers, processed into small interfering RNAs, and loaded into protein complexes to effectuate gene silencing after the recognition of different complementary target RNAs and or DNA (Fig. 1) (Voinnet 2009; Krol et al. 2010).

Although there are three major classes of small RNAs, miRNAs play specific roles in the regulation of gene expression in plants. MiRNA genes originated from inverted duplications and random sequences in the genome (Felippes et al. 2008; Voinnet 2009). They are transcribed by RNA Pol II into long primary polyadenylated RNA molecules and processed into mature miRNAs by Dicer proteins (Parizotto et al. 2004). In plants, several biological experiments indicate that miRNAs play key roles during development and in response to environmental stresses (Fig. 2) (Sunkar 2010). The growing number of miRNAs has revealed the high complexity of genomes and biochemical and metabolic pathways in plants. Different miRNAs can act as regulators, from very early developmental phases to the reproductive phase (Chen 2009). Although the study of the regulatory roles of miRNAs uncovered a new field in plant biology, the roles of several miRNAs remain to be discovered.

Here, in a concise review, the regulatory action of miRNAs in development and response to abiotic stress will be discussed. Briefly, the new sequencing technologies will be discussed as they apply to the characterisation and identification of new miRNAs.

In plants, mutations in the genes involved in biogenesis and the regulatory roles of miRNAs produce strong effects on development. These effects demonstrate the crucial role of miRNAs in development (Ramachandran and Chen 2008; Chen 2009; Xie et al. 2010). The AGO genes, especially the miR168a and miR168b-regulated AGO1, have a fundamental role in the stabilisation and regulatory action of other miRNAs (Vaucheret et al. 2004). In *Arabidopsis thaliana*, mutations in the MIR168a gene did not affect plant development under normal growth conditions. However, under stressful conditions, *A. thaliana* plants had less vigour and flowered early (Vaucheret 2009). Although some miRNA families are numerous, there are few examples in the literature that uncover functions for individual members (Chen 2009). One good example is the TF (transcription factor) coding genes NAC and CUC that are regulated by the miR164 family in *A. thaliana*, which are important in root and shoot development (Baker et al. 2005; Guo et al. 2005; Nikovics et al. 2006; Sieber et al. 2007; Raman et al. 2008). Triple mutants of *miR164abc* revealed that the genes athMIR164a and athMIR164b partially overlap athMIR164c function during floral development, as the phenotype became more severe in the triple mutant (Sieber et al. 2007). Individual mutants for athMIR164a and athMIR164b result in plants with more roots, which enhances the functional role of the miR164 family (Guo et al. 2005). In addition, the availability of nitrogen affects the development of roots regulated by the auxin receptor module AFB3/miR393 in *A. thaliana* (Vidal et al. 2010). To allow for proper root development, the SHR and SCR proteins activate the MIRNA165a and MIR166b genes, which in turn negatively regulate the TF HD-ZipIII (Carlsbecker et al. 2010). Mutant rice plants insensitive for auxin revealed the numerous miRNAs and complex regulatory signals involved in root development (Meng et al. 2009).
It is already known that miR156 regulates the SPL genes and that plants overexpressing miR156 are semi-dwarf, have altered numbers of leaves, and have longer vegetative phase (Xie et al. 2006b; Wang et al. 2008; Zhang et al. 2011c). The downregulated expression of miR156 from the juvenile to the adult phase is in contrast to the upregulation of miR172, which is an important regulator of the flowering repressors APETALA2, TOE1 and TOE2 (Aukerman and Sakai 2003; Wu et al. 2009; Zhu and Helliwell 2010). Interestingly, *cg1* mutants showed an overexpression of miR156 and reduced levels of miR172, suggesting

that the regulatory roles for these two miRNAs in the transition from the juvenile to the reproductive phase in maize and other plants is conserved (Chuck et al. 2007). Recently, transcriptional signals that modulate miR156 regulatory action were found to be crucial for phase change in plants (Yang et al. 2011). In maize, it was determined that the spatial expression of miR156, which regulates *ZmTSH4*, is crucial for the establishment of the lateral meristems (Chuck et al. 2010). Strikingly, the posttranscriptional regulation of *OsSPL14* by miR156 defines the rice plant architecture, with mutants displaying a reduced number of tillers and more branches in the panicles (Jiao et al. 2010; Miura et al. 2010). Throughout the different developmental stages of soybean seeds, 26 new miRNAs and their target genes were identified using deep sequencing and degradome approaches (Song et al. 2011).

Hormone signalling and gene expression under miRNA control have deterministic roles in plant development (Liu and Chen 2009; Liu et al. 2009a). In *A. thaliana*, miR159-targeting members of the GAMYB family regulates germination and anther formation (Reyes and Chua 2007). The overexpression of miR159 and the inhibition of MYB gene expression delayed flowering and caused male sterility (Millar and Gubler 2005). In Arabidopsis plants, *miR159ab* deregulation of the GAMYB-like genes resulted in reduction of the cell proliferation and programmed cell death (Alonso-Peral et al. 2010). Jasmonic acid biosynthesis is regulated by the TCP proteins, which are TFs. These TFs have functional roles in development and leaf senescence and are regulated by miR319 (Schommer et al. 2008). Functional genes in the auxin signalling pathway (ARFs) are miRNA targets. Plants with miR160-resistant forms of the *ARF10*, *ARF16* and *ARF17* genes showed pleiotropic effects in shoots and roots (Mallory et al. 2005; Liu et al. 2007). The overexpression of miR160 resulted in plants with less sensitivity to giberelic acid during germination (Liu et al. 2007). In flowering during stamens and gynoecium development, ARF6 and ARF8 are miR167 targets (Wu et al. 2006). The SCR family is targeted by miR171c to promote the proper development of auxiliary meristems during branching (Wang et al. 2010). Cell proliferation in *A. thaliana* is attenuated by the upregulation of miR396, which downregulates the GRF genes that are crucial regulators in the cell cycle (Rodriguez et al. 2010). The occurrence of stomata, crucial for plant transpiration, depends partially on miR824 targeting to the AGL16 gene (Kutter et al. 2007).

MiRNAS IN RESPONSE TO ABIOTIC STRESSES

In addition to the role of miRNAs in plant development, under abiotic stresses, they are dramatically affected and regulate several coding genes in plants (Reyes et al. 2010; Sunkar 2010). An understanding of how miRNAs act when they regulate gene expression and which coding genes are miRNA targets during stress responses, such as drought, salinity, metals, temperature and nutrient homeostasis, will help in the generation of more tolerant plants (Sunkar 2010).

THE REGULATORY ROLE OF MIRNAS IN PLANTS UNDER DROUGHT, SALINITY, ALUMINIUM, AND LOW TEMPERATURES

It has been suggested that miR393 is one of the key miRNAs during stress responses because of its altered expression in *A. thaliana*, *Oryza sativa*, *Medicago truncatula*, *Phaseolus vulgaris* and other plants under drought, salinity, low temperature, and aluminium stress conditions (Sunkar and Zhu 2004; Zhao et al. 2007; Liu et al. 2008; Arenas-Huertero et al. 2009; Trindade et al. 2010; Lima et al. 2011). However, the molecular evidence that miR393 regulates its targets in several environmental conditions remains to be considered. Recently, *Arabidopsis* plants overexpressing osaMIR393 became more tolerant to salt excess, suggesting a regulatory role in salinity tolerance (Gao et al. 2011). It is known that miRNAs from the miR169 family respond differently to drought, salinity, low temperatures and aluminium in plants (Zhao et al. 2007; Liu et al. 2008; Zhou et al. 2008a; Zhao et al. 2009a). In response to salinity and drought stresses in rice, the expression of NF-YA genes is modulated by members of the miR169 family (Zhao et al. 2009a). In *A. thaliana*, *nf-ya* plants and plants overexpressing miR169 are more sensitive to drought (Li et al. 2008). On the contrary in tomato, plants overexpressing miR169c, which targets a gene involved in the opening and closing of stomata, are more tolerant to drought (Zhang et al. 2011b). A reduction in the expression of miR530a, miR1445, miR1446a-e and miR1447 in *Populus trichocarpa* was detected in plants under drought and salinity, which is different from the downregulation of miR1450 under drought conditions and upregulation under high salinity (Lu et al. 2008). In *Triticum dicoccoides*, the ancestor of cultivated wheat, the upregulation of miR1450 revealed an inverse response

when compared with *Populus trichocarpa* under drought conditions (Kantar et al. 2011b). Although the gene MIR1450 is present in both monocot and dicots, the expression patterns suggest regulatory differences under drought (Lu et al. 2008; Kantar et al. 2011b). The formation of the superoxide anion O^{2-} in response to stresses is converted into less toxic molecules by SOD1 and SOD2 proteins, whose mRNAs are targeted by miR398 (Sunkar et al. 2006; Jagadeeswaran et al. 2009; Trindade et al. 2010; Kantar et al. 2011b). The inverse correlation between miRNAs miR156, miR166, miR171, miR408 and their targets were detected in barley plants under drought (Kantar et al. 2010). In different tissues from different developmental stages in rice plants under drought conditions in soil, miRNAs miR156, miR171 and miR408 were also detected (Zhou et al. 2010). In *Medicago truncatula*, miR408 acts to regulate plantacyanin genes in response to drought (Trindade et al. 2010). In acid soils, the availability of aluminium in low pH conditions inhibits root growth, which affects plant development dramatically (Ryan et al. 2011). Comparing *japonica* and *indica* subspecies, we have characterised the expression of miRNAs in rice plants treated with aluminium. Using RT-qPCR, it was possible to detect sixteen differentially expressed miRNAs in rice roots, which reveals a complex miRNA response in rice under aluminium stress. The inverse regulation of miR528 and its targets L-ascorbate oxidase and copper ion binding protein genes was also observed. This finding corresponds to the first report on the characterisation of the miRNA response in plants under aluminium stress (Lima et al. 2011).

MiRNAs AND THEIR REGULATORY ROLE IN THE RESPONSE TO UV-B RADIATION, HYPOXIA, AND OXIDATIVE STRESSES

The redox state of the cellular environment and the generation of ROS as a consequence of UV-B radiation and hypoxia reprograms plant responses due to eminent irreversible damage (Blokhina and Fagerstedt 2010; De Gara et al. 2010). The induction of miR166, miR396, miR395 and miR528 in maize plants under low oxygen points to a role for miRNA in morphological alterations and in sulphur and oxidative metabolism (Zhang et al. 2008). The repression of miR398 and the upregulation of SOD proteins has a crucial role in *Arabidopsis* plants under oxidative stress (Sunkar et al. 2006). The downregulation of miR395 and the induction of miR398, as well as the respective inversion of expression of

their targets in response to UV-B in *Populus tremula*, suggests that there are important differences in the stress-induced metabolic adjustments compared with *Arabidopsis* (Jia et al. 2009). By deep sequencing, the identification of miRNAs in rice plants under hydrogen superoxide treatment has broadened the roles for miRNAs in plants under oxidative stress (Li et al. 2010a).

THE IMPORTANCE OF MiRNAs IN NUTRIENT HOMEOSTASIS

The uptake of nutrients is a compulsory requirement of plants, and the homeostasis of nutrients is critical for the maintenance of growth and development (Giehl et al. 2009; Yang and Finnegan 2010). Sulphur is transported into the cell as sulphate and has a structural role in protein folding (Rausch and Wachter 2005). Under sulphate deficiency, miR395 down-regulates low affinity transporters and ATP sulphurilases (Jones-Rhoades and Bartel 2004). Interestingly, in *Arabidopsis* roots, both the sulphate transporter AST68 and miR395 were induced. The spatial expression patterns suggested that miR395 limits the expression of its targets in the phloem cells (Kawashima et al. 2009). Phosphate homeostasis is under miR399 regulation. Under low cellular phosphate levels, TF PHR1 activates miR399, which negatively regulates the *PHO2* gene, which has a role in protein degradation pathways (Bari et al. 2006). An alternative regulation in phosphate signalling is the expression of a non-coding RNA called IPS, which has a miR399 binding site with some mismatches that impair IPS cleavage by miR399 under ideal phosphate conditions. The sequestration of miRNAs by IPS (target mimicry) blocks the downregulation of PHO2 by miR399 (Franco-Zorrilla et al. 2007). Although target mimicry needs further investigation in other plants, it is functional and widespread in *Arabidopsis* (Todesco et al. 2010). In addition, by deep sequencing, the detection of several miRNAs revealed a much more complex regulatory network in phosphate signalling (Hsieh et al. 2009; Gu et al. 2010).

