UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

‘‘Caracterização funcional dos genes *ASR* na resposta ao alumínio em arroz (*Oryza sativa*)’’

Rafael Augusto Arenhart

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular do Departamento de Genética da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para a obtenção do título de Doutor em Ciências, área de concentração: Genética e Biologia Molecular.

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Orientadores

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SUMÁRIO

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LISTA DE ABREVIATURAS

Al - Alumínio
AOS – espécies ativas de oxigênio (do inglês Active Oxigen Species)
APx – Ascorbato Peroxidase
ART1 – do inglês “Aluminium resistance transcription factor 1”
ASR – do Inglês “Abscisic Acid stress and Ripening”
cDNA – DNA complementar
CDS – sequência do DNA codificador (do inglês Coding DNA Sequence)
ChIP -Imunoprecipitação da Cromatina (do inglês Chromatin Immunoprecipitation)
cTP – peptídeo de transferência a cloroplasto (do inglês Chloroplast transfer peptide)
Cv – cultivar
DNA – ácido desoxiribonucleico (do Inglês Deoxiribonucleic Acid)
DW – peso seco (do inglês Dry weight)
FAO – Organização das Nações Unidas para a Agricultura e Alimentação (do inglês Food and Agriculture Organization)
FW – peso fresco (do inglês Fresh weight)
GA – Giberilina (do inglês Gibberellin)
GFP – Proteína Verde Fluorescente (do inglês Green Fluorescent Protein)
Gi – identificação do gene (do inglês Gene Identification)
GO – Ontologia do gene (do inglês Gene Ontology)
GUS – β-glicoronidase (do inglês Glucoronidase)
hpRNA – hairpin RNA
Mbp – Mega pares de bases
MS – Espectometria de massa (do inglês Mass Spectrometry)
µM - micromolar
mM – milimolar
OA – ácidos orgânicos (do inglês Organic Acids)
PCD – morte celular programada (do inglês Programmed Cell Death)
PCR – reação em cadeia da DNA polimerase (do inglês Polymerase Chain Reaction)

PEG - polietilenoglicol

PMSF – do inglês phenylmethanesulfonylfluoride

PSII – fotossistema II, do inglês “Photosystem II”

QA e QB – Plastoquinona A e B

QTL – Do inglês “Quantitative Trait Loci”

RBC – células da borda da raiz (do inglês Root Border Cells)

RNA – ácido ribonucleico (do inglês Ribonucleic Acid)

RNAi – RNA de interferência

ROS – Espécies reativas de oxigênio (do inglês Reactive Oxigen Species)

RT-qPCR – Reação em cadeia da DNA polimerase quantitative precedida de transcrição reversa (do inglês Reverse Transcription Quantitative PCR)

RWC – conteúdo relativo de água (do inglês Relative Water Content)

Sapa – Do inglês “Streptavidin Agarose Pull Down system”

Ssp - subespécie

STAR 1/2 - Do inglês “sensitive to Al rhizotoxicity1”

TCA - Ácido tricloroacético (do inglês Trichloroacetic acid)

TOF – tempo de vôo (do inglês Time of Flight)

UV – Ultravioleta

UTR – região não traduzida (do inglês Untranslated Region)

X-Gluc - ácido 5-Bromo-4-cloro-3-indolil b-D-glucurônico, substrato cromogênico para a proteína *Gus*

RESUMO

O arroz é considerado o cereal mais tolerante ao alumínio (Al). Entretanto, a variabilidade entre os genótipos leva a uma considerável diferença quanto ao grau de tolerância para cultivares distintas. Diversos estudos mostram que plantas de arroz toleram o Al por intermédio de mecanismos internos e externos. Neste trabalho foi analisada a modulação da expressão da família gênica *ASR* (Aba, Stress and Ripening) em função do tratamento com Al. O gene *ASR5* foi diferencialmente expresso em raízes de arroz tolerante ao Al (ssp Japonica cv Nipponbare). Entretanto, *ASR5* não respondeu a exposição ao Al em raízes de arroz sensíveis ao Al (ssp Indica cv Taim). Plantas transgênicas silenciadas para os genes *ASR* apresentaram um aumento da sensibilidade ao Al. Calos embriogênicos de arroz transformados com a fusão ASR5-GFP revelaram que a proteína ASR5 localiza-se no núcelo e no citoplasma. Em protoplastos transformados, ASR5 localizou-se nos cloroplastos. Usando uma abordagem proteômica, comparando plantas não-transformadas e plantas ASR5_RNAi, um total de 41 proteínas com padrões contrastantes foi identificado. No intuito de identificar genes com expressão alterada pelo Al em arroz, e buscar genes afetados pelo silenciamento de ASR5, foi realizado o sequenciamento total dos transcritos utilizando plantas de arroz não transformadas e ASR5_RNAi em duas condições: controle e tratamento com Al. Essas análises transcriptômicas revelaram que 961 genes responderam ao Al em raízes de plantas de arroz não transgênicas submetidas ao Al. Nas plantas ASR5_RNAi, apenas 309 genes responderam ao tratamento com Al. Entretanto, apenas 52 desses genes se sobrepuseram quando comparados ao grupo de genes modulados em plantas não transformadas, sugerindo que a planta ASR5_RNAi perdeu a capacidade de regular um conjunto de genes. Além disso, análises de imunoprecipitação da cromatina seguida de sequenciamento em massa, revelaram que ASR5 liga-se ao promotor do gene *STAR1*, entre outros, regulando sua expressão em resposta ao Al. Os resultados mostram pela primeira vez que ASR5 atua como fator de transcrição em arroz e que está envolvido na regulação de genes responsivos ao Al conferindo tolerância frente à toxicidade do Al.

ABSTRACT

Among cereal crops, rice is considered the most aluminium (Al) tolerant species. However, variability among rice genotypes leads to remarkable differences in the degree of Al tolerance for distinct cultivars. A number of studies have demonstrated that rice plants achieve Al tolerance through internal and external mechanisms. We have analyzed expression changes of the rice *ASR* (Aba, Stress and Ripening) gene family as a function of Al treatment. The gene *ASR5* was differentially regulated in the Al-tolerant rice *ssp* Japonica cv Nipponbare. However, *ASR5* expression did not respond to Al exposure in Indica cv Taim rice roots, which are highly Al-sensitive. Transgenic plants carrying RNAi constructs that targeted the *ASR* genes displayed increased Al susceptibility in T1 plants. Rice embryogenic calli expressing an ASR5-GFP fusion revealed that ASR5 was localized in both the nucleus and cytoplasm. In transformed protoplasts, ASR5-GFP appears in chloroplast cells. Using a proteomic approach to compare non-transformed and ASR5_RNAi plants, a total of 41 proteins with contrasting expression patterns were identified. In order to identify genes with differential modulation under Al stress in rice, and search for genes affected by ASR5 silencing, RNA-seq was made in non-transformed plants and ASR5_RNAi plants in control conditions and after Al treatment. These analyses revealed that 961 genes responded to Al in non-transformed rice roots under Al stress. A total of 309 genes responded to Al in ASR5_RNAi plants. Only 52 genes overlapped between non transformed and ASR5_RNAi plants when comparing genes modulated by Al, showing that ASR-silenced plants lost the ability to modulate a set of genes in response to Al treatment. Furthermore, ChIP-Seq analysis revealed that ASR5 can bind to the promoter of *STAR1*, among other genes, regulating its expression under Al stress. These results show for the first time that ASR5 act as a transcription factor in rice and that it regulates Al-responsive genes conferring tolerance in rice against Al toxicity.

INTRODUÇÃO

Estresses abióticos causam a perda de milhões de dólares a cada ano devido à redução da produtividade de grãos, o que ameaça a sustentabilidade da agricultura (Mahajan e Tuteja, 2005). Em 1982, Boyer indicou que fatores ambientais poderão limitar a produção de grãos em mais de 70% (Boyer, 1982). Em 2007, um relatório da FAO mostrou que somente 3,5% da área terrestre global não é afetada por algum tipo de estresse ambiental (http://www.fao.org/docrep/010/a1075e/a1075e00.htm). Apesar da dificuldade em estimar com exatidão os efeitos de estresses abióticos, é evidente que estes continuam exercendo um significante impacto em plantas, baseado na percentagem da área afetada e pelo crescimento exponencial de publicações científicas relacionadas ao tópico (Cramer *et al*., 2011). É esperado que ocorra um declínio da produtividade de grãos em muitas áreas, devido à redução de terras cultiváveis, redução de fontes de água e mudanças climáticas (Lobell *et al*., 2011). Condições adversas ou estresses ambientais conduzem a uma série de mudanças morfológicas, fisiológicas, bioquímicas e moleculares causando danos aos vegetais, limitando seu crescimento e reduzindo a produtividade (Mahajan e Tuteja, 2005).

Ao longo da evolução, as plantas desenvolveram sofisticados mecanismos que as permite perceber estresses ambientais, ativando genes de resposta levando à mudanças na planta no intuito de tolerar ou resistir aos estresses (Gao *et al*., 2007). Entender a função dos genes é um dos maiores desafios da era pós-genômica (Cramer *et al*., 2011). A identificação e caracterização funcional dos genes poderão permitir o desenvolvimento de novas estratégias a fim de aumentar a tolerância à estresses em plantas (Cushman e Bohnert, 2000).

1. Arroz: modelo vegetal

Considerado um dos cereais mais importantes do mundo, o arroz é cultivado e consumido em todos os continentes. Cerca de 150 milhões de hectares de arroz são cultivados anualmente no mundo, chegando a uma

produção de cerca de 590 milhões de toneladas. Segundo dados da EMBRAPA (EMBRAPA, 2008), o Brasil está entre os dez principais produtores mundiais de arroz, com cerca de 11 milhões de toneladas ao ano.

O arroz é um organismo diplóide com 24 cromossomos e genoma de 390 megapares de bases (Mbp), sendo o menor entre os cereais, possuindo aproximadamente 32.000 genes com base em uma anotação acurada do genoma de *Oryza sativa* L. *ssp*. *Japonica* (Itoh *et al*., 2007).

O genoma do arroz possui sintenia com o genoma de outros cereais, como milho e trigo (Moore *et al*., 1995). Além disso, protocolos de transformação mediada por *Agrobacterium tumefaciens* já foram bem estabelecidos para o arroz ssp Japonica (Hiei *et al*., 1994).

2. Toxidez por Alumínio

O alumínio (Al) é o metal mais abundante da crosta terrestre e o terceiro elemento mais abundante da terra (Kochian *et al*., 2002). A grande maioria do Al está quelada por ligantes, ou ocorre em outras formas não tóxicas tais como aluminosilicatos ou precipitados (Driscoll e Schecher, 1990). Entretanto, em condição de solo ácido, o Al é liberado na forma iônica Al^{3+}, sendo esta solubilizada e incorporada pelas plantas (Huang *et al*., 2009). Este é um problema agravante, pois se estima que mais de 50 % dos solos aráveis do mundo são considerados solos ácidos (pH menor que 5.0) (von Uexkull e Mutert, 1995).

A acidificação do solo pode se desenvolver naturalmente devido à chuva ácida ou quando cátions básicos são lavados do solo. Além disso, a acidificação dos solos pode ser acelerada por más práticas agrícolas (Delhaize *et al*., 2012). O Al interfere em uma ampla gama de processos celulares. Potencialmente, a toxicidade por Al pode ocorrer pela sua interação com alvos apoplásticos como a parede celular, a membrana plasmática, e alvos simplásticos como o citosol (Kochian *et al*., 2004). A inibição do crescimento da raiz é o primeiro e mais visível sintoma da toxidez por Al e tem sido usada extensivamente para determinar graus de toxidez (Foy, 1988). O ápice da raiz em especial, é a parte mais sensível uma vez que é o local da divisão celular e elongação das células (Sivaguru e Horst,

1998). A exposição prolongada permite a interação do Al com o núcleo das células, resultando no bloqueio da divisão celular e do citoesqueleto (Silva *et al*., 2000).