Metals like copper and iron are also essential micronutrients to plants. MiRNAs miR398, miR408 and miR857 are part of a signalling network that functions in the regulation of copper levels in plant cells. Under copper deficiency, these miRNAs were induced and negatively regulated their targets (Yamasaki et al. 2007; Burkhead et al. 2009). Several miRNAs were up-regulated in response to low iron levels in *Arabidopsis* (Kong and Yang

2010). Interestingly, a member of the miR854 family that is induced in plants under iron deficiency conditions is also conserved in animals, and its targets can be regulated via translation inhibition (Arteaga-Vazquez et al. 2006; Kong and Yang 2010). In nutrient metabolism, miR169 regulates its target NF-YA genes, which have an important role in the balance of nitrogen in plants (Zhao et al. 2011).

NEXT GENERATION SEQUENCING TECHNOLOGIES: HOW TO DEAL WITH AN INCREASING AMOUNT OF DATA?

Deep sequencing technologies are revolutionising molecular biology (Brautigam and Gowik 2010), lowering the costs of sequencing and increasing throughput by several orders of magnitude (Paszkiewicz and Studholme 2010). A deep sequencing approach was successfully applied for *de novo* sequencing of plant genomes (Imelfort and Edwards 2009), metagenomics studies in grapevines (Coetzee et al. 2010), sequencing of natural strains in Arabidopsis (Ossowski et al. 2008), RNA sequencing of different tissues from soybean (Severin et al. 2010) and miRNA identification in many organisms. In addition, deep sequencing approaches applied to the characterisation of miRNA frequency and the identification of new miRNAs that regulate development and abiotic stress responses brought more complexity to regulatory networks in plants (Li et al. 2010a; Song et al. 2011). The growing number of miRNAs identified mainly by deep sequencing is increasing with sequence data in databases as miRBase (Kozomara and Griffiths-Jones 2010) and the Plant MicroRNA Database – PMRD (Zhang et al. 2010), a specific databank for plant miRNAs. This database allows for the retrieval of a target gene, promoter sequence, and expression profile for some miRNA genes. However, the rapid increase in molecular data in databases represents only the tip of the iceberg, and these data demand more laborious analyses for the identification of miRNA function. Figure 3 shows the abundance of reads and the approximate size of deep sequence results files from the three distinct experiments from our group. At least three improvements are necessary for this scenario: i) a very robust software framework is needed for dealing with such a large amount of data, ii) hardware that can store very large files is needed, and iii) some programming skills are required for dealing with such a huge amount of information. Finally, the evolution of software and hardware for the systematic organisation of these

data seems one step behind high throughput sequencing technology. Whether we are producing more data than we can analyse and attribute a function to raises a question: how to place all microRNAs in the complex gene expression networks?

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The regulatory role of miRNAs in plants is definitely a subject that will require much more investigation in plant biology. As presented in this review, several miRNAs have been determined to be involved in plant development and abiotic stress responses. They are positioned for the fine-tuning of distinct regulatory networks. In addition, the identification of new miRNAs and their targets adds more complexity to gene expression regulatory networks. The increasing number of miRNAs identified by deep sequencing, in different and multiple experimental conditions, points to a need for further biological investigation. To address the question raised in the previous section we consider that the latest strategies for the understanding of individual miRNA function as miRNA target mimicry (Franco-Zorrilla et al. 2007), identification of miRNA target genes by degradome approach (German et al. 2008; Li et al. 2010c), and silencing of miRNAs (Eamens and Wang 2011) are suitable molecular tools to unravel the functional role of the increasing number of miRNAs (Fig. 4). Finally, since miRNAs regulate numerous transcription factors during development and in response to different stresses, high throuput analysis as RNA-seq (deep sequencing of mRNAs), proteomics and metabolomics should be always considered as complementary approaches to investigate the global effects of the conservation and diversity of miRNA responses in different biological conditions and in different plant species.

ACKNOWLEDGMENTS

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REFERENCES

Alonso-Peral, M.M., Li, J., Li, Y., Allen, R.S., Schnippenkoetter, W., Ohms, S., White, R.G., Millar, A.A. (2010) The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in Arabidopsis. *Plant Physiology*, 154, 757-771.

Arenas-Huertero, C., Perez, B., Rabanal, F., Blanco-Melo, D., De la Rosa, C., Estrada-Navarrete, G., Sanchez, F., Covarrubias, A.A., Reyes, J.L. (2009) Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. *Plant Molecular Biology*, 70, 385-401.

Arteaga-Vazquez, M., Caballero-Perez, J., Vielle-Calzada, J.P. (2006) A family of microRNAs present in plants and animals. *The Plant Cell*, 18, 3355-3369.

Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *The Plant Cell*, 15, 2730-2741.

Baker, C.C., Sieber, P., Wellmer, F., Meyerowitz, E.M. (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. *Current Biology*, 15, 303-315.

Bari, R., Datt Pant, B., Stitt, M., Scheible, W.R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology*, 141, 988-999.

Blokhina, O. and Fagerstedt, K.V. (2010) Oxidative metabolism, ROS and NO under oxygen deprivation. *Plant Physiology and Biochemistry*, 48, 359-373.

Brautigam, A. and Gowik, U. (2010) What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. *Plant Biology (Stuttg)*, 12, 831-841.

Burkhead, J.L., Reynolds, K.A., Abdel-Ghany, S.E., Cohu, C.M., Pilon, M. (2009) Copper homeostasis. *New Phytologist*

Carlsbecker, A., Lee, J.Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vaten, A., Thitamadee, S., Campilho, A., Sebastian, J., Bowman, J.L., Helariutta, Y., Benfey, P.N. (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature*, 465, 316-321.

Chen, X. (2009) Small RNAs and their roles in plant development. *Annual Review of Cell and Developmental Biology*, 25, 21-44.

Chuck, G., Cigan, A.M., Saeteurn, K., Hake, S. (2007) The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. *Nature Genetics*, 39, 544-549.

Chuck, G., Whipple, C., Jackson, D., Hake, S. (2010) The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries. *Development*, 137, 1243-1250.

Coetzee, B., Freeborough, M.J., Maree, H.J., Celton, J.M., Rees, D.J., Burger, J.T. (2010) Deep sequencing analysis of viruses infecting grapevines: Virome of a vineyard. *Virology*, 400, 157-163.

De Gara, L., Locato, V., Dipierro, S., de Pinto, M.C. (2010) Redox homeostasis in plants. The challenge of living with endogenous oxygen production. *Respiratory Physiology and Neurobiology*, 173 Suppl, S13-19.

Eamens, A.L. and Wang, M.B. (2011) Alternate approaches to repress endogenous microRNA activity in *Arabidopsis thaliana*. *Plant signaling & behavior*, 6,

Felippes, F.F., Schneeberger, K., Dezulian, T., Huson, D.H., Weigel, D. (2008) Evolution of *Arabidopsis thaliana* microRNAs from random sequences. *RNA*, 14, 2455-2459.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics*, 39, 1033-1037.

Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H., Ji, W., Chen, Q., Zhu, Y. (2011) osa-MIR393: a salinity- and alkaline stress-related microRNA gene. *Molecular Biology Reports*, 38, 237-242.

German, M.A., Pillay, M., Jeong, D.H., Hetawal, A., Luo, S., Janardhanan, P., Kannan, V., Rymarquis, L.A., Nobuta, K., German, R., De Paoli, E., Lu, C., Schroth, G., Meyers,

B.C., Green, P.J. (2008) Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. *Nature biotechnology*, 26, 941-946.

Giehl, R.F., Meda, A.R., von Wiren, N. (2009) Moving up, down, and everywhere: signaling of micronutrients in plants. *Current Opinion in Plant Biology*, 12, 320-327.

Gu, M., Xu, K., Chen, A., Zhu, Y., Tang, G., Xu, G. (2010) Expression analysis suggests potential roles of microRNAs for phosphate and arbuscular mycorrhizal signaling in *Solanum lycopersicum*. *Physiologia Plantarum*, 138, 226-237.

Guo, H.S., Xie, Q., Fei, J.F., Chua, N.H. (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. *The Plant Cell*, 17, 1376-1386.

Hsieh, L.C., Lin, S.I., Shih, A.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H., Chiou, T.J. (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. *Plant Physiology*, 151, 2120-2132.

Imelfort, M. and Edwards, D. (2009) De novo sequencing of plant genomes using second-generation technologies. *Briefings in Bioinformatics*, 10, 609-618.

Jagadeeswaran, G., Saini, A., Sunkar, R. (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. *Planta*, 229, 1009-1014.

Jamalkandi, S.A. and Masoudi-Nejad, A. (2009) Reconstruction of *Arabidopsis thaliana* fully integrated small RNA pathway. *Functional & Integrative Genomics*, 9, 419-432.

Jia, X., Ren, L., Chen, Q.J., Li, R., Tang, G. (2009) UV-B-responsive microRNAs in *Populus tremula*. *Journal of Plant Physiology*, 166, 2046-2057.

Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., Li, J. (2010) Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nature Genetics*, 42, 541-544.

Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Molecular Cell*, 14, 787-799.

Kantar, M., Lucas, S.J., Budak, H. (2011) miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. *Planta*, 233, 471-484.

Kantar, M., Unver, T., Budak, H. (2010) Regulation of barley miRNAs upon dehydration stress correlated with target gene expression. *Functional and Integrative Genomics*, 10, 493-507.

Kawashima, C.G., Yoshimoto, N., Maruyama-Nakashita, A., Tsuchiya, Y.N., Saito, K., Takahashi, H., Dalmay, T. (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *The Plant Journal*, 57, 313-321.

Kong, W.W. and Yang, Z.M. (2010) Identification of iron-deficiency responsive microRNA genes and cis-elements in Arabidopsis. *Plant Physiology and Biochemistry*, 48, 153-159.

Kozomara, A. and Griffiths-Jones, S. (2010) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Research*, 39, D152-157.

Krol, J., Loedige, I., Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics*, 11, 597-610.

Kutter, C., Schob, H., Stadler, M., Meins, F., Jr., Si-Ammour, A. (2007) MicroRNA-mediated regulation of stomatal development in Arabidopsis. *The Plant Cell*, 19, 2417-2429.

Li, T., Li, H., Zhang, Y.X., Liu, J.Y. (2010) Identification and analysis of seven H2O2-responsive miRNAs and 32 new miRNAs in the seedlings of rice (*Oryza sativa* L. ssp. *indica*). *Nucleic Acids Research*

Li, W.X., Oono, Y., Zhu, J., He, X.J., Wu, J.M., Iida, K., Lu, X.Y., Cui, X., Jin, H., Zhu, J.K. (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *The Plant Cell*, 20, 2238-2251.

Lima, J.C., Arenhart, R.A., Margis-Pinheiro, M., Margis, R. (2011) Aluminum triggers broad changes in microRNA expression in rice roots. *Genetics and Molecular Research* 'in press'

Liu, H.H., Tian, X., Li, Y.J., Wu, C.A., Zheng, C.C. (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, 14, 836-843.

Liu, P.P., Montgomery, T.A., Fahlgren, N., Kasschau, K.D., Nonogaki, H., Carrington, J.C. (2007) Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *The Plant Journal*, 52, 133-146.

Liu, Q. and Chen, Y.Q. (2009) Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. *Biochemical and Biophyscal Research Communications*, 384, 1-5.

Liu, Q. and Paroo, Z. (2010) Biochemical principles of small RNA pathways. *Annual Review of Biochemistry*, 79, 295-319.

Liu, Q., Zhang, Y.C., Wang, C.Y., Luo, Y.C., Huang, Q.J., Chen, S.Y., Zhou, H., Qu, L.H., Chen, Y.Q. (2009) Expression analysis of phytohormone-regulated microRNAs in rice, implying their regulation roles in plant hormone signaling. *FEBS Letters*, 583, 723-728.

Lu, S., Sun, Y.H., Chiang, V.L. (2008) Stress-responsive microRNAs in Populus. *The Plant Journal*, 55, 131-151.

Mallory, A.C., Bartel, D.P., Bartel, B. (2005) MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *The Plant Cell*, 17, 1360-1375.

Meng, Y., Huang, F., Shi, Q., Cao, J., Chen, D., Zhang, J., Ni, J., Wu, P., Chen, M. (2009) Genome-wide survey of rice microRNAs and microRNA-target pairs in the root of a novel auxin-resistant mutant. *Planta*, 230, 883-898.

Millar, A.A. and Gubler, F. (2005) The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are MicroRNA-regulated genes that redundantly facilitate anther development. *The Plant Cell*, 17, 705-721.

Miura, K., Ikeda, M., Matsubara, A., Song, X.J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., Ashikari, M. (2010) OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nature Genetics*, 42, 545-549.

Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M., Laufs, P. (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. *The Plant Cell*, 18, 2929-2945.

Ossowski, S., Schneeberger, K., Clark, R.M., Lanz, C., Warthmann, N., Weigel, D. (2008) Sequencing of natural strains of *Arabidopsis thaliana* with short reads. *Genome Research*, 18, 2024-2033.

Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C., Voinnet, O. (2004) In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes and Development*, 18, 2237-2242.

Paszkiewicz, K. and Studholme, D.J. (2010) De novo assembly of short sequence reads. *Briefings in Bioinformatics*, 11, 457-472.

Ramachandran, V. and Chen, X. (2008) Small RNA metabolism in Arabidopsis. *Trends in Plant Science*, 13, 368-374.

Raman, S., Greb, T., Peaucelle, A., Blein, T., Laufs, P., Theres, K. (2008) Interplay of miR164, CUP-SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in *Arabidopsis thaliana*. *The Plant Journal*, 55, 65-76.

Rausch, T. and Wachter, A. (2005) Sulfur metabolism: a versatile platform for launching defence operations. *Trends in Plant Science*, 10, 503-509.

Reyes, J.L., Arenas-Huertero, C., Sunkar, R. (2010) Cloning of stress-responsive microRNAs and other small RNAs from plants. *Methods in Molecular Biology*, 639, 239-251.

Reyes, J.L. and Chua, N.H. (2007) ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *The Plant Journal*, 49, 592-606.

Rodriguez, R.E., Mecchia, M.A., Debernardi, J.M., Schommer, C., Weigel, D., Palatnik, J.F. (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. *Development*, 137, 103-112.

Ryan, P.R., Tyerman, S.D., Sasaki, T., Furuichi, T., Yamamoto, Y., Zhang, W.H., Delhaize, E. (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *Journal of Experimental Botany*, 62, 9-20.

Schommer, C., Palatnik, J.F., Aggarwal, P., Chetelat, A., Cubas, P., Farmer, E.E., Nath, U., Weigel, D. (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biology*, 6, e230.

Severin, A.J., Woody, J.L., Bolon, Y.T., Joseph, B., Diers, B.W., Farmer, A.D., Muehlbauer, G.J., Nelson, R.T., Grant, D., Specht, J.E., Graham, M.A., Cannon, S.B., May, G.D., Vance, C.P., Shoemaker, R.C. (2010) RNA-Seq Atlas of Glycine max: a guide to the soybean transcriptome. *BMC Plant Biology*, 10, 160.

Sieber, P., Wellmer, F., Gheyselinck, J., Riechmann, J.L., Meyerowitz, E.M. (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development*, 134, 1051-1060.

Song, Q.X., Liu, Y.F., Hu, X.Y., Zhang, W.K., Ma, B., Chen, S.Y., Zhang, J.S. (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. *BMC Plant Biology*, 11, 5.

Sunkar, R. (2010) MicroRNAs with macro-effects on plant stress responses. *Seminars in Cell and Developmental Biology*, 21, 805-811.

Sunkar, R., Kapoor, A., Zhu, J.K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. *The Plant Cell*, 18, 2051-2065.

Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *The Plant Cell*, 16, 2001-2019.

Todesco, M., Rubio-Somoza, I., Paz-Ares, J., Weigel, D. (2010) A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. *PLoS Genetics*, 6, e1001031.

Trindade, I., Capitao, C., Dalmay, T., Fevereiro, M.P., Santos, D.M. (2010) miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta*, 231, 705-716.

Vaucheret, H. (2009) AGO1 homeostasis involves differential production of 21-nt and 22-nt miR168 species by MIR168a and MIR168b. *PLoS ONE*, 4, e6442.

Vaucheret, H., Vazquez, F., Crete, P., Bartel, D.P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes and Developmental*, 18, 1187-1197.

Vidal, E.A., Araus, V., Lu, C., Parry, G., Green, P.J., Coruzzi, G.M., Gutierrez, R.A. (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proceedings of the Natinal Academy of Sciences of the U S A*, 107, 4477-4482.

Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell*, 136, 669-687.

Wang, J.W., Schwab, R., Czech, B., Mica, E., Weigel, D. (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in *Arabidopsis thaliana*. *The Plant Cell*, 20, 1231-1243.

Wang, L., Mai, Y.X., Zhang, Y.C., Luo, Q., Yang, H.Q. (2010) MicroRNA171c-targeted SCL6-II, SCL6-III, and SCL6-IV genes regulate shoot branching in Arabidopsis. *Molecular Plant*, 3, 794-806.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., Poethig, R.S. (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell*, 138, 750-759.

Wu, M.F., Tian, Q., Reed, J.W. (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. *Development*, 133, 4211-4218.

Xie, K., Wu, C., Xiong, L. (2006) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant Physiology*, 142, 280-293.

Xie, Z., Khanna, K., Ruan, S. (2010) Expression of microRNAs and its regulation in plants. *Seminars in Cell and Developmental Biology*, 21, 790-797.

Yamasaki, H., Abdel-Ghany, S.E., Cohu, C.M., Kobayashi, Y., Shikanai, T., Pilon, M. (2007) Regulation of copper homeostasis by micro-RNA in Arabidopsis. *Journal of Biological Chemistry*, 282, 16369-16378.

Yang, L., Conway, S.R., Poethig, R.S. (2011) Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development*, 138, 245-249.

Yang, X.J. and Finnegan, P.M. (2010) Regulation of phosphate starvation responses in higher plants. *Annals of Botany*, 105, 513-526.

Zhang, X., Zou, Z., Gong, P., Zhang, J., Ziaf, K., Li, H., Xiao, F., Ye, Z. (2011a) Over-expression of microRNA169 confers enhanced drought tolerance to tomato. *Biotechnol Letters*, 33, 403-409.

Zhang, X., Zou, Z., Zhang, J., Zhang, Y., Han, Q., Hu, T., Xu, X., Liu, H., Li, H., Ye, Z. (2011b) Over-expression of sly-miR156a in tomato results in multiple vegetative and reproductive trait alterations and partial phenocopy of the sft mutant. *FEBS Letters*, 585, 435-439.

Zhang, Z., Wei, L., Zou, X., Tao, Y., Liu, Z., Zheng, Y. (2008) Submergence-responsive MicroRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. *Annals of Botany*, 102, 509-519.

Zhang, Z.H., Yu, J.Y., Li, D.F., Zhang, Z.Y., Liu, F.X., Zhou, X., Wang, T., Ling, Y., Su, Z. (2010) PMRD: plant microRNA database. *Nucleic Acids Research*, 38, D806-D813.

Zhao, M., Ding, H., Zhu, J.K., Zhang, F., Li, W.X. (2011) Involvement of miR169 in the nitrogen-starvation responses in Arabidopsis. *New Phytologist*, 'in press'.

Zhao, B., Ge, L., Liang, R., Li, W., Ruan, K., Lin, H., Jin, Y. (2009) Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Molecular Biology*, 10, 29.

Zhao, B.T., Liang, R.Q., Ge, L.F., Li, W., Xiao, H.S., Lin, H.X., Ruan, K.C., Jin, Y.X. (2007) Identification of drought-induced microRNAs in rice. *Biochemical and Biophysical Research Communications*, 354, 585-590.

Zhou, L., Liu, Y., Liu, Z., Kong, D., Duan, M., Luo, L. (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *Journal of Experimental Botany*, 61, 4157-4168.

Zhou, X., Wang, G., Sutoh, K., Zhu, J.K., Zhang, W. (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochimical and Biophysical Acta*, 1779, 780-788.

Zhu, Q.H. and Helliwell, C.A. (2010) Regulation of flowering time and floral patterning by miR172. *Journal of Experimental Botany*, 62, 487-495.

Fig. 1. General view of the small RNA pathway in plants. Cis/Trans-Acting small interfering RNAs (TAS) Pathway: A TAS gene is transcribed by RNA Pol II into a TAS precursor, later this precursor is recognized by a complementary siRNA and sliced by Argonaute (AGO) proteins into small RNA which serves as a template for RNA Dependent RNA Polimerases (RDR). This siRNA duplex directs cleavage of the TAS precursor in *cis*, or another target mRNAs in *trans*. MicroRNA Pathway: A MIR gene, usually located in intergenic or intronic region, is transcribed by RNA Pol II into a precursor RNA named pri-microRNA, which is stabilized and cleaved by a protein complex composed by Dicer-like proteins (DCL) and Hyponastic Leaves (HYL) into pre-microRNA which is further processed by the same complex plus Serrate (SE) into a mature microRNA. The HUA Enhancer (HEN) methylates the resulting mature microRNA form in the 2'-hydroxy termini of the 3' terminal region. This methylated mature form is exported to cytoplasm through HASTY protein (HST). Once in the cytoplasm, AGO proteins recognize one strand of the mature microRNA and direct it to the target gene.

Later, the AGO can induce the slicing of mRNA target or repress the translation complex. The other microRNA strand is directed to the Exosome and degradated by Small RNA Degrading Nuclease (SDN). Natural Acting Small RNAs (NAT) Pathway: Overlapping genes can be transcribed by RNA Pol II, resulting in a NAT precursor complementary to a siRNA, which serves as a template to the RDR proteins. The DCL protein cleaves this double-stranded precursor into dsRNAs, which are exported to the cytosol by HST protein. The NAT-siRNAs loaded into AGO complexes induce mRNA degradation in the same way as for microRNA pathway.

Fig. 2. Mature miRNAs act in plant development, in response to abiotic stresses, and also in the control of nutrient homeostasis. In the presented model, miRNAs and their target genes are modulated in an inverse way for the fine-tuning of plant development, in response to abiotic stresses, and in the regulation of nutrient homeostasis. Depending on the plant species, the miRNA/target genes are modulated differently in the same biological conditions. Only miRNA targets already confirmed by expression analysis and/or degradome sequencing were included in this diagram.

Fig. 3. Small RNA sequencing results from the Illumina Solexa Genome Analyzer or Hi-Seq2000 platforms. Bars indicate the sizes of the output files (bytes) of each platform. TB: TeraBytes. The line shows the abundance of reads (units) of each platform. RNA samples were extracted from different tissues of *Oryza sativa*, *Glycine max* and *Jatropha curcas* and submitted for deep sequencing. A. Raw data received from deep sequencing. B. Data consisting of the nucleotide range lengths of putative microRNAs.

Fig. 4. General representation of the degradome sequencing approach, miRNA target mimicry, and silencing of miRNAs. The degradome sequencing approach has been used to detect miRNA target genes through deep sequencing of cleaved mRNAs and mapping of cleaved sites. Target mimicry is a suitable molecular tool that is based on the expression of a transgene carrying a non-real target of a miRNA that is partially complementary to the miRNA. This RNA contains three to four nucleotides that are not complementary to a core region in the middle of the miRNA, which causes the sequestration of miRNAs, blocks cleavage and up-regulates the real target mRNAs. Silencing of miRNAs triggered by

artificial miRNAs targeting the mature and or precursor miRNA, directs cleavage and RNA silencing of the precursor miRNAs and upregulation of miRNA target RNAs.

Fig.1.