A alta variabilidade da tolerância ao Al em plantas indica que algumas espécies de plantas desenvolveram mecanismos especiais (externos e internos) de sobrevivência a condições de estresses (Ma *et al*., 2002). Mecanismos externos incluem a ligação diferencial do Al à parede celular, permeabilização seletiva da membrana plasmática, formação de barreiras induzidas pelas plantas por meio do aumento do pH na rizosfera e exudação de ácidos orgânicos (AO) e compostos fenólicos (Kochian *et al*., 2004). Ácidos orgânicos como malato, citrato e oxalato têm importante papel na detoxificação de Al^{3+} por formarem complexos no citosol ou na interface raiz-solo protegendo as raízes das plantas (Ryan *et al*., 2001). Mecanismos internos incluem quelação do Al no citosol e compartimentalização em vacúolos descritos em algumas espécies (Ma *et al*., 1997; Zheng *et al*., 1998). Os mecanismos responsáveis pela tolerância de Al são variáveis entre as diversas espécies e cultivares e, possivelmente, controlados por diferentes genes e diferentes rotas metabólicas (Wu *et al*., 2000).

Uma vez que mudanças na expressão gênica controlam os processos fisiológicos normais e são também efetores nas respostas celulares aos estresses bióticos e abióticos, estudos visando identificar os genes de resposta ao estresse por Al forneceram uma descrição inicial dos genes potencialmente importantes envolvidos nesse tipo de estresse (Chandran *et al*, 2008; Maron *et al*, 2008; Goodwin e Sutter, 2009; Yamaji *et al*, 2009; Sawaki *et al*, 2009; Eticha *et al*, 2010; Duressa *et al*, 2011).

A tecnologia desenvolvida nos últimos anos através de análises em larga escala, permitiu a identificação de diversos genes responsivos a Al em Arabidopsis (Kumari *et al*., 2008; Goodwin e Sutter, 2009), Álamo (Grisel *et al*., 2010), *Medicago truncatula* (Chandran *et al*., 2008), feijão comum (Eticha *et al*., 2010), milho (Maron *et al*., 2008; Mattiello *et al*., 2010), soja (Duressa *et al*., 2010; You *et al*., 2011) e trigo (Guo *et al*., 2007; Houde e Diallo, 2008).

O arroz é o cereal mais resistente ao Al nas condições de campo (Foy, 1988). Como espécie, o arroz é de duas a cinco vezes mais tolerante que o trigo, a

cevada e o milho (Famoso *et al*., 2010). Em arroz, alguns genes envolvidos nesta tolerância já foram identificados, porém, ainda há a falta de análises em grande escala. O gene *ART1* está envolvido na regulação de vários genes responsáveis pela detoxificação do Al (Yamaji *et al*., 2009). Pelo menos quatro genes regulados por ART1 já foram caracterizados. *STAR1* e *STAR2* codificam uma proteína do tipo "ATP-Binding" e uma proteína de domínio transmembrana, respectivamente. Este complexo STAR1-STAR2 transporta UDP-glicose, modificando a parede celular, mascarando sítios de ligação do Al (Huang *et al*., 2009). *Nrat1* codifica uma proteína transportadora de Al (Xia *et al*., 2010) e *FRDL4*, uma proteína envolvida na secreção de citrato (Yokosho *et al*., 2011). Estes genes, envolvidos em ambos os mecanismos (internos e externos) de defesa ao Al, sugerem que o arroz pode lidar com o Al usando novos e/ou ambos os mecanismos.

3. ASR (*ABA Stress and Ripening*)

Genes *ASR* (do inglês, *ABA, Stress and Ripening*) foram descritos inicialmente em tomate (Iusem *et al*., 1993) e, em seguida, identificados em diversas plantas como milho (Zivy *et al*., 1998), arroz (Vaidyanathan *et al*., 1999), cana-de-açúcar (Sugiharto *et al*., 2002), melão (Hong, 2002), uva (Çakir et al., 2003), lírio (Yang *et al*., 2005) e *Gingko biloba* (Shen *et al*., 2005), sendo amplamente distribuído no reino vegetal, porém, ausente na planta modelo *Arabidopsis thaliana* (Maskin *et al*., 2001).

Os genes *ASR* são induzidos por estresse em plantas, e seus níveis de expressão são rapidamente aumentados em resposta à salinidade e à seca (Amitai-Zeigerson, 1995; Padmanabhan *et al*., 1997; Zivy *et al*., 1998; Kawasaki *et al*., 2001; Sugiharto *et al*., 2002; Yang *et al*., 2005). Entretanto, os padrões de expressão variam em diferentes tecidos e nas diferentes espécies (Padmanabhan *et al*., 1997). O gene *ASR* é também regulado pelo desenvolvimento, sendo expresso durante a polinização (Doczi *et al*., 2002) e em frutos em desenvolvimento (Canel et al., 1995; Hong, 2002; Çakir et al., 2003; Chen et al., 2011).

Em tomate, a família *ASR* é composta de quatro genes, os quais são diferencialmente expressos em folhas e raízes em reposta a estresse hídrico (Maskin *et al*., 2001). O gene *ASR1* de tomate é expresso em frutos, flores, folhas, raízes, gemas e sementes em plantas submetidas a condições normais de crescimento. Em contraste, os outros membros da família não mostram ampla distribuição no padrão de expressão constitutiva, sendo restritos a órgãos específicos (Maskin *et al*., 2001). Kalifa *et al* (Kalifa *et al*., 2004a) mostraram que a proteína ASR1 de tomate liga-se a uma sequência consenso de DNA, fato que está de acordo com sua localização nuclear. As proteínas ASR de outras espécies também estão localizadas no núcleo, onde regulam promotores específicos (Çakir et al., 2003; Yang et al., 2005), sugerindo que estas proteínas atuam como fatores de transcrição e que, provavelmente, ligam-se a genes codificadores de proteínas envolvidos no transporte de hexoses e genes responsivos ao ABA.

Segundo Yang *et al* (Yang *et al*., 2005), ASR1 de tomate contém duas regiões altamente conservadas. A primeira região contém resíduos de histidina na extremidade amino-terminal, possuindo uma sequencia específica de ligação de DNA dependente de zinco (Kalifa *et al*., 2004b). A segunda região está localizada na região carboxi-terminal e possui a assinatura ABA/WDS descrita para proteínas induzidas por ABA, estresse a amadurecimento (Canel *et al*., 1995), e um sinal de localização nuclear putativo (Çakir et al., 2003).

Recentemente, Konrad & Zvi (Konrad and Bar-Zvi, 2008) mostraram que a ASR1 de tomate possui atividade similar às chaperonas, protegendo proteínas da desnaturação causada por calor e ciclos de congelamento e descongelamento.

No primeiro trabalho envolvendo genes *ASR* de arroz, Vaidyanathan *et al* (Vaidyanathan *et al*., 1999) caracterizaram o padrão de expressão de *ASR1* em resposta a ABA e estresse osmótico. A proteína codificada pelo gene *ASR1* (recentemente nomeada de *ASR5* por Frankel *et al.,* 2006) é hidrofílica, com potencial de substrato para proteína cinase, e possuí um sítio de miriostilação na posição glicina-68 conservada entre todas as proteínas ASR alinhadas (Vaidyanathan *et al*., 1999), o que sugere um importante papel na função da

proteína. O resíduo miriostil pode se ligar a pacotes hidrofóbicos e conferir estabilidade estrutural à proteína (Zheng *et al*., 1993).

Em arroz, análises *in silico* revelaram seis cópias de genes *ASR*, dispersas em diferentes cromossomos, todas confirmadas por ESTs (Frankel *et al*., 2006) (Tabela 1).

O mRNA primário *ASR1* de arroz possui quatro formatos diferentes (S*plicings* alternativos*)*, cujos transcritos originam quatro proteínas diferentes. Os genes *ASR3* e *ASR4* estão localizados em *tandem* e possuem íntrons conservados com o mesmo comprimento, sugerindo uma recente duplicação gênica, enquanto que *ASR2* está localizado a mais de 200 kbp de distância de *ASR3* e *ASR4*. *ASR6* possui grande divergência dos outros genes *ASR* de arroz, mas se assemelha a *ASR4* de tomate em análise filogenética realizada por Frankel *et al* (2006). A sequência genômica de *ASR1* localiza-se no cromossomo 2 (Loc_Os02g33820.1); *ASR2, ASR3 e ASR4* no cromossomo 1 (Loc_Os01g73250.1), (Loc_Os01g72900.1) e (Loc_Os01g72910.1) respectivamente, *ASR5* está posicionado no cromossomo 11 (Loc_Os11g06720.1), e *ASR6* no cromossomo 4 (Loc_Os04g34600.1).

Tabela 1. Localização, tamanho do íntron (em pb), e da proteína (em aminoácidos) dos genes *ASR* em arroz (monocotiledônea) e tomate (dicotiledônea). Dados extraídos de Frankel *et al* (2006).

	Cromossomo	**Tamanho do íntron**	**Tamanho da proteína (aa)**	**ESTs**	**Em *tandem* com**
Arroz					
ASR1	II	*splicing*	63/71/91/105	sim	
ASR2	I	440	182	sim	
ASR3	I	131	105	sim	*ASR4*
ASR4	I	131	96	sim	*ASR3*
ASR5	XI	119	138	sim	
ASR6	IV	84	229	sim	
Tomate					
ASR1	IV	687	110	sim	?
ASR2	IV	111	114	sim	?
ASR3	IV	476	108	sim	?
ASR4	IV	111	297	sim	?

(aa) = aminoácidos

Splicing = diferentes formas de transcritos

ESTs = Expressed sequence tags

Takasaki *et al* (Takasaki *et al*., 2008), em trabalho de identificação de proteínas reguladas por giberilina (GA), demonstraram que a expressão de *ASR5* é regulada em nível de proteína, mas não em nível de mRNA, durante a expansão das bainhas das folhas em arroz, indicando um envolvimento dessa proteína no crescimento da planta.

Experimentos de superexpressão têm mostrado o uso potencial de genes *ASR* para aumentar a tolerância de plantas contra estresses abióticos. A superexpressão do gene *ASR1* de tomate em plantas de tabaco aumentou a

tolerância destas em reposta à salinidade (Kalifa *et al*., 2004a). A superexpressão do ortológo de *ASR* de lírio (*LLA23*) em *A. thaliana* aumentou a tolerância da planta à seca e a salinidade, bem como ao frio (Yang *et al*., 2005; Hsu *et al*., 2011). Superexpressando *ASR* de banana em *Arabidopsis*, (Dai *et al*., 2011) foram obtidas plantas mais tolerantes a estresse osmótico.

Usando uma abordagem proteômica, Yang *et al* (Yang *et al*., 2007) identificaram proteínas responsivas ao Al em raízes de arroz. Dentre essas proteínas, ASR5 apresentou aumento de sua expressão em reposta ao tratamento com Al.

Até o momento não há estudos da regulação da expressão da família gênica ASR, em nível de mRNA em resposta ao Al e a outros tipos de estresses abióticos.

A aplicação de corretivos de solos ácidos, apesar de ser uma estratégia efetiva para a redução da toxidez por Al, não é uma opção econômica e sustentável em longo prazo, e o desenvolvimento de variedades de cereais resistentes ao Al deve ser considerada (Yang *et al*., 2007). A identificação e a caracterização de genes e proteínas ligadas a mecanismos de resistência a Al poderão prover informações moleculares para uso no desenvolvimento de cultivares com melhor adaptabilidade a solos ácidos (Kochian *et al*., 2005).

Objetivos

O objetivo deste trabalho foi fornecer informações sobre o mecanismo molecular que governa a tolerância a alumínio em plantas de arroz, pela caracterização da expressão da família gênica *ASR* com ênfase em *ASR5*, para uso potencial no desenvolvimento de plantas com tolerância a este estresse.

Objetivos específicos:

1. Avaliar o padrão de expressão relativa dos transcritos da família de genes *ASR* em plantas de arroz submetidas ao estresse por Al através de PCR em tempo real;
2. Construir vetores binários de transformação de plantas e obter plantas transgênicas para o estudo e análise da localização subcelular do produto gênico da família *ASR;*
3. Silenciar os genes *ASR* em plantas transgênicas de arroz expressando construções *hairpin* para os genes alvo;
4. Avaliar o efeito do silenciamento dos genes *ASR* nas plantas transgênicas submetidas ao estresse provocado pelo Al.
5. Construir vetores binários de transformação de plantas e obter plantas transgênicas para o estudo e análise dos promotores do gene *ASR5*;
6. Realizar o sequenciamento total dos transcritos de raízes de arroz não transgênicos e silenciado para ASR5 em condições controle e tratadas com Al a fim de verificar o perfil de expressão dos genes alvos potenciais regulados por ASR5;
7. Realizar análises proteômicas comparativas das proteínas expressas em plantas não transgênicas e plantas silenciadas para ASR5;
8. Clonar, expressar e purificar a proteína ASR5 a fim de preparar proteínas recombinantes e anticorpos;
9. Construir bibliotecas de DNA que apresente afinidade de ligação à proteína ASR5, utilizando a estratégia de imunoprecipitação da cromatina (Chromatin Immunoprecipitation - CHIP), em plantas de arroz submetidas ao tratamento com Al;
10. Realizar o sequenciamento das bibliotecas de DNA obtidas no item 9, utilizando sequenciamento em massa;
11. Identificar os genes alvos potenciais da proteína ASR5 através de ferramentas de bioinformática pela comparação dos dados obtidos

nos itens 6 e 9 e os bancos de dados do genoma de arroz disponíveis;

12. Selecionar um gene alvo candidato representado nas bibliotecas (Plantas tratadas com Al), para validar a ligação entre o fator de transcrição codificado pelo gene *ASR5* e a sequência promotora alvo, através da técnica de Sapa – Streptavidin Agarose Pull Down system;
13. Descrever a rede de ativação transcricional, ainda que parcial, regulada pelas proteínas ASR de arroz.

Resultados

Os resultados serão apresentados em três capítulos. O capítulo 1 é dedicado à análise dos padrões de expressão dos genes *ASR* em resposta ao Al, ao estudo da localização subcelular da proteína ASR5, bem como à avaliação proteômica de plantas de arroz silenciadas para os genes *ASR.* O capítulo 2 descreve uma hipótese de regulação por *ASR5* de genes nucleares que codificam proteínas cloroplastídicas. O capítulo 3 é dedicado à análise do promotor do gene *ASR5,* à confirmação da proteína ASR5 como fator de transcrição em arroz regulando genes específicos de resposta ao Al, bem como a uma análise em larga escala dos transcritos expressos em plantas de arroz não transgênico e silenciadas para os genes ASR em condições controle e em resposta ao Al.

CAPÍTULO 1

Involvement of *ASR* genes in aluminium tolerance mechanisms in rice

Este estudo resultou em um artigo intitulado: ‘‘**Involvement of *ASR* genes in aluminium tolerance mechanisms in rice**’’, aceito para publicação no periódico *Plant, Cell & Environment*

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Involvement of *ASR* genes in aluminium tolerance mechanisms in rice

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ABSTRACT

Among cereal crops, rice is considered the most tolerant to aluminium (Al). However, variability among rice genotypes leads to remarkable differences in the degree of Al tolerance for distinct cultivars. A number of studies have demonstrated that rice plants achieve Al tolerance through an unknown mechanism that is independent of root tip Al exclusion. We have analysed expression changes of the rice *ASR* gene family as a function of Al treatment. The gene *ASR5* was differentially regulated in the Al-tolerant rice ssp. Japonica cv. Nipponbare. However, *ASR5* expression did not respond to Al exposure in Indica cv. Taim rice roots, which are highly Al sensitive. Transgenic plants carrying RNAi constructs that targeted the *ASR* genes were obtained, and increased Al susceptibility was observed in T1 plants. Embryogenic calli of transgenic rice carrying an ASR5-green fluorescent protein fusion revealed that ASR5 was localized in both the nucleus and cytoplasm. Using a proteomic approach to compare non-transformed and ASR-RNAi plants, a total of 41 proteins with contrasting expression patterns were identified. We suggest that the ASR5 protein acts as a transcription factor to regulate the expression of different genes that collectively protect rice cells from Al-induced stress responses.

Key-words: abiotic stress; aluminium; ASR gene family; RNAi.

INTRODUCTION

Aluminium (Al) is the most abundant metal, accounting for approximately 7% of Earth's mass. Regardless of its abundance, Al is not considered an essential nutrient; however, it can occasionally stimulate plant growth or induce other desirable effects when present at low concentrations (Foy 1983). Most Al is chelated by ligands or is present in non-toxic forms, such as aluminosilicates or precipitates. Al-induced toxicity can occur through solubilization of Al in soils under highly acidic conditions (pH below 5.0) (Famoso *et al.* 2010). It has been estimated that approximately 50% of arable land is negatively impacted by Al toxicity that results from acidic soil (Panda, Baluska & Matsumoto 2009). Al toxicity is considered a primary limiting factor in regard to agricultural productivity (Matsumoto 2000) because it inhibits root growth and mineral absorption (Liu & Luan 2001), leading to a stunted root system that negatively impacts the uptake of water and nutrients. There are many potential cellular locations that could be damaged through interaction with Al, including the cell wall, the surface of the plasma membrane, the cytoskeleton and the nucleus (Panda *et al.* 2009). For example, it has been demonstrated that Al binds strongly to the cell wall of root epidermal and cortical cells (Delhaize, Ryan & Randall 1993). However, some plants are able to tolerate toxic levels of Al in acidic soils. These plants have evolved mechanisms to detoxify Al that is both present internally and externally (Kochian, Pineros & Hoekenga 2005). To achieve internal detoxification, plants accumulate Al inside vacuoles, where it is chelated with organic acids (OA), such as citrate and oxalate (Ma 2007). In contrast, the majority of Al-tolerant plants exclude Al from the root tip by releasing OA at sites of high Al concentration; examples of these acids include malate, citrate and oxalate (Ma, Ryan & Delhaize 2001; Kochian, Hoekenga & Pineros 2004). In species such as sorghum and wheat, the OA–Al complex prevents Al from entering the cell (Sasaki *et al.* 2004; Magalhaes *et al.* 2007), which reduces the concentration and potential toxicity of Al at the growing root tip (Ma *et al.* 2001).

Rice is considered the most Al-tolerant crop (Fageria 1989; Duncan & Baligar 1990); however, there is variability among different rice genotypes, resulting in widely varied tolerance levels among different cultivars (Ferreira 1995). In two independent studies, Ma *et al.* (2002) and Yang *et al.* (2008) observed no OA exudation and increased Al accumulation in the root apex of Al-susceptible rice strains relative to Al-tolerant strains. These results demonstrate that rice plants achieve high levels of Al tolerance through a novel mechanism that does not involve root tip Al exclusion (Famoso *et al.* 2010).

Although genetic studies in rice have identified more than 10 quantitative trait loci for Al tolerance, the

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responsible genes have only recently been cloned (Huang *et al.* 2009). The genes *STAR1* and *STAR2* were isolated from an Al-tolerant cultivar irradiated with γ-rays (Ma *et al.* 2005). The disruption of either gene resulted in hypersensitivity to Al toxicity. *STAR1* encodes a nucleotide-binding domain protein, and *STAR2* encodes a transmembrane domain protein of a bacterial-type ATP-binding cassette (ABC) transporter. Analyses indicated that *STAR1* and *STAR2* form a complex that functions as an ABC transporter that is required for detoxification of Al in rice. The ABC transporter transports uridine diphosphate (UDP)-glucose, which may be used to modify the cell wall (Huang *et al.* 2009). Yamaji *et al.* (2009) isolated the zinc finger transcription factor ART1, which regulates multiple rice genes implicated in Al tolerance. Genes regulated by ART1 include *STAR1*, *STAR2* and *Nrat1*, the latter of which is a specific transporter that mediates the sequestration of trivalent Al ions into vacuoles to achieve Al detoxification (Xia *et al.* 2010).

Using a proteomic approach, Yang *et al.* (2007) identified some proteins responsive to Al in rice roots; ASR5 was more highly expressed in these roots. The *ASR* (abscisic acid, stress and ripening) gene was first described in tomato (Iusem *et al.* 1993). Subsequently, *ASR* genes were found to be widely distributed in the vegetal kingdom, having been identified in potato (Silhavy *et al.* 1995), pinus (Chang *et al.* 1996), maize (Riccardi *et al.* 1998), rice (Vaidyanathan, Kuruvila & Thomas 1999), sugarcane (Sugiharto *et al.* 2002), grape (Cakir *et al.* 2003) and others. Nevertheless, *ASR* genes do not occur in the genome of *Arabidopsis thaliana* (Maskin *et al.* 2001). *ASR* genes are expressed during fruit ripening and are induced in response to ABA and various abiotic stresses, including water and salt stresses (Carrari, Fernie & Iusem 2004). Kalifa *et al.* (2004a) demonstrated that tomato ASR1 proteins were present as unstructured monomers localized in the cytosol and as structured homodimers in the nucleus, where they can bind DNA. Cytosolic tomato ASR1 performs a chaperone-like activity and can stabilize a number of proteins, protecting them from denaturation induced by repeated freeze/thaw cycles (Konrad & Bar-Zvi 2008). Furthermore, a grape ASR protein binds to the promoter of a hexose transporter gene (Cakir *et al.* 2003), suggesting that it may be a transcription factor that is involved in sugar metabolism. *In silico* analyses mapped the locations of six copies of *ASR* genes in the rice genome in different chromosomes; these loci were confirmed by expressed sequence tags (ESTs) (Frankel *et al.* 2006).

In this study, we analysed changes in gene expression of the rice *ASR* family in response to Al treatment. We found that all members of the *ASR* gene family display a variable degree of expression, indicating that *ASR* genes of the tolerant Japonica rice (cv. Nipponbare) are differentially regulated in response to Al. Conversely, *ASR5* did not respond to Al exposure in Indica rice roots (cv. Taim), which unlike the Nipponbare cultivar, is highly sensitive to Al (Freitas *et al.* 2006). According to Ma *et al.* (2002), Japonica varieties are often more resistant to Al than the Indica varieties. In addition to gene expression analyses, transgenic plants carrying RNAi constructs targeting the *ASR* genes were made, and an increased Al susceptibility was observed in T1 plants. In addition, transgenic embryogenic calli of rice carrying an ASR5-green fluorescent protein (GFP) fusion protein revealed that ASR5 is located in both the nucleus and the cytoplasm, suggesting that ASR5 may act as a transcription factor.

MATERIALS AND METHODS

Plant material and growth conditions

For Al treatments, rice seeds from Japonica Nipponbare and Indica Taim backgrounds were germinated on filter paper for 4 d in the dark at 28 °C. The seedlings were grown in a hydroponic solution (Baier, Somers & Gusiafson 1995) for 12 d in a growth chamber at 28 °C under 12 h of light. The hydroponic solution was replaced every 4 d. After 12 d, seedlings were treated with 150 μM $AlCl_3$. Root tissue samples were collected at 4 and 8 h after the start of treatment. Identical conditions were used for experiments involving the use of 450 μM $AlCl_3$. Samples were collected after 8 h of treatment; roots and shoots were collected from Nipponbare plants and only roots were collected from Taim. For gene expression analysis at the root base and apex, rice plants were cultivated in the same conditions described earlier and samples of Nipponbare roots were divided into two segments: apex (0.5 cm) and base (4 ± 0.5 cm). Control plants were not treated with $AlCl_3$. All plants were grown in acidic conditions (pH 4.5).

To analyse responses to cold stress, 2-week-old plants (cv. Nipponbare) were transferred to growth chambers and exposed to a temperature of 4 °C for 12 h. Control plants were maintained at 28 °C. For ultraviolet (UV) light treatment, 2-week-old plants (cv. Nipponbare) were transferred to the growth chamber and exposed to two 4 h treatments of continuous UV-B illumination (0.25 kJ m^{-2} min^{-1}) at a 20 h interval, and subsequently maintained under normal light during the recovery period. Analyses were performed 24 h following initial UV irradiation. For drought stress, cv. Nipponbare seedlings were grown to the four-leaf stage in soil with a normal supply of water. Subsequently, control plants were watered normally and stressed seedlings were not watered for 14 d.

Quantitative real-time PCR (RT-qPCR)

Tissue samples were collected and immediately frozen in liquid nitrogen; total RNA was then extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was carried out using the Moloney murine leukemia virus (M-MLV) RT reverse transcriptase enzyme (Promega, Madison, WI, USA). The RT reaction was performed in a final volume of 50 μL. A 1:10 solution of total cDNA was prepared (stock solution). For RT-qPCR reactions, the stock solution was diluted at a ratio of 1:100.