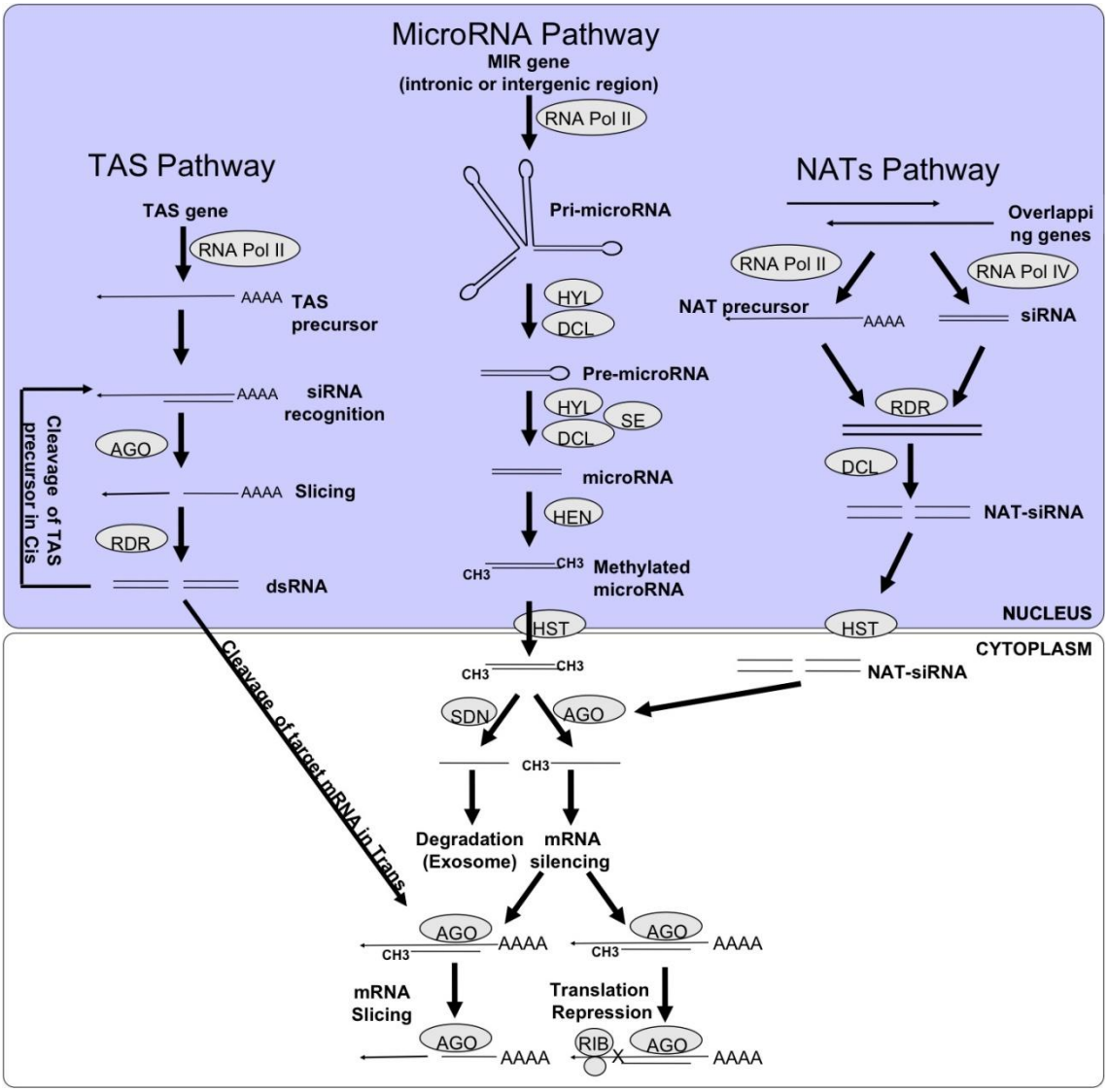

MicroRNA Pathway
MIR gene
(intronic or intergenic region)
RNA Pol II
Pri-microRNA
HYL
DCL
Pre-microRNA
HYL
SE
DCL
microRNA
HEN
CH3
CH3
Methylated
microRNA
HST
TAS Pathway
TAS gene
RNA Pol II
AAAA
TAS
precursor
AAAA
siRNA
recognition
AGO
AAAA
Slicing
RDR
dsRNA
Cleavage of TAS
precursor in Cis
NATs Pathway
Overlappi
ng genes
RNA Pol II
RNA Pol IV
NAT precursor
AAAA
siRNA
RDR
DCL
NAT-siRNA
HST
NUCLEUS
CYTOPLASM
NAT-siRNA
CH3
CH3
SDN
AGO
CH3
Degradation
(Exosome)
mRNA
silencing
Cleavage of target mRNA in Trans
AGO
CH3
AAAA
AGO
CH3
AAAA
mRNA
Slicing
Translation
Repression
AGO
AAAA
RIB
AGO
AAAA

Fig. 2.

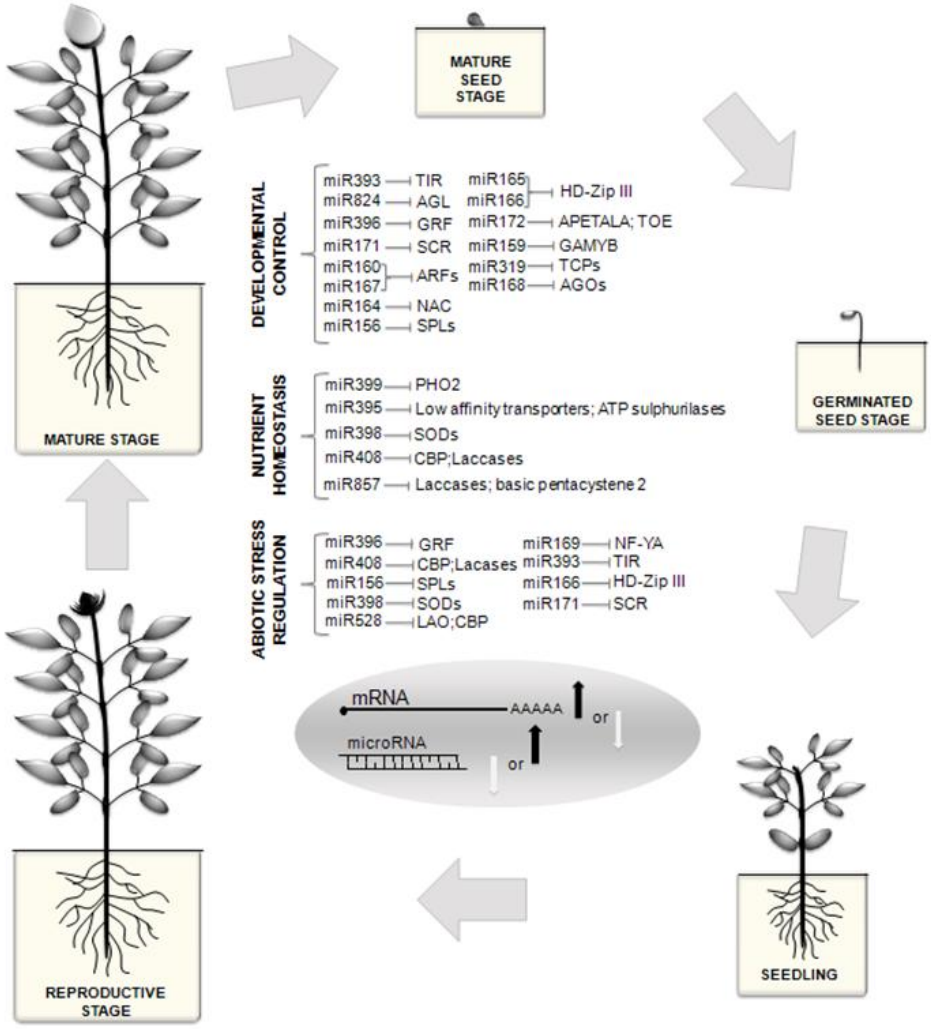
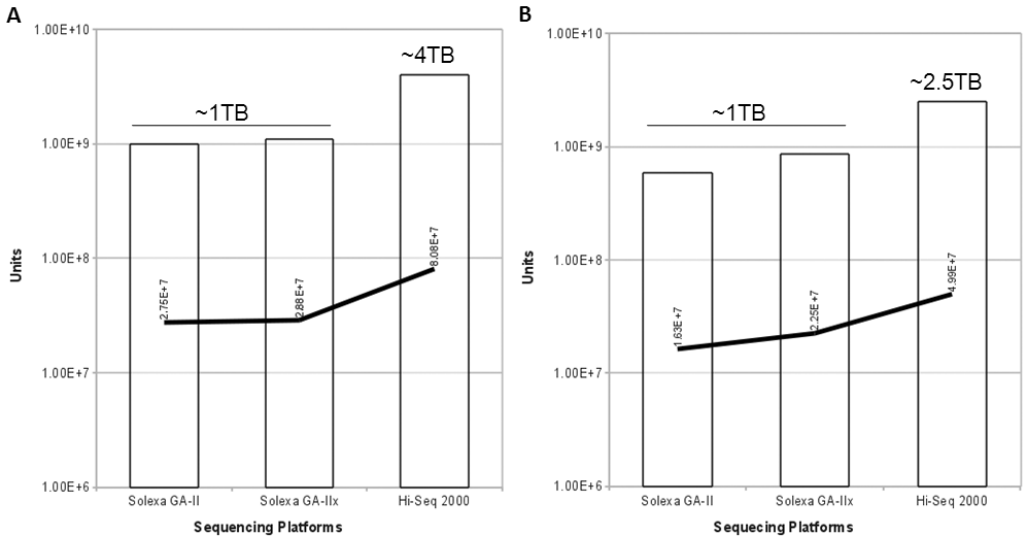

MATURE SEED STAGE
GERMINATED SEED STAGE
SEEDLING
REPRODUCTIVE STAGE
MATURE STAGE
DEVELOPMENTAL CONTROL
miR393 TIR
miR824 AGL
miR396 GRF
miR171 SCR
miR160 ARFs
miR167
miR164 NAC
miR156 SPLs
miR165 miR166 HD-Zip III
miR172 APETALA; TOE
miR159 GAMYB
miR319 TCPs
miR168 AGOs
NUTRIENT HOMEOSTASIS
miR399 PHO2
miR395 Low affinity transporters; ATP sulphurilases
miR398 SODs
miR408 CBP;Laccases
miR857 Laccases; basic pentacystene 2
ABIOTIC STRESS REGULATION
miR396 GRF
miR408 CBP;Lacases
miR156 SPLs
miR398 SODs
miR528 LAO;CBP
miR169 NF-YA
miR393 TIR
miR166 HD-Zip III
miR171 SCR
mRNA
AAAAA
or
microRNA
or

Fig. 3


A
1.00E+10
1.00E+9
1.00E+8
1.00E+7
1.00E+6
Units
~1TB
~4TB
2.75E+7
2.88E+7
6.08E+7
Solexa GA-II
Solexa GA-IIx
Hi-Seq 2000
Sequencing Platforms
B
1.00E+10
1.00E+9
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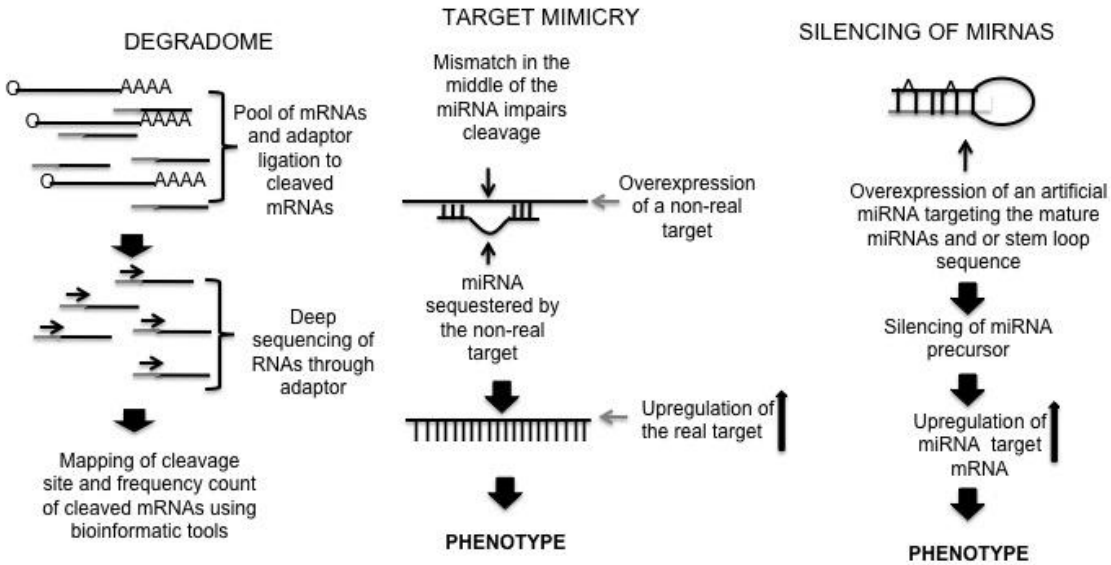
Solexa GA-II
Solexa GA-IIx
Hi-Seq 2000
Sequecing Platforms

Fig. 4.