The protocol for RT-qPCR is summarized as follows: an initial step of 5 min at 94 °C followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. Samples were maintained for 2 min at 40 °C to promote re-annealing and were then warmed from 55 to 99 °C to generate relative denaturing curve data for the amplification products. RT-qPCR was carried out with 10 μL of the diluted cDNA (1:100), 2.0 μL of 10× PCR buffer (Tris–HCL at 100 mM at pH 8.0, KCl at 500 mM), 1.2 μL $MgCl_2$ 50 mM, 0.1 μL 5 mM deoxy-nucleotide triphosphates (dNTPs), 0.2 μL of each individual 10 μM primer solution, 4.25 μL H_2O, 2.0 μL SYBR GREEN (1:100 000) and 0.05 μL *Platinum Taq DNA Polymerase* (5 U/μL, Invitrogen), in a final volume of 20 μL. All reactions were performed in four technical replications, and calculations were performed using the $2^{-\Delta\Delta C}$ method (Livak & Schmittgen 2001). Quantitative PCR was performed using specific primer pairs for *ASR1* to *ASR6*: *ASR1* 5′-TGGTGGACTACGACAAGGAGA and 5′-GCCACCTCCTCCTTCACC-, *ASR2* 5′- CATGGCGGCTACGGCTAC and 5′- GGTCCTTCTTCGCCTGGT, *ASR3* 5′- CACCACAAGAACGACGACAA and 5′-TGTGATGCTCGTGGATGG-, *ASR4* 5′- CGACTATCGCAAGGAGGAGA and 5′- CGATCCCTTCCTTCATCTTG, *ASR5* 5′- CCAGGACGAGTACGAGAGGT and 5′- CGATCTCCTCCGTGATCTTG, *ASR6* 5′- GCCCGGAGAAGTACAGGAAG and 5′- GCCCTCCTCGATCCTGTG, *APx1* 5′- CTACAAGGAGGCCCACCTCA and 5′- CCGCATTTCATACCAACACA and *APx2* 5′- ACCTGAGGTCCCCTTCCA and 5′- CTCTCCTTGTGGCATCTTCC. The primers *eFα* 5′-TTTCACTCTTGGTGTGAAGCAGAT and 5′-GACTTCCTTCACGATTTCATCGTAA, *FDH* 5-′CAAAATCAGCTGGTGCTTCTC and 5′-TTCCAATGCATTCAAAGCTG and *Actin* 5′-GACTCTGGTGATGGTGTCAGC and 5′-GGCTGGAAGAGGACCTCAGG were used as reference genes against which the amount of mRNA present in each sample was normalized. The primers *STAR1* (Huang *et al.* 2009) 5′-TCGCATTGGCTCGCACCCT and 5′-TCGTCTTCTTCAGCCGCACGAT were used as positive controls for Al stresses. The efficiency of amplification was calculated for all samples and all different primer pairs using the LinReg program (Ramakers *et al.* 2003). Only samples with efficiency values higher than 85% were considered for expression analysis, with corrections according to primer efficiencies as previously described (Pfaffl 2001). The quantities of amplified products were compared using an Applied Biosystems (Foster City, CA, USA) 7500 Real-Time PCR System.

Construction of plant vector and plant transformation

A chimeric gene for producing RNA with a hairpin structure (hpRNA) was constructed based on the sequence of the ASR5 locus (LOC_Os11g06720.1). The following primers were used to amplify the 417 bp region corresponding to the full ASR5 coding sequence: 5′-CACCATGGCGGAGGAGAAGCAC and 5′-TCAGCCGAAGAGGTGGTG. PCR products were cloned into the Gateway vector pANDA in an inverted repeat configuration, in which the chimeric gene is under the control of the maize ubiquitin promoter with an intron placed upstream of the inverted repeats (Miki & Shimamoto 2004).

To determine the subcellular localization of the ASR5 protein in transgenic rice calli, the complete coding sequence was fused with the GFP coding sequence at the N-terminus into a modified Gateway vector pH 7FWG2 (Karimi, Inze & Depicker 2002). Restriction enzymes were used to replace the 35S promoter with the maize ubiquitin promoter. *Agrobacterium*-mediated transformation of rice calli was performed as described previously (Upadhyaya *et al.* 2000) using Nipponbare cultivar. For transient expression of GFP-ASR5 in rice protoplasts, the complete coding sequence of ASR5 was fused to the GFP coding sequence at its N-terminus and cloned into the Gateway vector pART7-YFP (Galvan-Ampudia & Offringa 2007). The amplified cDNA was introduced into appropriate plasmids by Gateway technology. The resulting vector was used to perform protoplast transformation.

Protoplast isolation was performed essentially as described (Chen *et al.* 2006) and protoplast transformation was performed according to the methodology described in a previous report (Tao, Cheung & Wu 2002). Transformed protoplasts were incubated in the dark for 24–48 h at 27 °C prior to imaging. Fluorescence microscopy was performed with an Olympus FluoView 1000 confocal laser-scanning microscope (Center Valley, PA, USA) equipped with a set of filters capable of distinguishing enhanced green and yellow fluorescent proteins (EGFP and EYFP, respectively) and plastid autofluorescence. Images were captured with a high-sensitivity photomultiplier tube detector.

Characterization of RNAi transgenic plants

T1 or T2 generation and non-transformed (NT) rice seeds were germinated in moistened filter paper at 28 °C in the dark. Four-day-old seedlings were then transferred to plastic pots containing only Baier's solution (Baier *et al.* 1995) or Baier's solution supplemented with 450 μM $AlCl_3$ or 25 μM of cadmium ($CdCl_2$). Plants were maintained for 12 d, and root length was then measured. The pH (4.5 for both treatments) was monitored daily, and the nutrient solution was replaced every 4 d. Relative root elongation in cadmium or in Al experiments was defined as the percentage of the root elongated by $CdCl_2$ or Al compared to the control ($CdCl_2$-and Al-free).

For drought stresses, T1 generation plants and NT plants were grown in soil for 5 months with a normal supply of water and then subjected to drought for 15 d without watering.

The relative water content (RWC) of leaves was determined by the procedure described by Cairo (1995). A total of 20 leaf discs of 12 mm in diameter were collected randomly, and fresh weight (FW1) was determined with an analytical balance. The discs were then transferred to a Petri dish containing distilled water and placed on the laboratory

bench for a period of 12 h under constant illumination (40 μmol m^{-2} s^{-1}). After this period, leaf discs were removed from the Petri dish, placed on filter paper and subjected to light pressure to eliminate excess water. The discs were immediately weighed again to determine measured mass (FW2). To determine the dry tissue mass [dry weight (DW)], the discs were placed in paper bags and exposed to greenhouse ventilation with an air temperature of 75 °C for a period of 48 h. The RWC calculations were performed according to the following equation:

$$\text{RWC}\,(\%) = \text{FW1} - \text{DW}/\text{FW2} - \text{DW} \times 100$$

The humidity percentage of leaf tissues was determined according to the relationship described by Slavik (1974):

$$\text{Humidity}\,\% = \text{FW1} - \text{DW}/\text{FW1} \times 100.$$

Western blotting

Twelve-day-old rice leaves of NT and RNAi plants were macerated and homogenized in 0.5 M Tris–HCl (pH 8.3), 2% Triton X-100, 20 mM $MgCl_2$, 2% β-mercaptoethanol, 1 mM Phenylmethanesulfonylfluoride (PMSF), 2.5% Polyethylene glycol (PEG) and 1 mM ethylenediaminetetraacetic acid (EDTA); samples were incubated at 4 °C for 1 h. Protein extracts were centrifuged and precipitates were discarded. Samples of each lysate were loaded to yield 50 μg of protein, which was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in 15% gel. ASR5 protein was detected with a rabbit polyclonal ASR5 antibody (1:500). To prepare the antibody, ASR5 full-length cDNA was cloned into a pGEX (GE, Fairfield, CT, USA) vector; the recombinant clone was introduced into *Escherichia coli* (BL21). ASR5 expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein was purified with Glutathione Sepharose 4B (GE). The purified protein was injected into a rabbit, and the serum obtained from that animal contained the antibody. Goat anti-rabbit IgG (1:1000) conjugated to alkaline phosphatase was used as the secondary antibody. The bands were detected with a premixed BCIP/NBT substrate solution (Sigma, St Louis, MO, USA) and recorded on X-ray film.

Two-dimensional gel electrophoresis and mass spectrometry

Leaves of 6-month-old plants, both NT and expressing RNAi, were macerated in liquid nitrogen and homogenized in a buffer containing 0.5 M Tris–HCl, pH 8.3, 2% Triton X-100, 20 mM $MgCl_2$, 2% β-mercaptoethanol, 1 mM PMSF, 2.5% PEG and 1 mM EDTA; samples were then incubated at 4 °C for 2 h. The samples were centrifuged and the precipitate was discarded. An acetone solution containing trichloroacetic acid was added to the supernatant to yield a final concentration of 10%, and the mixture was incubated for 12 h at –20 °C to allow protein precipitation to occur. The resulting precipitate was washed with cold acetone and dissolved in a solution containing 7 M urea and 2 M thiourea (Acquadro *et al.* 2009). The amount of protein extracted for each sample was quantified *via* the Bradford (1976) method.

Approximately 500 μg of extracted protein was used for analysis in two-dimensional gel electrophoresis. In the first dimension, proteins were separated according to their isoelectric points under a pH gradient ranging from 4 to 7. In the second dimension, proteins were separated according to their molecular weight in 12.5% polyacrylamide gel containing SDS. The gels were stained with Coomassie brilliant blue R-250 from Thermo Scientific (Rockford, IL, USA) and analysed to obtain images of spots of interest, which were subsequently excised and digested with 10 ng/uL of trypsin. The peptide solution was separated with a multidimensional chromatographic system. The eluted fractions were analysed in a mass spectrometer with electrospray ionization, and two mass analysers – a quadrupole (Q) associated with a tube in which one measures the ion time of flight (TOF) and an ion detector. In conjunction with the online Q-TOF, a capillary chromatography system [Acquity ultra performance liquid chromatography (UPLC)] was used to submit samples in a reverse phase column. To perform MS analysis, the peptides eluted from the column were ionized and then their mass/charge ratio (m/z) was determined. The spectra-generated MS/MS was processed in Mascot Distiller software and compared against the database of the National Center for Biotechnology Information using Mascot software (http://www.matrixscience.com).

Statistical analyses

Data represent mean ±standard error of the mean. Data were analysed by analysis of variance (ANOVA) followed by Duncan's post hoc test, using the statistical program SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, USA) (http://www.spss.com). The values were considered statically significant when $P < 0.05$.

In silico analyses

In silico analyses were performed using the Microarray platform (RiceXPro, available at http://ricexpro.dna.affrc.go.jp/index.html) and the BLAST (bl2seq) algorithm for aligning amino acid sequences (available at http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi).

RESULTS

Expression profile of ASR genes in rice

The expression patterns of different *ASR* genes following Al exposure (150 μM) were investigated using quantitative PCR. In Nipponbare plants, a rice cultivar tolerant to excess levels of Al, the expression of *ASR* genes was up-regulated in response to Al treatment (Fig. 1). Transcript levels corresponding to the genes *ASR1*, *ASR4* and *ASR5* were

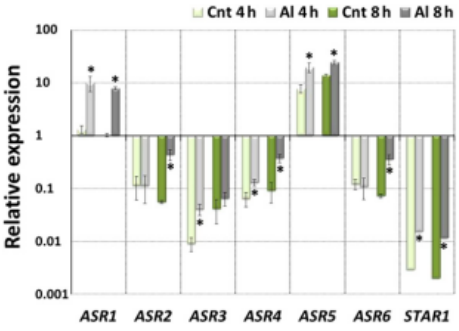

Figure 1. The *ASR* gene family responds to aluminium stress. Determination of rice *ASR* family expression levels in response to treatment with Al. Relative expression was plotted using *ASR1* gene expression levels in control plants as calibrator. Total RNA was extracted from roots and used for cDNA synthesis. Analyses were conducted using RT-qPCR. Roots of Nipponbare cultivar were collected after 4 and 8 h of treatment with Al (150 μM). Values represent the means ± SD ($N = 4$). Asterisks indicate significantly different means: *$P < 0.05$.

up-regulated after 4 and 8 h of treatment, while *ASR2* and *ASR6* showed an increase in their transcript levels only after 8 h of treatment; similarly, *ASR3* was up-regulated only after 4 h of Al treatment.