DEGRADOME
AAAA
AAAA
AAAA
Pool of mRNAs and adaptor ligation to cleaved mRNAs
Deep sequencing of RNAs through adaptor
Mapping of cleavage site and frequency count of cleaved mRNAs using bioinformatic tools
TARGET MIMICRY
Mismatch in the middle of the miRNA impairs cleavage
Overexpression of a non-real target
miRNA sequestered by the non-real target
Upregulation of the real target
PHENOTYPE
SILENCING OF MIRNAS
Overexpression of an artificial miRNA targeting the mature miRNAs and or stem loop sequence
Silencing of miRNA precursor
Upregulation of miRNA target mRNA
PHENOTYPE

CAPÍTULO V: Conclusões, considerações finais e perspectivas

CONCLUSÕES E CONSIDERAÇÕES FINAIS

O papel regulatório dos microRNAs ao longo do desenvolvimento e em resposta a diferentes condições biológicas está confirmado pelos diversos trabalhos na literatura que demonstram, ao menos, uma resposta diferencial ao nível de expressão dos microRNAs e na ação regulatória sobre seus alvos (Li et al., 2010c; Meng et al., 2010b; Xie et al., 2010)

A RESPOSTA DOS miRNAS EM PLANTAS DE ARROZ TRATADAS COM ALUMÍNIO

A presença do alumínio em solos ácidos pode ocasionar danos irreversíveis nas plantas (Ryan et al., 2011). A compreensão dos mecanismos fisiológicos, bioquímicos e genéticos das respostas ao alumínio deve tornar possível a identificação dos pontos chave nas respostas de tolerância e sensibilidade e ao desenvolvimento de plantas mais tolerantes. Os resultados desta tese demonstraram que diversos microRNAs respondem de forma diferente ao tratamento com alumínio. Comparando-se as plantas de arroz *japonica* cv. Nipponbare e arroz *indica* cv. TAIM sugere-se que parte da resposta dos microRNAs ao alumínio é diferente, pois dos 19 microRNAs diferencialmente expressos nas raízes do arroz *japonica*, somente 9 foram detectados como diferencialmente expressos no arroz *indica*. A confirmação, por RT-qPCR, de dois alvos do miR528, especialmente o gene L-Ascorbato Oxidase revela que a resposta ao alumínio via microRNAs deve estar relacionada com a regulação da divisão celular. É relatado na literatura que a proteína L-Ascorbato Oxidase está relacionada ao bloqueio do ciclo celular e que a inversão da expressão do miR528 e do gene alvo L-Ascorbato Oxidase em arroz pode estar ligada `a regulação da divisão celular (Xue et al., 2009). Este dado molecular pode estar relacionado ao fato de que um dos primeiros fenótipos da resposta ao alumínio em plantas é o crescimento inibido das raízes (Panda et al., 2009). Especula-se que o miR808 deve ter um papel inverso em relação `a tolerância a altos níveis de alumínio em arroz, pois seu perfil de expressão é oposto comparando-se as plantas de arroz *indica* e *japonica*. Através da análise de microarranjos, foram identificados diversos microRNAs como responsivos ao cádmio em arroz. Apesar de os dados da confirmação da expressão de alguns microRNAs

não refletirem a correlação inversa com os alvos, foi confirmada a expressão diferencial do miR166 na resposta ao cádmio por RT-qPCR (Ding et al., 2011). No nosso trabalho, o miR166k foi detectado como diferencialmente expresso em raízes de arroz *japonica* tratadas com Al. Apesar de não termos confirmado o gene HD Zip como alvo do miR166 em resposta ao Al, já foi relatado na literatura o envolvimento deste microRNA regulando genes HD ZIP no desenvolvimento de raízes em Arabidopsis (Carlsbecker et al., 2010). Embora a resposta diferencial ao Al e cádmio dos microRNAs possa ser diferente, seria interessante investigar melhor o papel do miR166 em resposta ao Al em arroz. Outros microRNAs como o miR415, miR426, miR809a e o miR813 também podem ter um papel diferencial no mecanismo de tolerância ao alumínio em arroz. Porém, serão necessários mais experimentos, como a confirmação dos prováveis alvos que estes microRNAs regulam em resposta ao alumínio. Alguns dos microRNAs identificados como diferencialmente expressos em resposta ao alumínio, também respondem de forma diferencial em outras condições biológicas, como frente a estresses por sal, seca e frio (Sunkar 2010; Trindade et al., 2010; Gao et al., 2011). É interessante observar que as respostas das plantas aos diferentes estímulos por seca, sal, frio, hormônios e metais, tem similaridades quanto a presença de microRNAs de diferentes famílias nas rotas de sinais que culminam nas respostas da plantas (Sunkar 2010). Isto sugere que o papel de um mesmo microRNA não está relacionado somente a um tipo de resposta, mas a um papel mais universal nas respostas das plantas a diferentes estímulos. De outro modo, é relevante mencionar que um determinado microRNA pode ou não se comportar da mesma forma nas respostas das plantas a diferentes estímulos ou até mesmo em plantas de diferentes espécies. Por exemplo, plantas de arroz e Arabidopsis superexpressando o osa-miR393 tornaram-se mais sensíveis ao estresse por sal (Gao et al., 2011). Interessantemente, plantas de Arabidopsis superexpressando o miR169 tornaram-se mais sensíveis a seca, enquanto que plantas de tomateiro superexpressando o miR169c tornaram-se mais tolerantes (Li et al., 2008; Zhang et al., 2011b).

O DESENVOLVIMENTO DE RAÍZES LATERAIS EM PLANTAS É REGULADO POR miRNAS

Em um trabalho publicado recentemente foi discutido que o papel dos microRNAs no desenvolvimento de raízes está intimamente ligado `as respostas aos circuitos regulatórios em alça (feedback-loops), nutrientes e a hormônios como a auxina (Meng et al., 2010b). Em Arabidopsis, a superexpressão do miR164 ocasionou uma diminuição no numero de raízes laterais. Por outro lado, a superexpressão do gene do fator transcricional NAC1, alvo do miR164, contendo mutações no sitio de ligação do microRNA gerou plantas com mais raízes laterais (Guo et al., 2005).

Os dados preliminares da caracterização das plantas de arroz superexpressando o miR164 demonstram que há uma diminuição na presença de raízes laterais nas plantas transgênicas. Porém, outros dados como número de raízes laterais e a confirmação dos alvos do miR164 ainda serão obtidos a fim de confirmar a ação regulatória do miR164.

Em relação `a expressão espacial dos microRNAs, pode-se observar que o miR164a e o miR164d estão localizados nas raízes laterais. Já o miR164f é expresso também na raiz primária. Os cortes realizados manualmente revelaram que a expressão do miR164f está concentrada no estelo, região que compreende os tecidos do floema, xilema e periciclo. É citado na literatura que o início da formação de raízes laterais é dependente da ativação das células do periciclo (Fukaki et al., 2007). Portanto, sugere-se que a expressão do miR164f nas células do periciclo tem papel regulatório no correto estabelecimento das raízes laterais. Especula-se que a expressão do miR164a na endoderme e a expressão do miR164f no estelo pode revelar um padrão específico de regulação e função dos diferentes membros da família miR164 em diferentes camadas e tipos celulares nas raízes de plantas de arroz. Porém, os resultados ainda não são suficientes para corroborar qualquer hipótese a respeito. É necessária uma análise de todos os membros da família miR164 para confirmar a hipótese acima.

O PAPEL DOS miRNAS NA REGULAÇÃO DO DESENVOLVIMENTO E NAS RESPOSTAS A ESTRESSES ABIÓTICOS

As plantas possuem um programa genético ao longo do desenvolvimento em resposta aos mais variados estresses, cuja regulação ocorre ao nível transcricional e pós-transcricional (Kaufmann et al., 2010; Xie et al., 2010). Os microRNAs desempenham papel crucial no desenvolvimento e nas respostas das plantas a estresses (Sunkar 2010; Xie et al., 2010). Em relação ao papel regulatório e função biológica, alguns casos melhor estudados podem ser discutidos aqui. Os microRNAs miR156 e miR172, cujas funções estão tanto relacionadas `a regulação da transição da fase jovem para a fase adulta das plantas, quanto nas respostas a estresses têm sugerido uma crucial importância destes, como reguladores tanto ao longo do desenvolvimento quanto nas respostas a estresses (Wu and Poethig 2006; Wu et al., 2009; Zhou et al., 2010). Os miR164, miR390, miR166 e miR165 são exemplos de microRNAs envolvidos nas redes de sinais que regulam o desenvolvimento de raízes (Guo et al., 2005; Carlsbecker et al., 2010; Meng et al., 2010b). Outro exemplo bem caracterizado em relação `a resposta de microRNAs a estresses é o do miR398, que regula os genes que codificam as proteínas superóxido dismutases envolvidas nas respostas ao estresse oxidativo. Em condições de estresse, este microRNA funciona como um ativador de genes importantes na detoxificação de espécies reativas de oxigênio que podem ser danosas `as células (Jagadeeswaran et al., 2009; Trindade et al., 2010; Kantar et al., 2011b). Diversos microRNAs conservados entre plantas monocotiledôneas e dicotiledôneas regulam diferentes fatores transcricionais ao longo do desenvolvimento e em resposta a diferentes estresses. Isto sugere que em plantas, o crescimento e o desenvolvimento em condições de estresse são, em parte, modulados por vias reguladas por microRNAs (Sunkar 2010).

As novas tecnologias de seqüenciamento em massa revolucionaram a biologia molecular (Brautigam and Gowik 2010), porém tornaram mais complexa a compreensão do papel regulatório dos microRNAs. Através do uso destas tecnologias é possível notar que diversos microRNAs funcionam tanto como reguladores do crescimento e desenvolvimento e como moléculas reguladoras das respostas das plantas aos mais variados estresses. O uso destas tecnologias tornou possível a identificação e caracterização da expressão de centenas de microRNAs e outros pequenos RNAs nas mais

diversas condições biológicas nas quais podemos testar as plantas. Porém, atribuir papeis regulatórios aos diferentes microRNAs e seus alvos nas complexas redes de sinais ao longo do desenvolvimento e em resposta a estresses ainda é uma tarefa que demandará tempo.

PERSPECTIVAS

- Identificar e analisar a frequência de microRNAs em plantas de arroz sob altas concentrações de alumínio por sequencimento em massa;
- Utilizar a abordagem de seqüenciamento do "degradoma" de RNAs para identificar os alvos dos microRNAs diferencialmente expressos em plantas de arroz tratadas com alumínio;
- determinar o padrão de expressão espacial dos demais membros da família miR164 em raízes de plantas de arroz;
- confirmar o fenótipo das linhagens de arroz superexpressando o miR164b através da determinação do tamanho e número de raízes laterais;

- determinar o número de cópias do transgene nas diferentes linhagens de arroz obtidas;

- confirmar os alvos do miR164 em plantas de arroz por 5'RACE;

REFERÊNCIAS dos capítulos I e V

Allen, E, Xie, Z, Gustafson, AM, Carrington, JC. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121: 207-221.

Allen, E, Xie, Z, Gustafson, AM, Sung, GH, Spatafora, JW, Carrington, JC. (2004) Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*. *Nat Genet* 36: 1282-1290.

Alonso-Peral, MM, Li, J, Li, Y, Allen, RS, Schnippenkoetter, W, Ohms, S, White, RG, Millar, AA. (2010) The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in Arabidopsis. *Plant Physiol* 154: 757-771.

Arenas-Huertero, C, Perez, B, Rabanal, F, Blanco-Melo, D, De la Rosa, C, Estrada-Navarrete, G, Sanchez, F, Covarrubias, AA, Reyes, JL. (2009) Conserved and novel miRNAs in the legume *Phaseolus vulgaris* in response to stress. *Plant Mol Biol* 70: 385-401.

Arteaga-Vazquez, M, Caballero-Perez, J, Vielle-Calzada, JP. (2006) A family of microRNAs present in plants and animals. *Plant Cell* 18: 3355-3369.

Aukerman, MJ and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* 15: 2730-2741.

Baker, CC, Sieber, P, Wellmer, F, Meyerowitz, EM. (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. *Curr Biol* 15: 303-315.

Bari, R, Datt Pant, B, Stitt, M, Scheible, WR. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol* 141: 988-999.

Bartel, DP. (2007) MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 131: 11-29.

Bartels, S, Gonzalez Besteiro, MA, Lang, D, Ulm, R. (2010) Emerging functions for plant MAP kinase phosphatases. *Trends Plant Sci* 15: 322-329.

Blokhina, O and Fagerstedt, KV. (2010) Oxidative metabolism, ROS and NO under oxygen deprivation. *Plant Physiol Biochem* 48: 359-373.

Boualem, A, Laporte, P, Jovanovic, M, Laffont, C, Plet, J, Combier, JP, Niebel, A, Crespi, M, Frugier, F. (2008) MicroRNA166 controls root and nodule development in *Medicago truncatula*. *Plant J* 54: 876-887.

Boudsocq, M. (2010) [Plant calcium sensors in osmotic signaling]. *Biol Aujourdhui* 204: 21-31.

Boudsocq, M, Barbier-Brygoo, H, Lauriere, C. (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J Biol Chem* 279: 41758-41766.

Brautigam, A and Gowik, U. (2010) What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. *Plant Biol (Stuttg)* 12: 831-841.

Burkhead, JL, Reynolds, KA, Abdel-Ghany, SE, Cohu, CM, Pilon, M. (2009) Copper homeostasis. *New Phytol*

Carlsbecker, A, Lee, JY, Roberts, CJ, Dettmer, J, Lehesranta, S, Zhou, J, Lindgren, O, Moreno-Risueno, MA, Vaten, A, Thitamadee, S, Campilho, A, Sebastian, J, Bowman, JL, Helariutta, Y, Benfey, PN. (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* 465: 316-321.

Chen, F, He, G, He, H, Chen, W, Zhu, X, Liang, M, Chen, L, Deng, XW. (2010) Expression analysis of miRNAs and highly-expressed small RNAs in two rice subspecies and their reciprocal hybrids. *J Int Plant Biol* 52: 971-980.

Chen, X. (2005) MicroRNA biogenesis and function in plants. *FEBS Lett* 579: 5923-5931.

Chen, X. (2009) Small RNAs and their roles in plant development. *Annu Rev Cell Dev Biol* 25: 21-44.