When a higher concentration of Al was used to treat Nipponbare plants (450 μM), the expression of *ASR5* was increased relative to plants treated with 150 μM of Al. In shoots and roots, *ASR5* expression increased after 8 h of treatment with 450 μM Al. In contrast, in the roots of the Al-sensitive cultivar Taim (ssp. Indica), *ASR5* expression did not respond to 450 μM Al treatment (Fig. 2a). To confirm the phenotype of the Taim cultivar respective to the tolerance or sensitivity to Al, we have performed experiments comparing the growth of both Nipponbare and Taim plants in the presence of 450 μM Al. Our results show that in response to Al, Taim roots elongated only 18.23%, whereas Nipponbare elongated 59.57% (Fig. 2b,c), indicating that Taim cultivar is significantly more sensitive to Al compared to the Nipponbare cultivar.

Relative to control roots, *ASR5* mRNA expression in Japonica rice was not significantly different in either of the two root segments (apex and base) Al. However, the expression of *ASR5* transcripts increased in both segments in response to Al treatment, although stronger expression was observed in the root tip (Fig. 3). To confirm the effect of Al on gene expression, *STAR1* was used as a positive control (Huang *et al*. 2009). As *ASR* genes are known to be involved in plant responses to several abiotic stresses, the expression patterns of *ASR5* in response to cold, drought and UV light were also analysed (Supporting Information Fig. S1). The expression of *ASR5* gene did not respond in roots maintained at 4 °C, but increased in leaves when plants were subjected to drought for 14 d. In UV-treated plants, *ASR5* transcript levels decreased drastically when relative to control plants.

Using a microarray platform (RiceXPro) to analyse gene expression patterns for Japonica rice grown in natural field conditions (Sato *et al*. 2010), we observed that *ASR1* and *ASR5* were expressed in leaves and roots at the vegetative stage and that there was reduced expression at the onset of inflorescence. In later stages of flower development, higher transcript levels were detected in palea, lemma and ovaries (Supporting Information Fig S2).

ASR-silenced plants are sensitive to Al and drought

Transgenic plants were obtained, which expressed RNAi constructs targeting ASR5. Expression analysis of transgenic rice plants demonstrated knockdown of both *ASR1* and *ASR5* genes. Besides, these genes were not able to respond anymore to Al (Fig. 4a). Western blot analyses showed a decrease in the ASR5 protein levels (Fig. 4b). Under normal conditions, there were no differences between the ASR RNAi plants and NT plants with regard to height, leaf number and root development. However, the onset of flowering was delayed by 15 d in transgenic plants, which also presented an abnormal panicle development and reduced seed number relative to NT plants (Fig. 4c). In addition, transgenic plants showed a reduction in the number of leaf trichomes, palea and lemma (Fig. 4d). T1 generation seeds were germinated and grown for 12 d in a solution containing 450 μM Al. Increased Al sensitivity was observed in T1 plants accompanied by strongly inhibited root elongation (Fig. 5a,b). In shoots, however, there was no difference in fresh weight between NT and RNAi line (Fig. 5c). Furthermore, ASR silencing affects specific Al response since roots of NT plants and ASR RNAi were both strongly inhibited using cadmium (Supporting Information Fig. S3). Transgenic plants subjected to drought for 15 d also showed greater susceptibility to this condition (Fig. 6). These results indicate that ASR5 knockdown affects rice plants by increasing their sensitivity to abiotic stresses.

Global protein levels were affected in ASR-RNAi plants

Using a proteomic approach to compare NT and ASR-RNAi plants, a total of 41 proteins were identified, 11 of which showed increased expression versus 30 that were down-regulated in ASR-RNAi plants. Of these proteins, 32% are involved in photosynthesis, 20% in carbohydrate metabolism and 17% in response to stress. The other proteins are functionally associated with amino acid metabolism, phosphate metabolism, development, protein degradation, nucleotide binding, cellular composition and electron transport (Table 1) (Supporting Information Fig. S4).

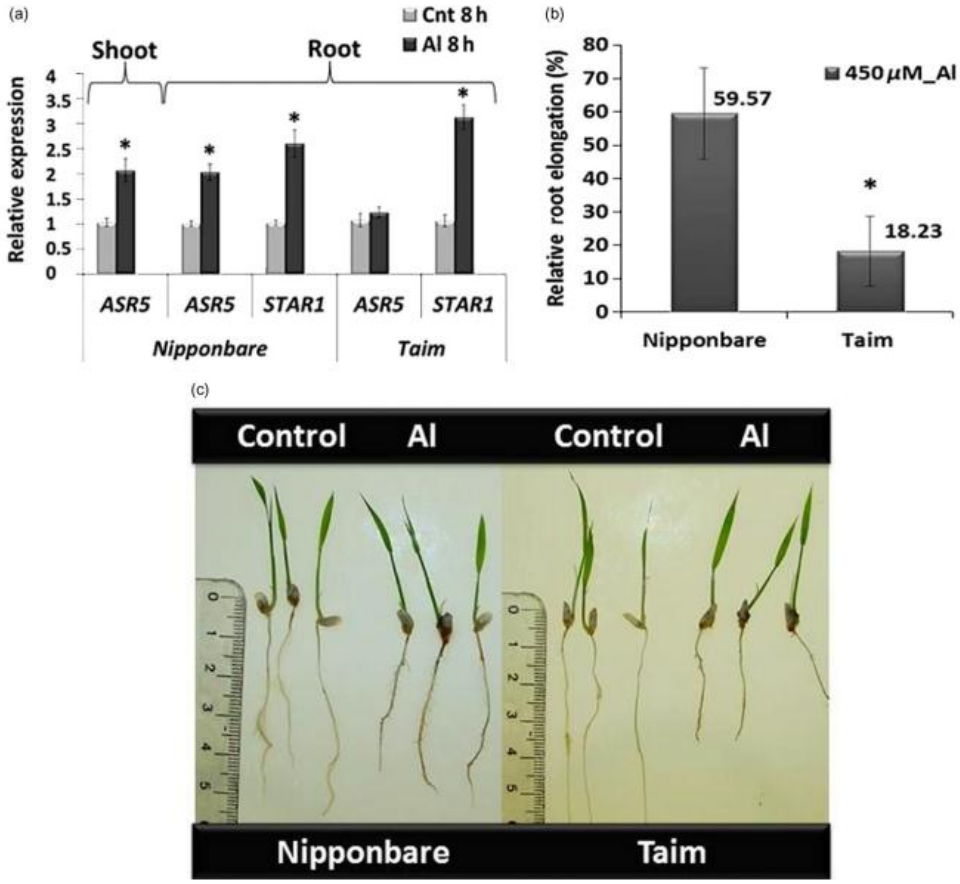

Figure 2. *ASR5* does not respond to aluminium stress in an Al-sensitive rice cultivar. (a) Total RNA was extracted from roots and shoots, and used for cDNA synthesis. Analyses were conducted via RT-qPCR. Relative expression was plotted using *ASR5* and *STAR1* expression levels in control plants as calibrator. Roots and shoots of Nipponbare cultivar and roots of Taim cultivar were collected after 8 h of treatment with Al (450 μM). (b) Relative root elongation (RRE) for Nipponbare and Taim plants. (c) Nipponbare and Taim plants growth for 12 d in a solution containing 450 μM Al. Values represent the means ± SD ($N = 4$). Asterisks indicate significantly different means: $^*P < 0.05$.

Cytosolic ascorbate peroxidase gene knockdown rendered rice plants more sensitive to high Al concentrations

Among the stress-related genes down-regulated in ASR-RNAi plants, APx1 and APx2 protein levels were markedly reduced. The down-regulation of cytosolic ascorbate peroxidase (APx) in response to ASR gene knockdown (displayed in Table 1) prompted us to examine how plants containing RNAi sequences targeting these peroxidases (APx1/2s plants) could respond to toxic concentrations of Al. These Apx1/2s plants were previously obtained by our group (Rosa *et al.* 2010). Treatment with 750 μM Al was conducted in hydroponics for 14 d. Seedlings with reduced expression of cytosolic *APx* genes (Apx1/2s plants) showed greater sensitivity to this concentration of Al. The aerial parts of Apx1/2s plants developed necrotic lesions in high extension of foliar volume. Conversely, unlike RNAi plants, NT plants leaves had expanded after 14 d of stress (Supporting Information Fig. S5a). The plants (both transgenic and NT) did not differ when grown in control conditions (Rosa *et al.* 2010). In wild-type tolerant cultivar

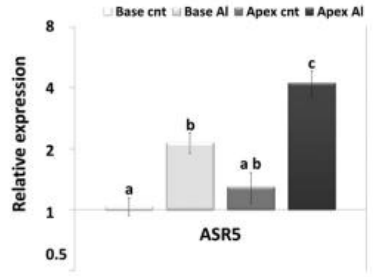

Figure 3. *ASR5* expression levels increase significantly in root apex after Al exposure. Total RNA was extracted from roots, and used for cDNA synthesis. Analyses were conducted using RT-qPCR. Relative expression was plotted in a logarithmic scale and *ASR5* expression levels in root base of control plants was used as calibrator. After 8 h of treatment with Al (450 μM), roots of Nipponbare cultivar were collected and divided into two segments: base and apex (0.5 cm). Bars with different letters are significantly different (ANOVA, $P < 0.05$).

Nipponbare, APx1 and APx2 increase its levels of transcripts in response to Al (Rosa *et al.* 2010), whereas in the sensitive cultivar Taim, APx1 and APx2 did not respond to Al (Supporting Information Fig. S5b).

ASR5 protein is localized in nucleus, cytoplasm and chloroplasts

To identify the subcellular localization of the ASR5 protein *in vivo*, the full-length cDNA of *ASR5* was fused at its N-terminus to the coding sequence of GFP. A modified pH 7FWG2 vector (see Materials and Methods) was used for stable expression and the pART vector was used to achieve transient expression. In stable expression analyses, ASR5-GFP fusion proteins expressed in transgenic rice embryogenic calli were present in the nucleus and cytoplasm (Fig. 7a). NT embryogenic calli were used as negative control. Conversely, transient expression analyses of rice protoplasts obtained from green leaf showed that ASR5 resides in chloroplasts (Fig. 7b). For this experiment, a vector containing only GFP was used as positive control and an empty vector was used as a negative control. These results indicate that ASR5 is located in multiple cellular compartments.

Analysis of ASR5 nuclear localization signal

Most ASR proteins have a nuclear localization signal (NLS) in their C-terminal region that is composed of two sets of basic amino acids conserved between different members of monocots and dicots and separated by a segment composed

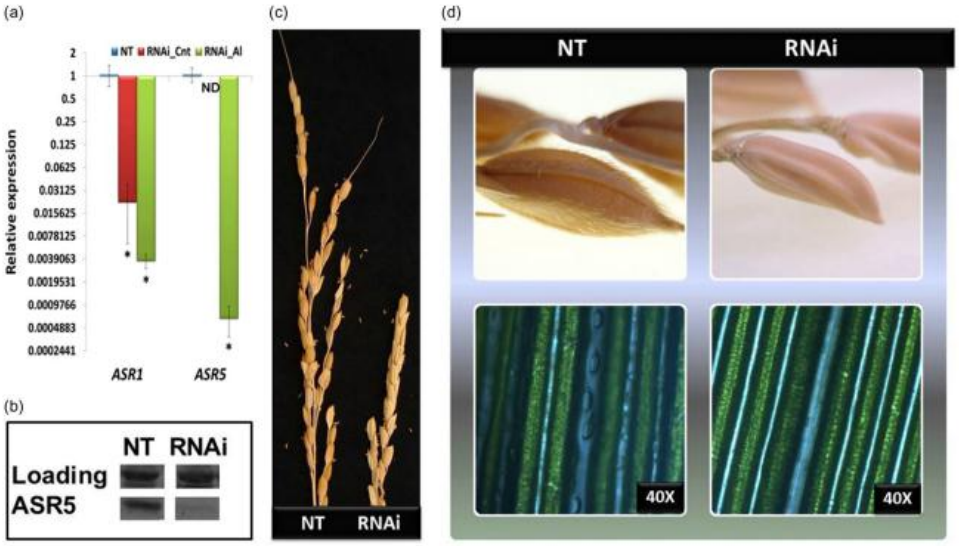

Figure 4. Characterization of plants expressing the RNAi construct. (a) Total RNA was extracted from leaves, and used for cDNA synthesis. Analyses were conducted using RT-qPCR. Measurements were performed on 3-week-old leaves of ASR RNAi plants in control condition (red bars), ASR RNAi plants in Al condition (450 μM for 8h – green bars) and non-transformed (NT) plants (blue bars). Values represent the means ± SD ($N = 4$). Asterisks indicate significantly different means: *$P < 0.05$. ND means non-detected. (b) Western blot analysis of ASR5 protein expression in RNAi and NT rice plants. (c) Abnormal panicle development in T1 RNAi plants compared to NT plants. (d) Reduction of trichomes in leaves, palea and lemma of T1 RNAi plants compared with NT plants.