Chen, XM. (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* 303: 2022-2025.

Chitwood, DH, Nogueira, FT, Howell, MD, Montgomery, TA, Carrington, JC, Timmermans, MC. (2009) Pattern formation via small RNA mobility. *Genes Dev* 23: 549-554.

Chuck, G, Cigan, AM, Saeteurn, K, Hake, S. (2007) The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. *Nat Genet* 39: 544-549.

Chuck, G, Whipple, C, Jackson, D, Hake, S. (2010) The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries. *Development* 137: 1243-1250.

Coetzee, B, Freeborough, MJ, Maree, HJ, Celton, JM, Rees, DJ, Burger, JT. (2010) Deep sequencing analysis of viruses infecting grapevines: Virome of a vineyard. *Virology* 400: 157-163.

Collins, NC, Shirley, NJ, Saeed, M, Pallotta, M, Gustafson, JP. (2008) An ALMT1 gene cluster controlling aluminum tolerance at the Alt4 locus of rye (*Secale cereale* L). *Genetics* 179: 669-682.

Coudert, Y, Perin, C, Courtois, B, Khong, NG, Gantet, P. Genetic control of root development in rice, the model cereal. *Trends Plant Sci* 15: 219-226.

Cui, X, Xu, SM, Mu, DS, Yang, ZM. (2009) Genomic analysis of rice microRNA promoters and clusters. *Gene* 431: 61-66.

De Gara, L, Locato, V, Dipierro, S, de Pinto, MC. (2010) Redox homeostasis in plants. The challenge of living with endogenous oxygen production. *Respir Physiol Neurobiol* 173 Suppl: S13-19.

Delhaize, E, Gruber, BD, Ryan, PR. (2007) The roles of organic anion permeases in aluminium resistance and mineral nutrition. *FEBS Lett* 581: 2255-2262.

Delhaize, E and Ryan, PR. (1995) Aluminum Toxicity and Tolerance in Plants. *Plant Physiol* 107: 315-321.

Dello Ioio, R, Nakamura, K, Moubayidin, L, Perilli, S, Taniguchi, M, Morita, MT, Aoyama, T, Costantino, P, Sabatini, S. (2008) A genetic framework for the control of cell division and differentiation in the root meristem. *Science* 322: 1380-1384.

Des Marais, DL and Juenger, TE. (2010) Pleiotropy, plasticity, and the evolution of plant abiotic stress tolerance. *Ann N Y Acad Sci* 1206: 56-79.

Ding, Y, Chen, Z, Zhu, C. (2011) Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*). *J Exp Bot*

Duressa, D, Soliman, K, Chen, D. (2010) Identification of Aluminum Responsive Genes in Al-Tolerant Soybean Line PI 416937. *Int J Plant Genom* 2010:

Eamens, AL and Wang, MB. (2011) Alternate approaches to repress endogenous microRNA activity in *Arabidopsis thaliana*. *Plant Signal Behav* 6:

Faiyue, B, Al-Azzawi, MJ, Flowers, TJ. (2010a) The role of lateral roots in bypass flow in rice (*Oryza sativa* L.). *Plant Cell Environ* 33: 702-716.

Faiyue, B, Vijayalakshmi, C, Nawaz, S, Nagato, Y, Taketa, S, Ichii, M, Al-Azzawi, MJ, Flowers, TJ. (2010b) Studies on sodium bypass flow in lateral rootless mutants lrt1 and lrt2, and crown rootless mutant crl1 of rice (*Oryza sativa* L.). *Plant Cell Environ* 33: 687-701.

Felippes, FF, Schneeberger, K, Dezulian, T, Huson, DH, Weigel, D. (2008) Evolution of *Arabidopsis thaliana* microRNAs from random sequences. *RNA* 14: 2455-2459.

Franco-Zorrilla, JM, Valli, A, Todesco, M, Mateos, I, Puga, MI, Rubio-Somoza, I, Leyva, A, Weigel, D, Garcia, JA, Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet* 39: 1033-1037.

Fukaki, H, Okushima, Y, Tasaka, M. (2007) Auxin-mediated lateral root formation in higher plants. *Int Rev Cytol* 256: 111-137.

Furukawa, J, Yamaji, N, Wang, H, Mitani, N, Murata, Y, Sato, K, Katsuhara, M, Takeda, K, Ma, JF. (2007) An aluminum-activated citrate transporter in barley. *Plant Cell Physiol* 48: 1081-1091.

Gao, P, Bai, X, Yang, L, Lv, D, Pan, X, Li, Y, Cai, H, Ji, W, Chen, Q, Zhu, Y. (2011) osa-MIR393: a salinity- and alkaline stress-related microRNA gene. *Mol Biol Rep* 38: 237-242.

Garcia, D, Collier, SA, Byrne, ME, Martienssen, RA. (2006) Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway. *Curr Biology* 16: 933-938.

German, MA, Pillay, M, Jeong, DH, Hetawal, A, Luo, S, Janardhanan, P, Kannan, V, Rymarquis, LA, Nobuta, K, German, R, De Paoli, E, Lu, C, Schroth, G, Meyers, BC, Green, PJ. (2008) Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. *Nat Biotechnol* 26: 941-946.

Giehl, RF, Meda, AR, von Wiren, N. (2009) Moving up, down, and everywhere: signaling of micronutrients in plants. *Curr Opin Plant Biol* 12: 320-327.

Griffiths-Jones, S. (2004) The microRNA Registry. *Nucleic Acids Res* 32: D109-111.

Griffiths-Jones, S. (2006) miRBase: the microRNA sequence database. *Methods Mol Biol* 342: 129-138.

Griffiths-Jones, S, Saini, HK, van Dongen, S, Enright, AJ. (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res* 36: D154-158.

Grisel, N, Zoller, S, Kunzli-Gontarczyk, M, Lampart, T, Munsterkotter, M, Brunner, I, Bovet, L, Metraux, JP, Sperisen, C. (2010) Transcriptome responses to aluminum stress in roots of aspen (*Populus tremula*). *BMC Plant Biol* 10: 185.

Gu, M, Xu, K, Chen, A, Zhu, Y, Tang, G, Xu, G. (2010) Expression analysis suggests potential roles of microRNAs for phosphate and arbuscular mycorrhizal signaling in Solanum lycopersicum. *Physiol Plant* 138: 226-237.

Guerra-Assuncao, JA and Enright, AJ. (2010) MapMi: automated mapping of microRNA loci. *BMC Bioinform* 11: 133.

Guo, HS, Xie, Q, Fei, JF, Chua, NH. (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. *Plant Cell* 17: 1376-1386.

Gutierrez, L, Bussell, JD, Pacurar, DI, Schwambach, J, Pacurar, M, Bellini, C. (2009) Phenotypic plasticity of adventitious rooting in Arabidopsis is controlled by

complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA abundance. *Plant Cell* 21: 3119-3132.

Hackenberg, M, Sturm, M, Langenberger, D, Falcon-Perez, JM, Aransay, AM. (2009) miRanalyzer: a microRNA detection and analysis tool for next-generation sequencing experiments. *Nucleic Acids Res* 37: W68-76.

Han, Y, Cao, H, Jiang, J, Xu, Y, Du, J, Wang, X, Yuan, M, Wang, Z, Xu, Z, Chong, K. (2008) Rice ROOT ARCHITECTURE ASSOCIATED1 binds the proteasome subunit RPT4 and is degraded in a D-box and proteasome-dependent manner. *Plant Physiol* 148: 843-855.

Hirayama, T and Shinozaki, K. (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant Journal* 61: 1041-1052.

Hoekenga, OA, Maron, LG, Pineros, MA, Cancado, GM, Shaff, J, Kobayashi, Y, Ryan, PR, Dong, B, Delhaize, E, Sasaki, T, Matsumoto, H, Yamamoto, Y, Koyama, H, Kochian, LV. (2006) AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. *Proc Natl Acad Sci U S A* 103: 9738-9743.

Hossain, MA, Cho, JI, Han, M, Ahn, CH, Jeon, JS, An, G, Park, PB. (2010) The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. *J Plant Physiol* 167: 1512-1520.

Hsieh, LC, Lin, SI, Shih, AC, Chen, JW, Lin, WY, Tseng, CY, Li, WH, Chiou, TJ. (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. *Plant Physiol* 151: 2120-2132.

Huang, CF, Yamaji, N, Ma, JF. (2010) Knockout of a bacterial-type ATP-binding cassette transporter gene, AtSTAR1, results in increased aluminum sensitivity in Arabidopsis. *Plant Physiol* 153: 1669-1677.

Huang, CF, Yamaji, N, Mitani, N, Yano, M, Nagamura, Y, Ma, JF. (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. *Plant Cell* 21: 655-667.

Ikeda, M and Ohme-Takagi, M. (2009) A novel group of transcriptional repressors in Arabidopsis. *Plant Cell Physiol* 50: 970-975.

Imelfort, M and Edwards, D. (2009) De novo sequencing of plant genomes using second-generation technologies. *Brief Bioinform* 10: 609-618.

Inukai, Y, Sakamoto, T, Ueguchi-Tanaka, M, Shibata, Y, Gomi, K, Umemura, I, Hasegawa, Y, Ashikari, M, Kitano, H, Matsuoka, M. (2005) Crown rootless1, which is essential for crown root formation in rice, is a target of an AUXIN RESPONSE FACTOR in auxin signaling. *Plant Cell* 17: 1387-1396.

IRGSP. (2005) The map-based sequence of the rice genome. *Nature* 436: 793-800.

Islam, MS and Wang, MH. (2009) Expression of dehydration responsive element-binding protein-3 (DREB3) under different abiotic stresses in tomato. *BMB Rep* 42: 611-616.

Iyer-Pascuzzi, A, Simpson, J, Herrera-Estrella, L, Benfey, PN. (2009) Functional genomics of root growth and development in Arabidopsis. *Curr Opin Plant Biol* 12: 165-171.

Iyer-Pascuzzi, AS and Benfey, PN. (2009) Transcriptional networks in root cell fate specification. *Biochim Biophys Acta* 1789: 315-325.

Jagadeeswaran, G, Saini, A, Sunkar, R. (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. *Planta* 229: 1009-1014.

Jahn, CE, McKay, JK, Mauleon, R, Stephens, J, McNally, KL, Bush, DR, Leung, H, Leach, JE. (2011) Genetic Variation in Biomass Traits among 20 Diverse Rice Varieties. *Plant Physiol* 155: 157-168.

Jamalkandi, SA and Masoudi-Nejad, A. (2009) Reconstruction of *Arabidopsis thaliana* fully integrated small RNA pathway. *Func Integr Genomics* 9: 419-432.

Jeanteur, P. (2010) [miRNAs and cancer]. *Bull Cancer* 97: 1231-1239.

Jia, X, Ren, L, Chen, QJ, Li, R, Tang, G. (2009) UV-B-responsive microRNAs in *Populus tremula*. *J Plant Physiol* 166: 2046-2057.

Jiao, Y, Wang, Y, Xue, D, Wang, J, Yan, M, Liu, G, Dong, G, Zeng, D, Lu, Z, Zhu, X, Qian, Q, Li, J. (2010) Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nat Genet* 42: 541-544.

Johnson, C, Bowman, L, Adai, AT, Vance, V, Sundaresan, V. (2007) CSRDB: a small RNA integrated database and browser resource for cereals. *Nucleic Acids Res* 35: D829-833.

Jones-Rhoades, MW and Bartel, DP. (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Mol Cell* 14: 787-799.

Jones-Rhoades, MW, Bartel, DP, Bartel, B. (2006) MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* 57: 19-53.

Kantar, M, Lucas, SJ, Budak, H. (2011b) miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. *Planta* 233: 471-484.

Kantar, M, Unver, T, Budak, H. (2010) Regulation of barley miRNAs upon dehydration stress correlated with target gene expression. *Func Integr Genomics* 10: 493-507.

Kaufmann, K, Pajoro, A, Angenent, GC. (2010) Regulation of transcription in plants: mechanisms controlling developmental switches. *Nat Rev Genet* 11: 830-842.

Kawashima, CG, Yoshimoto, N, Maruyama-Nakashita, A, Tsuchiya, YN, Saito, K, Takahashi, H, Dalmay, T. (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. *Plant Journal* 57: 313-321.

Khush, GS. (1997) Origin, dispersal, cultivation and variation of rice. *Plant Mol Biol* 35: 25-34.

Kochian, LV, Hoekenga, OA, Pineros, MA. (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu Rev Plant Biol* 55: 459-493.

Kong, WW and Yang, ZM. (2010) Identification of iron-deficiency responsive microRNA genes and cis-elements in Arabidopsis. *Plant Physiol Biochem* 48: 153-159.

Kozomara, A and Griffiths-Jones, S. (2010) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* 39: D152-157.

Krol, J, Loedige, I, Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 11: 597-610.