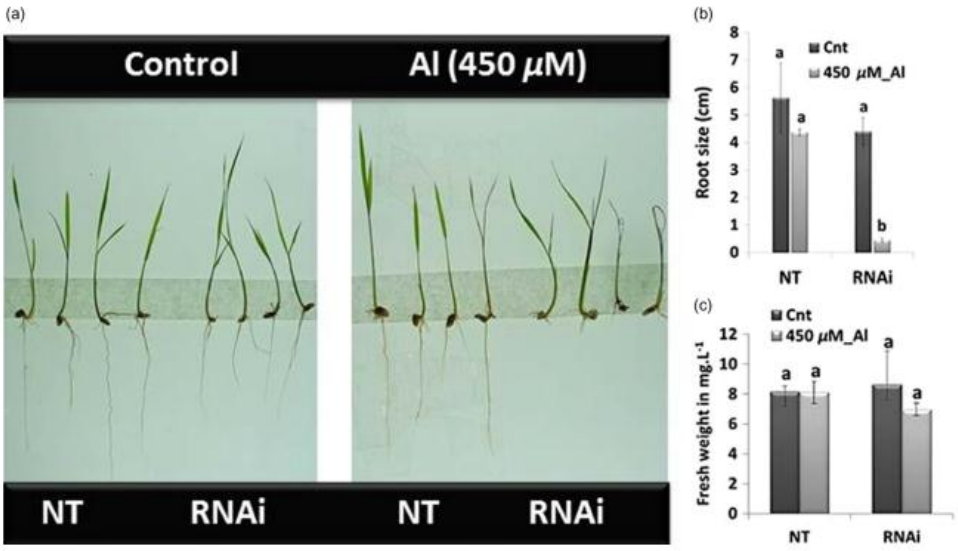

Figure 5. ASR-silenced plants are sensitive to aluminium. (a) T1 generation (RNAi) and non-transformed (NT) growth for 12 d in a solution containing 450 μM Al. (b) Differences in root length for ASR RNAi and NT plants. (c) Differences in shoot fresh weight for ASR RNAi and NT plants. Bars with different letters are significantly different (ANOVA, $P < 0.05$).

of approximately 10 unconserved amino acids, or a continuous set of basic amino acids (Padmanabhan, Dias & Newton 1997; Cakir *et al*. 2003; Kalifa *et al*. 2004a). With the exception of ASR2, all of the rice ASR proteins contain basic lysine (K) residues, which is a characteristic of NLSs that is relatively well conserved in lily ASR – LLA23 (Wang *et al*. 2005) (Supporting Information Fig. S6). These residues are divided into two sets represented by the letters A and B, the latter of which is found only in the ASR5 protein (Supporting Information Fig. S6).

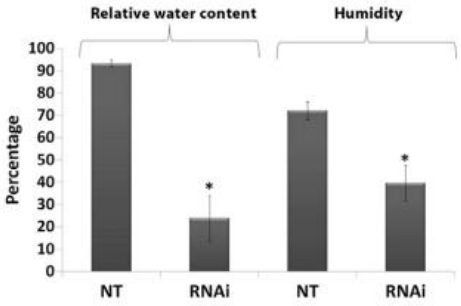

Figure 6. ASR-silenced plants are sensitive to drought. Analyses of relative water content and percentage of humidity in 5-month-old T1 generation (RNAi) and non-transformed (NT) plants subjected to drought for 15 d. Asterisks indicate significantly different means: *$P < 0.05$.

DISCUSSION

The *ASR* gene family is well established as being involved in responses to ABA and abiotic stresses, including drought and salinity; *ASR* genes are also involved in the process of fruit ripening (Carrari *et al*. 2004; Kalifa *et al*. 2004a; Konrad & Bar-Zvi 2008). Here, we have demonstrated that, in addition to *ASR5*, the whole rice *ASR* gene family is up-regulated at the transcriptional level when rice plants are subjected to high Al concentrations. Rice ASR5 was previously identified as a protein that responded to Al (Yang *et al*. 2007). Moreover, global transcriptional analyses comparing the Al responses of two wheat (*Triticum aestivum* L.) near-isogenic lines of variable Al tolerance revealed an early increase in *ASR1* transcript expression (Guo *et al*. 2007). Therefore, based on their expression profiles, the involvement of *ASR* genes in response to Al is common to both rice and wheat. Although some literature reports have demonstrated the Al-induced *ASR* gene response, the direct involvement of this gene family in regard to Al tolerance has not been previously reported. Therefore, our effort to compare *ASR* mRNA accumulation in response to Al in two varieties of rice with contrasting Al sensitivity represents the first study

Table 1. Differentially expressed proteins in leafs of non-transformed and ASR5-RNAi plants identified by mass spectrometry (electrospray ionization-MS/MS)

			Theoretic		Observed				
No spot[a]	Δ Expression[b]	Protein name	pI	P. M. (kDa)	pI	P. M. (kDa)	Score[c]	S. P. (%)[d]	Accession (NCBI)[e]
Protein degradation									
1	↓	Heat shock protein 70 (hypothetical protein OsJ_17967)	5.12	74	4.77	78	962	36	gi|222631026
2	↓	Mitochondrial chaperonin (Os12g0277500)	5.12	61	4.93	73	699	43	gi|115488160
Photosynthesis									
3	↑	ATP synthase alpha/beta chain, C-terminal domain (Os01g0711000)	5.03	54	5.21	70	231	28	gi|115439527
4	↓	ATP synthase CF1 alpha subunit	5.95	56	6.17	70	1234	54	gi|11466784
5	↓	Ribulose bisphosphate carboxylase large chain	6.22	53	6.51	66	935	40	gi|11466795
6	↓	ATP synthase gamma chain (Os07g0513000)	8.6	40	6.21	56	405	34	gi|115472339
7	↑	Putative oxygen-evolving enhancer protein 1, chloroplast precursor (Os01g0501800)	6.1	35	5.30	48	1286	61	gi|115436780
8	↓	Putative ferredoxin-NADP(H) oxidoreductase	7.98	41	5.96	52	359	36	gi|41052915
9	↑	Chlorophyll *a-b* binding protein	4.73	24	4.93	37	529	60	gi|108864186
10	↑	Chloroplast 23 kDa polypeptide of photosystem II	5.56	20	5.90	34	900	67	gi|164375543
11	↑	Putative photosystem I reaction centre subunit IV	9.64	16	5.75	23	430	61	gi|34394725
12	↓	Putative ribulose bisphosphate carboxylase large chain precursor (Os05g0427800)	4.88	16	4.61	18	56	13	gi|115464019
13	↓	Ribulose bisphosphate carboxylase, small chain	5.89	15	6.11	15	551	88	gi|56966763
14	↓	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	7	26	5.50	25	314	36	gi|290585768
15	↓	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	7	26	6.19	29	245	30	gi|290585768
Carbohydrate metabolism									
16	↓	Putative transketolase	6.12	81	5.69	78	693	29	gi|28190676
17	↓	Sedoheptulose-1,7-bisphosphatase precursor	5.83	43	4.9	57	662	40	gi|27804768
18	↑	Lactate/malate dehydrogenase, NAD binding domain (Os10g0478200)	5.75	36	5.94	56	388	49	gi|115482534
19	↓	Lactate/malate dehydrogenase, alpha/beta C-terminal domain (Os05g0574400)	8.22	36	6.68	55	612	70	gi|115465579
20	↑	Glycolate oxidase (Os03g0786100)	8.48	40	6.93	57	529	44	gi|115455773
21	↓	Putative uridylyltransferase related	5.72	30	4.63	51	200	34	gi|187608845
22	↓	Putative ribose-5-phosphate isomerase	4.91	27	4.71	38	266	45	gi|34393836
23	↓	Ribulose-5-phosphate-3-epimerase	8.93	29	6.14	35	209	20	gi|4105561
Nucleotide binding									
24	↓	Ribulose 1:5-bisphosphate carboxylase/oxygenase activase small isoform precursor	5.59	52	4.86	64	675	61	gi|62733297
25	↓	Chloroplast translational elongation factor Tu	5.68	56	5.58	63	879	49	gi|218191089

Table 1. *Continued*

No spot[a]	Δ Expression[b]	Protein name	Theoretic		Observed		Score[c]	S. P. (%)[d]	Accession (NCBI)[e]
			pI	P. M. (kDa)	pI	P. M. (kDa)			
Cellular component									
26	↑	Actin (Os11g0163100)	5.31	42	5.43	60	167	27	gi|115484337
27	↓	Fibrillin-like protein	5.04	34	4.64	48	155	22	gi|29367475
28	↓	PAP fibrillin family domain containing protein (Os11g0595200)	8.92	28	5.03	46	141	14	gi|115486133
Amino acid metabolism									
29	↓	Glutamine synthetase, catalytic domain (Os02g0735200)	5.51	39	5.56	58	198	19	gi|115448531
30	↓	Putative glycine cleavage system H protein (Os10g0516100)	4.92	17	4.45	18	162	28	gi|115482934
Phosphate metabolism									
31	↓	Putative inorganic pyrophosphatase	5.8	32	5.04	48	121	16	gi|46805452
Stress response									
32	↓	Glyoxalase (hypothetical protein OsJ_26316)	5.53	35	5.53	51	330	41	gi|125602450
33	↑	Class III peroxidase (OsPrx111)	5.77	33	5.73	51	461	44	gi|20286
34	↓	Chloroplastic lipocalin (Os04g0626400)	6.23	38	5.01	47	65	11	gi|115460690
35	↓	Cytosolic ascorbate peroxidase – OsAPx1 (Os03g0285700)	5.42	27	5.67	37	381	54	gi|115452337
36	↑	Manganese superoxide dismutase	6.5	25	6.07	31	425	56	gi|601869
37	↑	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	5.79	21	5.71	19	617	68	gi|42408425
38	↓	Putative superoxide dismutase 1	6.24	28	6.23	22	139	36	gi|108708142
Transport of electrons									
39	↓	Oxidoreductase NAD-binding domain (Os07g0328300)	5.44	31	4.58	45	364	48	gi|115445869
40	↑	2Fe-2S iron-sulphur cluster binding domain (anti-disease protein 1)	4.37	15	4.04	24	191	57	gi|18698985
Development									
41	↓	Germin-like protein 1	6.01	22	6.01	26	185	15	gi|4239821

[a]Numbers correspond to two dimensional (2-DE) gels.
[b]Increase (↑) or reduction (↓) of proteins in response to ASR silencing.
[c]Mascot score.
[d]% of protein sequencing.
[e]Accession number of NCBI.

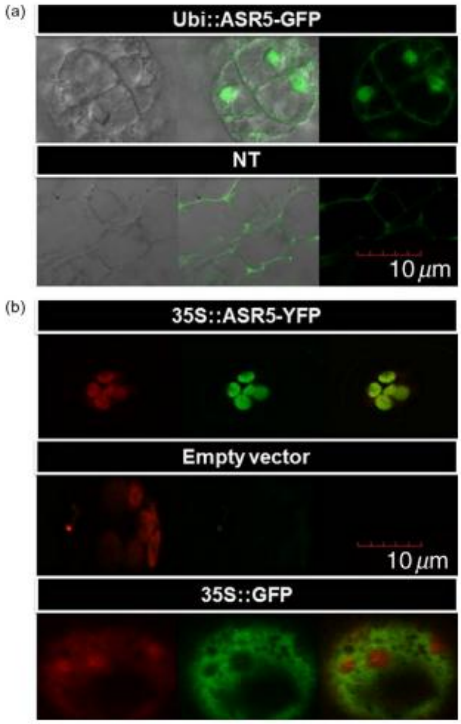

Figure 7. ASR5 is localized in nuclei, cytoplasm and chloroplasts. (a) Localization of UBI-ASR5::GFP in rice embryogenic calli. Green fluorescent signal indicates cell wall autofluorescence in non-transformed rice. (b) Localization of ASR5 in the chloroplasts of rice protoplast visualized by transient expression of 35S-ASR5::YFP. Negative and positive control groups with 35S-gus empty and 35S-gus:YFP^{C}, respectively.

of the functional role of *ASR* genes in this plant defence mechanism.