Kushwaha, H, Gupta, S, Singh, VK, Rastogi, S, Yadav, D. (2010) Genome wide identification of Dof transcription factor gene family in sorghum and its comparative phylogenetic analysis with rice and Arabidopsis. *Mol Biol Rep*

Kutter, C, Schob, H, Stadler, M, Meins, F, Jr., Si-Ammour, A. (2007) MicroRNA-mediated regulation of stomatal development in Arabidopsis. *Plant Cell* 19: 2417-2429.

Laplaze, L, Benkova, E, Casimiro, I, Maes, L, Vanneste, S, Swarup, R, Weijers, D, Calvo, V, Parizot, B, Herrera-Rodriguez, MB, Offringa, R, Graham, N, Doumas, P, Friml,

J, Bogusz, D, Beeckman, T, Bennett, M. (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19: 3889-3900.

Larsen, PB, Cancel, J, Rounds, M, Ochoa, V. (2007) Arabidopsis ALS1 encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. *Planta* 225: 1447-1458.

Larsen, PB, Geisler, MJ, Jones, CA, Williams, KM, Cancel, JD. (2005) ALS3 encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in Arabidopsis. *Plant J* 41: 353-363.

Laufs, P, Peaucelle, A, Morin, H, Traas, J. (2004) MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. *Development* 131: 4311-4322.

Lee, RC, Feinbaum, RL, Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75: 843-854.

Leung, AK and Sharp, PA. (2010) MicroRNA functions in stress responses. *Mol Cell* 40: 205-215.

Li, T, Li, H, Zhang, YX, Liu, JY. (2010a) Identification and analysis of seven H2O2-responsive miRNAs and 32 new miRNAs in the seedlings of rice (*Oryza sativa* L. ssp. indica). *Nucleic Acids Res*

Li, WX, Oono, Y, Zhu, J, He, XJ, Wu, JM, Iida, K, Lu, XY, Cui, X, Jin, H, Zhu, JK. (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *Plant Cell* 20: 2238-2251.

Li, X, Yan, W, Agrama, H, Hu, B, Jia, L, Jia, M, Jackson, A, Moldenhauer, K, McClung, A, Wu, D. (2010b) Genotypic and phenotypic characterization of genetic differentiation and diversity in the USDA rice mini-core collection. *Genetica* 138: 1221-1230.

Li, YF, Zheng, Y, Addo-Quaye, C, Zhang, L, Saini, A, Jagadeeswaran, G, Axtell, MJ, Zhang, W, Sunkar, R. (2010c) Transcriptome-wide identification of microRNA targets in rice. *Plant J* 62: 742-759.

Lin, PC, Pomeranz, MC, Jikumaru, Y, Kang, SG, Hah, C, Fujioka, S, Kamiya, Y, Jang, JC. (2011) The Arabidopsis tandem zinc finger protein AtTZF1 affects ABA- and GA-mediated growth, stress and gene expression responses. *Plant J* 65: 253-268.

Liu, H, Wang, S, Yu, X, Yu, J, He, X, Zhang, S, Shou, H, Wu, P. (2005) ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J* 43: 47-56.

Liu, HH, Tian, X, Li, YJ, Wu, CA, Zheng, CC. (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA* 14: 836-843.

Liu, PP, Montgomery, TA, Fahlgren, N, Kasschau, KD, Nonogaki, H, Carrington, JC. (2007) Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *Plant J* 52: 133-146.

Liu, Q and Chen, YQ. (2009) Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. *Biochem Biophy Res Commun* 384: 1-5.

Liu, Q and Paroo, Z. (2010) Biochemical principles of small RNA pathways. *Annu Rev Biochem* 79: 295-319.

Liu, Q, Zhang, YC, Wang, CY, Luo, YC, Huang, QJ, Chen, SY, Zhou, H, Qu, LH, Chen, YQ. (2009a) Expression analysis of phytohormone-regulated microRNAs in rice, implying their regulation roles in plant hormone signaling. *FEBS Lett* 583: 723-728.

Liu, S, Wang, J, Wang, L, Wang, X, Xue, Y, Wu, P, Shou, H. (2009b) Adventitious root formation in rice requires OsGNOM1 and is mediated by the OsPINs family. *Cell Res* 19: 1110-1119.

Lu, S, Sun, YH, Chiang, VL. (2008) Stress-responsive microRNAs in Populus. *Plant J* 55: 131-151.

Lumba, S, Cutler, S, McCourt, P. (2010) Plant nuclear hormone receptors: a role for small molecules in protein-protein interactions. *Annu Rev Cell Dev Biol* 26: 445-469.

Ma, JF, Ryan, PR, Delhaize, E. (2001) Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci* 6: 273-278.

Magalhaes, JV, Liu, J, Guimaraes, CT, Lana, UG, Alves, VM, Wang, YH, Schaffert, RE, Hoekenga, OA, Pineros, MA, Shaff, JE, Klein, PE, Carneiro, NP, Coelho, CM, Trick, HN, Kochian, LV. (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. *Nat Genet* 39: 1156-1161.

Mallory, AC, Bartel, DP, Bartel, B. (2005) MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* 17: 1360-1375.

Mallory, AC, Dugas, DV, Bartel, DP, Bartel, B. (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr Biol* 14: 1035-1046.

Mallory, AC and Vaucheret, H. (2006) Functions of microRNAs and related small RNAs in plants. *Nat Genet* 38 Suppl: S31-36.

Maron, LG, Pineros, MA, Guimaraes, CT, Magalhaes, JV, Pleiman, JK, Mao, C, Shaff, J, Belicuas, SN, Kochian, LV. (2010) Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J* 61: 728-740.

Martinez, NJ, Ow, MC, Reece-Hoyes, JS, Barrasa, MI, Ambros, VR, Walhout, AJ. (2008) Genome-scale spatiotemporal analysis of *Caenorhabditis elegans* microRNA promoter activity. *Genome Res* 18: 2005-2015.

Matsumoto, H. (2000) Cell biology of aluminum toxicity and tolerance in higher plants. *Int Rev Cytol* 200: 1-46.

Mattiello, L, Kirst, M, da Silva, FR, Jorge, RA, Menossi, M. (2010) Transcriptional profile of maize roots under acid soil growth. *BMC Plant Biol* 10: 196.

McSteen, P. (2010) Auxin and monocot development. *Cold Spring Harb Perspect Biol* 2: a001479.

Meng, Y, Gou, L, Chen, D, Mao, C, Jin, Y, Wu, P, Chen, M. (2010a) PmiRKB: a plant microRNA knowledge base. *Nucleic Acids Res* 39: D181-187.

Meng, Y, Huang, F, Shi, Q, Cao, J, Chen, D, Zhang, J, Ni, J, Wu, P, Chen, M. (2009) Genome-wide survey of rice microRNAs and microRNA-target pairs in the root of a novel auxin-resistant mutant. *Planta* 230: 883-898.

Meng, Y, Ma, X, Chen, D, Wu, P, Chen, M. (2010b) MicroRNA-mediated signaling involved in plant root development. *Biochem Biophys Res Commun* 393: 345-349.

Meriga, B, Reddy, BK, Rao, KR, Reddy, LA, Kishor, PB. (2004) Aluminium-induced production of oxygen radicals, lipid peroxidation and DNA damage in seedlings of rice (*Oryza sativa*). *J Plant Physiol* 161: 63-68.

Millar, AA and Gubler, F. (2005) The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are MicroRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* 17: 705-721.

Miura, K, Ikeda, M, Matsubara, A, Song, XJ, Ito, M, Asano, K, Matsuoka, M, Kitano, H, Ashikari, M. (2010) OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nat Genet* 42: 545-549.

Moxon, S, Schwach, F, Dalmay, T, Maclean, D, Studholme, DJ, Moulton, V. (2008) A toolkit for analysing large-scale plant small RNA datasets. *Bioinformatics* 24: 2252-2253.

Nagasaki, H, Itoh, J, Hayashi, K, Hibara, K, Satoh-Nagasawa, N, Nosaka, M, Mukouhata, M, Ashikari, M, Kitano, H, Matsuoka, M, Nagato, Y, Sato, Y. (2007) The small interfering RNA production pathway is required for shoot meristem initiation in rice. *Proc Natl Acad Sci U S A* 104: 14867-14871.

Nakamura, A, Umemura, I, Gomi, K, Hasegawa, Y, Kitano, H, Sazuka, T, Matsuoka, M. (2006) Production and characterization of auxin-insensitive rice by overexpression of a mutagenized rice IAA protein. *Plant Journal* 46: 297-306.

Navarro, L, Dunoyer, P, Jay, F, Arnold, B, Dharmasiri, N, Estelle, M, Voinnet, O, Jones, JD. (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312: 436-439.

Nikovics, K, Blein, T, Peaucelle, A, Ishida, T, Morin, H, Aida, M, Laufs, P. (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. *Plant Cell* 18: 2929-2945.

Nogueira, FT, Madi, S, Chitwood, DH, Juarez, MT, Timmermans, MC. (2007) Two small regulatory RNAs establish opposing fates of a developmental axis. *Genes Dev* 21: 750-755.

Ossowski, S, Schneeberger, K, Clark, RM, Lanz, C, Warthmann, N, Weigel, D. (2008) Sequencing of natural strains of *Arabidopsis thaliana* with short reads. *Genome Res* 18: 2024-2033.

Panda, SK, Baluska, F, Matsumoto, H. (2009) Aluminum stress signaling in plants. *Plant Signal Behav* 4: 592-597.

Parizot, B, Laplaze, L, Ricaud, L, Boucheron-Dubuisson, E, Bayle, V, Bonke, M, De Smet, I, Poethig, SR, Helariutta, Y, Haseloff, J, Chriqui, D, Beeckman, T, Nussaume, L.

(2008) Diarch symmetry of the vascular bundle in Arabidopsis root encompasses the pericycle and is reflected in distich lateral root initiation. *Plant Physiol* 146: 140-148.

Parizotto, EA, Dunoyer, P, Rahm, N, Himber, C, Voinnet, O. (2004) In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev* 18: 2237-2242.

Parry, G, Calderon-Villalobos, LI, Prigge, M, Peret, B, Dharmasiri, S, Itoh, H, Lechner, E, Gray, WM, Bennett, M, Estelle, M. (2009) Complex regulation of the TIR1/AFB family of auxin receptors. *Proc Natl Acad Sci U S A* 106: 22540-22545.

Paszkiewicz, K and Studholme, DJ. (2010) De novo assembly of short sequence reads. *Brief Bioinform* 11: 457-472.

Peragine, A, Yoshikawa, M, Wu, G, Albrecht, HL, Poethig, RS. (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. *Genes Dev* 18: 2368-2379.

Peret, B, De Rybel, B, Casimiro, I, Benkova, E, Swarup, R, Laplaze, L, Beeckman, T, Bennett, MJ. (2009) Arabidopsis lateral root development: an emerging story. *Trends Plant Sci* 14: 399-408.

Pfaffl, MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.

Qiu, D, Xiao, J, Xie, W, Cheng, H, Li, X, Wang, S. (2009) Exploring transcriptional signalling mediated by OsWRKY13, a potential regulator of multiple physiological processes in rice. *BMC Plant Biol* 9: 74.

Ramachandran, V and Chen, X. (2008) Small RNA metabolism in Arabidopsis. *Trends Plant Sci* 13: 368-374.

Raman, S, Greb, T, Peaucelle, A, Blein, T, Laufs, P, Theres, K. (2008) Interplay of miR164, CUP-SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in *Arabidopsis thaliana*. *Plant J* 55: 65-76.

Rausch, T and Wachter, A. (2005) Sulfur metabolism: a versatile platform for launching defence operations. *Trends Plant Sci* 10: 503-509.

Reyes, JL, Arenas-Huertero, C, Sunkar, R. (2010) Cloning of stress-responsive microRNAs and other small RNAs from plants. *Methods Mol Biol* 639: 239-251.

Reyes, JL and Chua, NH. (2007) ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *Plant J* 49: 592-606.

Rodriguez, RE, Mecchia, MA, Debernardi, JM, Schommer, C, Weigel, D, Palatnik, JF. (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. *Development* 137: 103-112.

Ross, EJ, Stone, JM, Elowsky, CG, Arredondo-Peter, R, Klucas, RV, Sarath, G. (2004) Activation of the *Oryza sativa* non-symbiotic haemoglobin-2 promoter by the cytokinin-regulated transcription factor, ARR1. *J Exp Bot* 55: 1721-1731.

Rushton, PJ, Somssich, IE, Ringler, P, Shen, QJ. (2010) WRKY transcription factors. *Trends Plant Sci* 15: 247-258.

Ryan, PR, Raman, H, Gupta, S, Horst, WJ, Delhaize, E. (2009) A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. *Plant Physiol* 149: 340-351.