Our results reveal that the *ASR5* expression levels were not affected by Al treatment in the Al-sensitive cultivar (Taim), but were significantly increased in the Al-tolerant Nipponbare rice cultivar (Fig. 2). *ASR5* gene expression was induced in the salt-resistant rice cultivar 'Pokkali' in response to NaCl treatment, but not in the sensitive rice cultivar 'IR29' (Salakdeh *et al.* 2002). In our experiments, *ASR5* mRNA levels increased significantly in the root apex following exposure to Al (Fig. 3). According to Panda *et al.* (2009), the root is the most Al-sensitive plant region. Al phytotoxicity blocks cell division, and, as a result, root hair development is inhibited and the root apex becomes swollen and damaged (Clarkson 1965). The root apex and elongation zone are highly sensitive to Al and easily accumulate Al. As a result, more severe physical damage happens in these zones relative to the mature root regions (Panda *et al.* 2009). In contrast to other stresses, cold treatment did not affect *ASR5* expression. Nevertheless, it is possible that ASR5 plays a protective role in leaf tissues rather than roots, as Kim *et al.* (2009) has reported that *ASR5* transcripts are up-regulated in rice leaves after 3 and 6 h of stress. Furthermore, the overexpression of ASR5 increased the cold tolerance of transgenic rice. In other plants, ASR overexpression indicates the potential use of ASR as a gene for plant breeding. Stable expression of tomato ASR1 in tobacco and potato has been shown to increase salt tolerance and influence glucose metabolism, respectively (Kalifa *et al.* 2004b; Frankel *et al.* 2007). In *Arabidopsis*, overexpression of a lily ASR ortholog resulted in increased salt tolerance (Yang *et al.* 2005). Although many studies involving *ASR* genes have been performed previously, there are presently no reports characterizing the responses of the entire rice *ASR* family to abiotic stresses and, more specifically, Al treatment. These genes respond to different abiotic stresses and Al exposure, suggesting that they play an important role in protecting rice plants from environmental stresses.

To further evaluate the functional role of rice *ASR* genes, we generated transgenic plants carrying an RNAi construct that targeted the *ASR5* gene. *ASR5* knockdown was confirmed at the mRNA and protein levels (Fig. 4a,b), and *ASR1* was also knocked down at mRNA level (Fig. 4a). Both genes were not able to respond anymore in ASR RNAi plants stressed with Al. Due to sequence homology between all members of the ASR family, it is reasonable to expect that the ASR5-RNAi construct could also silence other *ASR* genes. However, as *ASR2*, *ASR3*, *ASR4* and *ASR6* showed 100-fold less transcripts than *ASR1* and *ASR5* (Fig 1), we were not able to detect expression of these transcripts in RNAi plants. These results suggested that the RNAi construct was able to reduce expression of the whole *ASR* gene family. ASR knockdown plants showed a delay in flowering, underwent abnormal panicle development and presented reductions in the seed number relative to NT plants (Fig. 4c). Transgenic plants also showed a reduction in the number of trichomes in leaf, as well as in the palea and lemma (Fig. 4d). Microarray analysis revealed that the *ASR* gene expression in reproductive tissues is developmentally regulated (Supporting Information Fig S2).

Leaf trichomes may be important to numerous biological functions, and trichome production has been shown to be a variable character with simple inheritance in several species (Karkkainen, Løe & Agren 2004). Furthermore, elevated trichome density has been shown to decrease rates of water loss in several species (Ehleringer & Mooney 1978; Ehleringer 1981; Perez-Estrada, Cano-Santana & Oyama 2000). This may partially explain the susceptibility of the RNAi rice plants to drought stress (Fig. 6).

ASR knockdown plants were more sensitive to Al exposure (Fig. 5a). The primary phenotype of Al exposure was dramatic root inhibition, which is the primary symptom of Al phytotoxicity (Fig. 5b). This result is consistent with the

expression pattern observed for the ASR RNAi plants, suggesting that ASR proteins play a functional role in the mechanism of Al tolerance. Using cadmium as a heavy metal stress, both NT and ASR RNAi plants showed strong inhibition of root elongation which suggests that ASR proteins are specific for Al response in rice (Supporting Information Fig. S3). In addition, rice plants were increasingly sensitive to drought, containing reduced water content and percentage humidity relative to control plants (Fig. 6). The involvement of *ASR* genes in the response to drought stress has been already proposed by other groups (Padmanabhan *et al*. 1997; Riccardi *et al*. 1998; Sugiharto *et al*. 2002); however, our results are the first confirmation of the involvement for these proteins in the mechanism of Al tolerance.

Most ASR proteins have a NLS at their C-terminus (Padmanabhan *et al*. 1997; Cakir *et al*. 2003; Kalifa *et al*. 2004a). Recently, Takasaki *et al*. (2008) identified ASR5 protein from rice in nuclear and cytosolic compartments of rice leaves, suggesting that ASR5 might localize in these two subcellular compartments. In our stable expression analyses with transgenic rice embryogenic calli expressing ASR5-GFP fusion proteins, we confirmed that ASR5 is present in both the nucleus and cytoplasm (Fig. 7a). These results are consistent with previous reports that have demonstrated that ASR proteins are located mainly in the nucleus where they could regulate specific promoters (Cakir *et al*. 2003; Wang *et al*. 2005; Yang *et al*. 2005).

Surprisingly, in transient expression analysis, ASR5 localized in chloroplasts of rice protoplasts obtained from green leaves (Fig. 7b). This is also the first report of a chloroplastic localization pattern for ASR proteins. The nuclear localization suggests that ASR5 might act as a transcription factor in rice plants as reported in grape (Cakir *et al*. 2003). ASR5 may do so specifically in the root apex of rice plants, where it could possibly regulate the expression of other genes involved in Al tolerance. However, the precise physiological explanation for the localization of ASR5 in chloroplasts is not clear. It is possible that ASR could regulate the expression of chloroplastic genes. Alternatively, ASR proteins may shuttle to the chloroplast as a chaperone for other proteins typically located there. A disturbance in the architecture of the chloroplast may occur in response to Al toxicity. Moreover, there is a decrease in the photosynthesis ratio as a result of reduced electron transport in photosystem II (Ali *et al*. 2008 and Zhang, Ryan & Tyerman 2001). It is not surprising that ASR might perform some function in the chloroplast, but it does seem unlikely that it is efficiently shuttled there, as the N-terminal regions of ASR proteins lack the peptide required to target proteins to the chloroplast. Therefore, further studies must be conducted to confirm this surprising subcellular localization biological significance.

To identify potential target genes of the ASR5 mechanism, we used a proteomic approach to compare the proteomic profiles of NT and ASR-RNAi plants. Several chloroplastic proteins were differentially expressed in transformed rice plants. At least three chloroplastic proteins with reduced expression were identified: Chloroplastic lipocalin (gi | 115436780), chloroplast translational elongation factor Tu (gi | 218191089) and heat shock protein 70 (gi | 222631026). Levesque-Tremblay, Havaux & Ouellet (2009) showed through mutational analyses that chloroplastic lipocalin protects *Arabidopsis* from oxidative stress. In contrast, Latijnhouwers, Xu & Muller (2010) showed an essential role for 70 kDa heat shock proteins in the development of *Arabidopsis* chloroplasts. Several up-regulated proteins identified in our analysis were also chloroplastic proteins. In total, five proteins exhibited increased expression: 23 kDa polypeptide of chloroplast photosystem II (gi | 164375543), putative photosystem I reaction centre subunit IV (gi | 34394725), putative oxygen-evolving enhancer protein 1, chloroplast precursor (gi | 115436780), chlorophyll *a-b* binding protein (gi | 108864186), putative superoxide dismutase (SOD) [Cu-Zn] and chloroplast precursor (gi | 42408425). Interestingly, the response of chlorophyll *a-b* binding protein to Al has already been reported in *Arabidopsis*: Its expression decreased in response to Al exposure (Richards *et al*. 1998).

At least two classes of enzymes of the antioxidant system were found to be reduced in ASR-RNAi plants: a putative SOD (gi | 108708142) and the cytosolic APx – APx1 and APx2 (LOC_Os03 g17690) (gi | 115452337). Al cannot catalyse redox reactions on its own, but the involvement of oxidative stress in Al toxicity has been suggested (Jones *et al*. 2006). According to Jones *et al*. (2006), Al triggers the accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O2-), which correlates positively with Al stress and ROS production. Furthermore, to alleviate oxidative damage during Al stress, several antioxidant enzymes are up-regulated, including SOD, APx and catalase (CAT) (Chen, Qi & Liu 2005; Kumari, Taylor & Deyholos 2008; Sharma & Dubey, 2007 and Panda & Matsumoto 2010).

In canola, the overexpression of manganese SOD conferred Al tolerance (Basu, Good & Taylor 2001). Yin *et al*. (2010) described that the overexpression of the dehydroascorbate reductase conferred tolerance to Al in tobacco. An Al-tolerant cultivar of *Melaleuca* trees showed higher antioxidant enzyme activity than their Al-sensitive counterparts during Al stress (Tahara *et al*. 2008); this demonstrates that an enhanced antioxidant capacity can enhance Al tolerance.

Under most conditions, plants can efficiently scavenge H_2O_2 by CAT or ascorbate glutathione cycles, wherein APX reduces it to H_2O. Ma, Jianmin & Shen (2007) showed that H_2O_2 production in response to Al stress was more pronounced in an Al-sensitive rice cultivar than in an Al-tolerant cultivar. The tolerant cultivar had significantly higher activities of CAT, APx, dehydroascorbate reductase, glutathione peroxidase, glutathione reductase and concentrations of reduced glutathione higher than those in the Al-sensitive cultivar. In a previous study, our group determined that the knockdown of both cytosolic APx (APx1/2s plants) resulted in a plant that was more tolerant in low concentration of Al (150 μM) (Rosa *et al*. 2010). Because

ASR-RNAi plants present lower level of both cytosolic APxs, we analysed the responses of APx1/2s plants to high concentration of Al. As expected, APx1/2s plants were more sensible to Al stress than the NT plants (Supporting Information Fig. S5a). Besides, *APx1* and *APx2* transcript levels responded to Al in the tolerant cultivar Nipponbare (Rosa *et al*. 2010) but did not responded to Al in the sensitive cultivar Taim (Supporting Information Fig. S5b). These data strongly suggest that ASR proteins may directly or indirectly regulate the expression of APx, helping to maintain the redox homeostasis during the onset of stress, and subsequently contributing to the tolerance of toxic Al levels. The increased sensitivity to high Al concentrations exhibited by APx1 and 2 knockdown plants contrasts with a previous report by Rosa *et al*. (2010), in which APx1/2s conferred elevated Al tolerance. However, in the study by Rosa *et al*., a lower Al concentration was used (150 μM). Because these plants have higher H_2O_2 content, they assume a state of constitutive acclimation that permits them to tolerate mild stresses, such as 150 μM Al. However, the low levels of cytosolic APx were not sufficient to compensate for more stressful conditions, resulting in a more Al-sensitive phenotype.

Although ASR proteins from lily present a NLS, they can be found in nuclei and in the cytoplasm (Wang *et al*. 2005). Among rice ASR proteins, the putative NLS of ASR5 is the only one that contains two lysine residues that comprise the set B (Supporting Information Fig. S6) and is more directly related to the lily ASR. With the exception of ASR2, all other rice proteins present the first two lysine residues conserved in set A (Supporting Information Fig. S6). Wang *et al*. (2005) showed that the set A is more important in determining the nuclear localization of lily ASR proteins and that replacement of the first two lysine residues with alanine accounts for the mutation that most severely affects nuclear translocation. The fourth lysine residue of the set A in lily ASR protein is replaced by a glutamine residue in the rice ASR6 protein. Nevertheless, ASR6 has a lysine residue at position 202 that regulates subcellular localization, raising the possibility of nuclear localization of this protein. ASR1, ASR3 and ASR4 have all the lysine residues which are similar to those of the set A and identical to lily ASR protein. Due to the importance of this set for the nuclear localization of lily ASR proteins, it is likely that these proteins behave similarly. ASR2 proteins from rice present only the second lysine residue in the conserved set, and lack three of the lysine residues within that grouping in addition to the two residues of the set B, raising the possibility that rice ASR2 localizes in cellular compartments other than the nucleus.