Ryan, PR, Tyerman, SD, Sasaki, T, Furuichi, T, Yamamoto, Y, Zhang, WH, Delhaize, E. (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *J Exp Bot* 62: 9-20.

Sakai, H, Honma, T, Aoyama, T, Sato, S, Kato, T, Tabata, S, Oka, A. (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* 294: 1519-1521.

Sang, T and Ge, S. (2007) Genetics and phylogenetics of rice domestication. *Curr Opin Genet Dev* 17: 533-538.

Santos, MO and Aragao, FJ. (2009) Role of SERK genes in plant environmental response. *Plant Signal Behav* 4:

Sasaki, T, Yamamoto, Y, Ezaki, B, Katsuhara, M, Ahn, SJ, Ryan, PR, Delhaize, E, Matsumoto, H. (2004) A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37: 645-653.

Sawaki, Y, Iuchi, S, Kobayashi, Y, Ikka, T, Sakurai, N, Fujita, M, Shinozaki, K, Shibata, D, Kobayashi, M, Koyama, H. (2009) STOP1 regulates multiple genes that protect arabidopsis from proton and aluminum toxicities. *Plant Physiol* 150: 281-294.

Schommer, C, Palatnik, JF, Aggarwal, P, Chetelat, A, Cubas, P, Farmer, EE, Nath, U, Weigel, D. (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biol* 6: e230.

Severin, AJ, Woody, JL, Bolon, YT, Joseph, B, Diers, BW, Farmer, AD, Muehlbauer, GJ, Nelson, RT, Grant, D, Specht, JE, Graham, MA, Cannon, SB, May, GD, Vance, CP, Shoemaker, RC. (2010) RNA-Seq Atlas of *Glycine max*: a guide to the soybean transcriptome. *BMC Plant Biol* 10: 160.

Sheldon, CC, Conn, AB, Dennis, ES, Peacock, WJ. (2002) Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. *Plant Cell* 14: 2527-2537.

Shi, Y, Zhao, X, Hsieh, J, Wichterle, H, Impey, S, Banerjee, S, Neveu, P, Kosik, KS. (2010) MicroRNA regulation of neural stem cells and neurogenesis. *J Neurosci*30: 14931-14936.

Shinozaki, K, Yamaguchi-Shinozaki, K, Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6: 410-417.

Sieber, P, Wellmer, F, Gheyselinck, J, Riechmann, JL, Meyerowitz, EM. (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* 134: 1051-1060.

Sivaguru, M, Fujiwara, T, Samaj, J, Baluska, F, Yang, Z, Osawa, H, Maeda, T, Mori, T, Volkmann, D, Matsumoto, H. (2000) Aluminum-induced 1-->3-beta-D-glucan inhibits cell-to-cell trafficking of molecules through plasmodesmata. A new mechanism of aluminum toxicity in plants. *Plant Physiol* 124: 991-1006.

Smale, ST. (2001) Core promoters: active contributors to combinatorial gene regulation. *Genes Dev* 15: 2503-2508.

Song, QX, Liu, YF, Hu, XY, Zhang, WK, Ma, B, Chen, SY, Zhang, JS. (2011) Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. *BMC Plant Biol* 11: 5.

Sunkar, R. (2010) MicroRNAs with macro-effects on plant stress responses. *Semin Cell Dev Biol* 21: 805-811.

Sunkar, R, Kapoor, A, Zhu, JK. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18: 2051-2065.

Sunkar, R and Zhu, JK. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* 16: 2001-2019.

Suryapriya, P, Snehalatha, A, Kayalvili, U, Krishna, R, Singh, S, Ulaganathan, K. (2009) Genome-wide analyses of rice root development QTLs and development of an online resource, Rootbrowse. *Bioinformation* 3: 279-281.

Teixeira, FK, Menezes-Benavente, L, Galvao, VC, Margis, R, Margis-Pinheiro, M. (2006) Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. *Planta* 224: 300-314.

Todesco, M, Rubio-Somoza, I, Paz-Ares, J, Weigel, D. (2010) A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. *PLoS Genet* 6: e1001031.

Trindade, I, Capitao, C, Dalmay, T, Fevereiro, MP, Santos, DM. (2010) miR398 and miR408 are up-regulated in response to water deficit in Medicago truncatula. *Planta* 231: 705-716.

Vaucheret, H. (2009) AGO1 homeostasis involves differential production of 21-nt and 22-nt miR168 species by MIR168a and MIR168b. *PLoS ONE* 4: e6442.

Vaucheret, H, Vazquez, F, Crete, P, Bartel, DP. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18: 1187-1197.

Vidal, EA, Araus, V, Lu, C, Parry, G, Green, PJ, Coruzzi, GM, Gutierrez, RA. (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 107: 4477-4482.

Vieweg, MF, Fruhling, M, Quandt, HJ, Heim, U, Baumlein, H, Puhler, A, Kuster, H, Andreas, MP. (2004) The promoter of the Vicia faba L. leghemoglobin gene VfLb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. *Mol Plant Microbe Interact* 17: 62-69.

Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell* 136: 669-687.

Wang, JW, Schwab, R, Czech, B, Mica, E, Weigel, D. (2008) Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in *Arabidopsis thaliana*. *Plant Cell* 20: 1231-1243.

Wang, JW, Wang, LJ, Mao, YB, Cai, WJ, Xue, HW, Chen, XY. (2005) Control of root cap formation by MicroRNA-targeted auxin response factors in Arabidopsis. *Plant Cell* 17: 2204-2216.

Wang, L, Mai, YX, Zhang, YC, Luo, Q, Yang, HQ. (2010) MicroRNA171c-targeted SCL6-II, SCL6-III, and SCL6-IV genes regulate shoot branching in Arabidopsis. *Mol Plant* 3: 794-806.

Wu, G, Park, MY, Conway, SR, Wang, JW, Weigel, D, Poethig, RS. (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* 138: 750-759.

Wu, G and Poethig, RS. (2006) Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. *Development* 133: 3539-3547.

Wu, MF, Tian, Q, Reed, JW. (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. *Development* 133: 4211-4218.

Xia, J, Yamaji, N, Kasai, T, Ma, JF. (2010) Plasma membrane-localized transporter for aluminum in rice. *Proc Natl Acad Sci U S A* 107: 18381-18385.

Xia, J, Yamaji, N, Ma, JF. (2011) Further characterization of an aluminum influx transporter in rice. *Plant Signal Behav* 6:

Xie, F and Zhang, B. (2010) Target-align: a tool for plant microRNA target identification. *Bioinformatics* 26: 3002-3003.

Xie, K, Wu, C, Xiong, L. (2006a) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant Physiol* 142: 280-293.

Xie, K, Wu, C, Xiong, L. (2006b) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant Physiol* 142: 280-293.

Xie, Z, Khanna, K, Ruan, S. (2010) Expression of microRNAs and its regulation in plants. *Semin Cell Dev Biol* 21: 790-797.

Xuan, P, Guo, M, Liu, X, Huang, Y, Li, W. (2011) PlantMiRNAPred: efficient classification of real and pseudo plant pre-miRNAs. *Bioinformatics*

Xue LJ, Zhang JJ and Xue HW (2009) Characterization and expression profiles of miRNAs in rice seeds. *Nucleic Acids Res*37: 916-930.

Yamaguchi-Shinozaki, K and Shinozaki, K. (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci* 10: 88-94.

Yamaji, N, Huang, CF, Nagao, S, Yano, M, Sato, Y, Nagamura, Y, Ma, JF. (2009) A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *Plant Cell* 21: 3339-3349.

Yamamoto, Y, Kobayashi, Y, Devi, SR, Rikiishi, S, Matsumoto, H. (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiol* 128: 63-72.

Yamasaki, H, Abdel-Ghany, SE, Cohu, CM, Kobayashi, Y, Shikanai, T, Pilon, M. (2007) Regulation of copper homeostasis by micro-RNA in Arabidopsis. *J Biol Chem* 282: 16369-16378.

Yang, JL, Li, YY, Zhang, YJ, Zhang, SS, Wu, YR, Wu, P, Zheng, SJ. (2008) Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. *Plant Physiol* 146: 602-611.

Yang, L, Conway, SR, Poethig, RS. (2011) Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development* 138: 245-249.

Yang, Q, Wang, Y, Zhang, J, Shi, W, Qian, C, Peng, X. (2007) Identification of aluminum-responsive proteins in rice roots by a proteomic approach: cysteine synthase as a key player in Al response. *Proteomics* 7: 737-749.

Yang, XJ and Finnegan, PM. (2010) Regulation of phosphate starvation responses in higher plants. *Ann Bot* 105: 513-526.

Yasuda, J. (2010) [Research and clinical applications of functional RNAs]. *Nippon Rinsho* 68 Suppl 8: 584-589.

Yoon, EK, Yang, JH, Lim, J, Kim, SH, Kim, SK, Lee, WS. (2010) Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development. *Nucleic Acids Res* 38: 1382-1391.

Zhang, J, He, Z, Tian, H, Zhu, G, Peng, X. (2007) Identification of aluminium-responsive genes in rice cultivars with different aluminium sensitivities. *J Exp Bot* 58: 2269-2278.

Zhang, M, Liu, W, Bi, YP. (2009a) [Dehydration-responsive element-binding (DREB) transcription factor in plants and its role during abiotic stresses]. *Yi Chuan* 31: 236-244.

Zhang, X, Zou, Z, Gong, P, Zhang, J, Ziaf, K, Li, H, Xiao, F, Ye, Z. (2011a) Over-expression of microRNA169 confers enhanced drought tolerance to tomato. *Biotechnol Lett*

Zhang, X, Zou, Z, Zhang, J, Zhang, Y, Han, Q, Hu, T, Xu, X, Liu, H, Li, H, Ye, Z. (2011c) Over-expression of sly-miR156a in tomato results in multiple vegetative and reproductive trait alterations and partial phenocopy of the sft mutant. *FEBS Lett* 585: 435-439.

Zhang, Y, Chen, C, Jin, XF, Xiong, AS, Peng, RH, Hong, YH, Yao, QH, Chen, JM. (2009b) Expression of a rice DREB1 gene, OsDREB1D, enhances cold and high-salt tolerance in transgenic Arabidopsis. *BMB Report* 42: 486-492.

Zhang, Z, Wei, L, Zou, X, Tao, Y, Liu, Z, Zheng, Y. (2008) Submergence-responsive MicroRNAs are potentially involved in the regulation of morphological and metabolic adaptations in maize root cells. *Ann Bot* 102: 509-519.

Zhang, ZH, Yu, JY, Li, DF, Zhang, ZY, Liu, FX, Zhou, X, Wang, T, Ling, Y, Su, Z. (2010) PMRD: plant microRNA database. *Nucleic Acids Res* 38: D806-D813.

Zhao, B, Ge, L, Liang, R, Li, W, Ruan, K, Lin, H, Jin, Y. (2009a) Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. *BMC Mol Biol* 10: 29.

Zhao, BT, Liang, RQ, Ge, LF, Li, W, Xiao, HS, Lin, HX, Ruan, KC, Jin, YX. (2007) Identification of drought-induced microRNAs in rice. *Biochem Biophys Res Commun* 354: 585-590.

Zhao, CR, Ikka, T, Sawaki, Y, Kobayashi, Y, Suzuki, Y, Hibino, T, Sato, S, Sakurai, N, Shibata, D, Koyama, H. (2009b) Comparative transcriptomic characterization of aluminum, sodium chloride, cadmium and copper rhizotoxicities in *Arabidopsis thaliana*. *BMC Plant Biol* 9: 32.

Zhao, Y, Hu, Y, Dai, M, Huang, L, Zhou, DX. (2009c) The WUSCHEL-related homeobox gene WOX11 is required to activate shoot-borne crown root development in rice. *Plant Cell* 21: 736-748.

Zheng, BS, Yang, L, Mao, CZ, Zhang, WP, Wu, P. (2006) QTLs and candidate genes for rice root growth under flooding and upland conditions. *Yi Chuan Xue Bao* 33: 141-151.

Zheng, BS, Yang, L, Zhang, WP, Mao, CZ, Wu, YR, Yi, KK, Liu, FY, Wu, P. (2003) Mapping QTLs and candidate genes for rice root traits under different water-supply conditions and comparative analysis across three populations. *Theor Appl Genet* 107: 1505-1515.

Zhou, L, Liu, Y, Liu, Z, Kong, D, Duan, M, Luo, L. (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *J Exp Bot* 61: 4157-4168.

Zhou, X, Ruan, J, Wang, G, Zhang, W. (2007) Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol* 3: e37.

Zhou, X, Wang, G, Sutoh, K, Zhu, JK, Zhang, W. (2008a) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim Biophys Acta* 1779: 780-788.

Zhu, QH and Helliwell, CA. (2010) Regulation of flowering time and floral patterning by miR172. *J Exp Bot* 62: 487-495.