The potential for the ASR5 protein to be a transcriptional factor indicates that this protein may act by regulating the expression of different genes that collectively contribute to the protection of the cell. Future challenges include the identification of the promoters of genes that are targeted by the ASR protein. This would help explain the involvement of ASR in determining the Al tolerance of rice plants. Our results highlight the potential of the *ASR* gene family as promising subjects for plant biotechnology studies that seek to obtain plants with greater resistance to Al, particularly in acidic soils.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression analysis of the *ASR5* gene in response to cold, UV-B irradiation and drought stresses. To achieve cold stress, seedlings were maintained for 12 h at 4 °C. Total RNA was extracted from roots and used for cDNA synthesis. For the experiment with UV treatment, seedlings were subjected to two treatments of UV-B illumination (for 4 h of continuously at 0.25 kJ m^{-2} min^{-1}) with 20 h intervals, and then maintained under normal light for recovery. Analyses were performed 24 h after the onset of UV irradiation when leaves were collected. For drought stress, plants were cultivated for 15 d under drought conditions. Analyses were conducted using RT-qPCR. Values represent the means +/– SD (N = 4). Asterisks indicate significantly different means: (*) $P < 0.05$.

Figure S2. Expression profiles of *ASR1* and *ASR5* gene expression derived from microarray experiment data. Expression profiles of (a) *ASR1* and (b) *ASR5*. The bar and line graphs represent raw signal intensity and normalized signal intensity for 'Os02g0543000' and 'Os11g0167800', respectively, which were derived from spatiotemporal profiling of various tissues and organs (RXP_0001), as is available at http://ricexpro.dna.affrc.go.jp/index.html.

Figure S3. ASR-silencing did not affect sensitivity to Cadmium in ASR RNAi plants. (a) T1 generation (ASR RNAi) and non-transformed (NT) plants of rice ssp. Japonica cv. Nipponbare and ssp. Indica cv. Taim growth for 12 d in a solution containing 25 μM Cd. (b) Relative Root Elongation (RRE) for ASR RNAi and NT plants. Bars with different letters are significantly different (ANOVA, $P < 0.05$).
Figure S4. Functional classification of differentially expressed proteins from leaves of RNAi plants by 2D-gel electrophoresis. The proteins identified were classified by function according to GO Slim (http://rice.plantbiology.msu.edu/index.shtml) and KEGG pathways (http://www.genome.jp/kegg/pathway.html).
Figure S5. Apx1/2s-silenced plants are sensitive to aluminium. (a) Growth of T1 generation (RNAi) and non-transformed (NT) plants for 14 d in a solution containing 750 μM of Al. (b) Total RNA was extracted from roots and shoots, and used for cDNA synthesis. Analyses were conducted via RT-qPCR. Relative expression was plotted using *APx1*, *APx2* and *STAR1* expression levels in control plants as calibrator. Roots of Taim cultivar were collected after 8 h of treatment with Al (450 μM). Values represent the means ± SD (N = 4). Asterisks indicate significantly different means: (*) $P < 0.05$.
Figure S6. *In silico* analyses of the ASR Nuclear localization signal. Comparison of the nuclear localization signal of rice ASR proteins with the lily ASR protein LLA23. Underlined letters indicate the basic amino acids of the putative nuclear localization signal, with the two sets represented by underlined regions of A and B. Identical amino acids are shaded in lily ASR sequence. The size, in number of amino acids, of each protein is indicated on the right.

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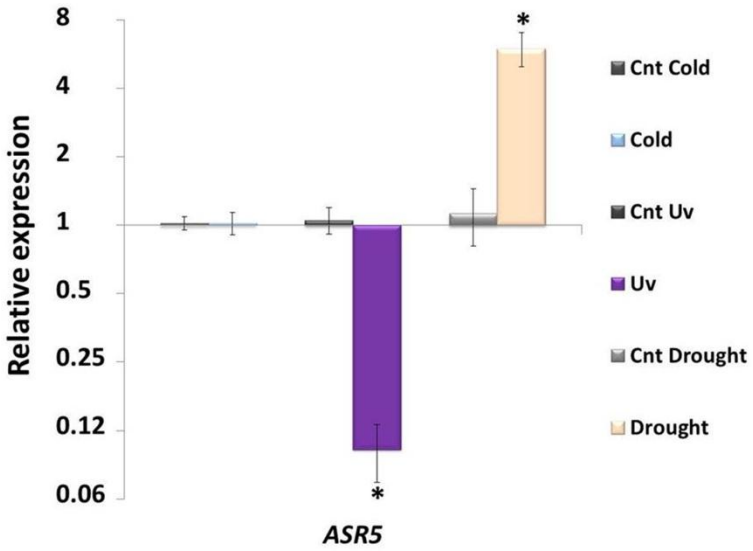
Supporting Information



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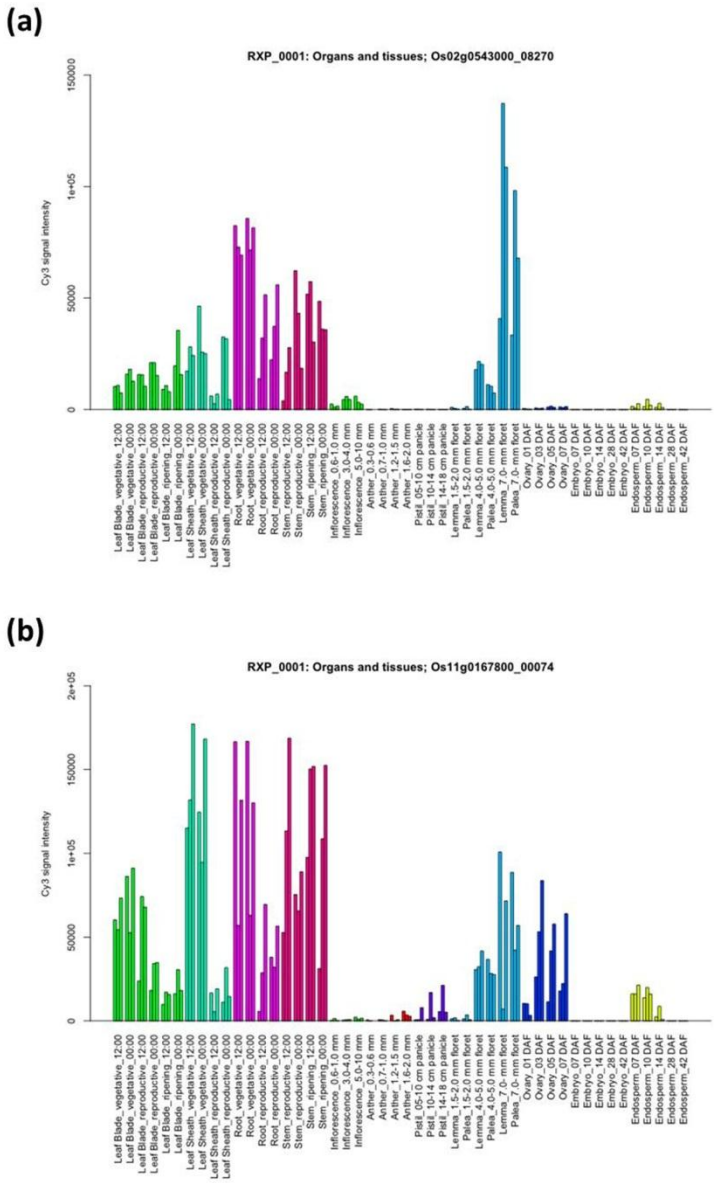

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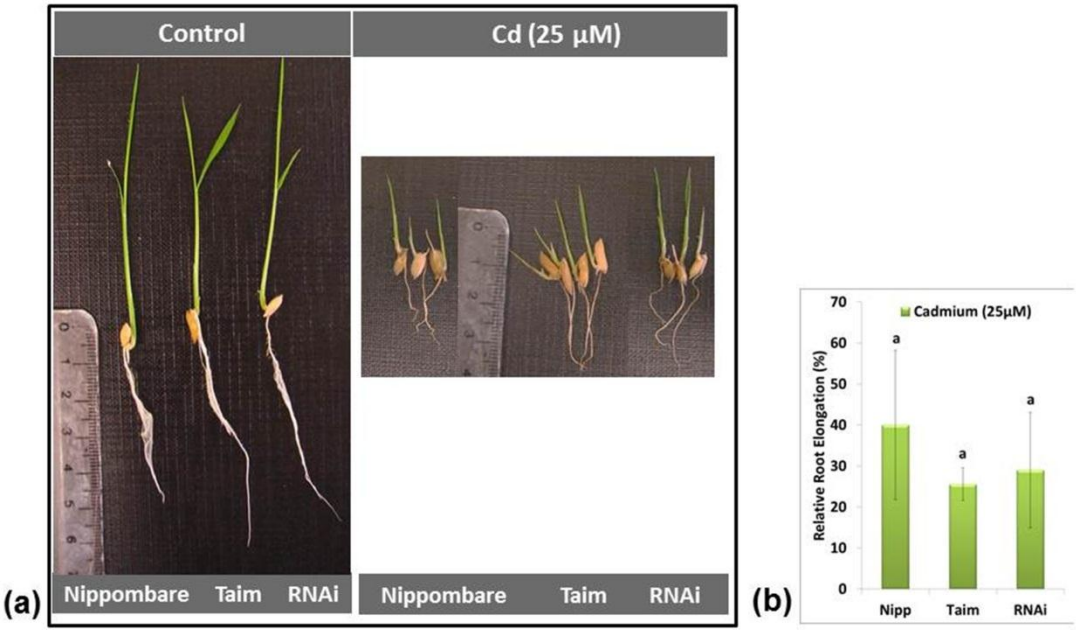

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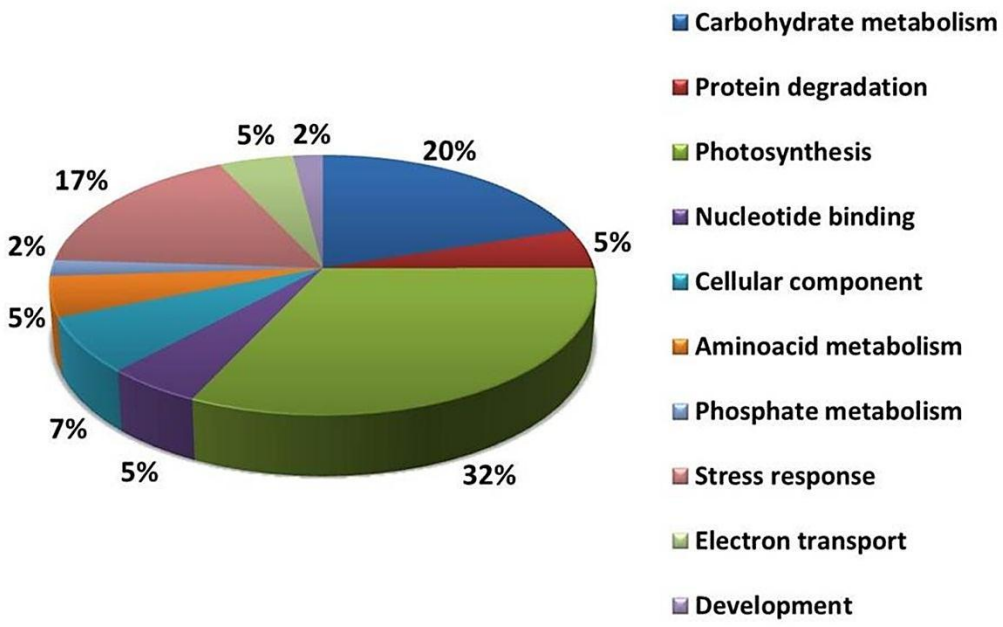

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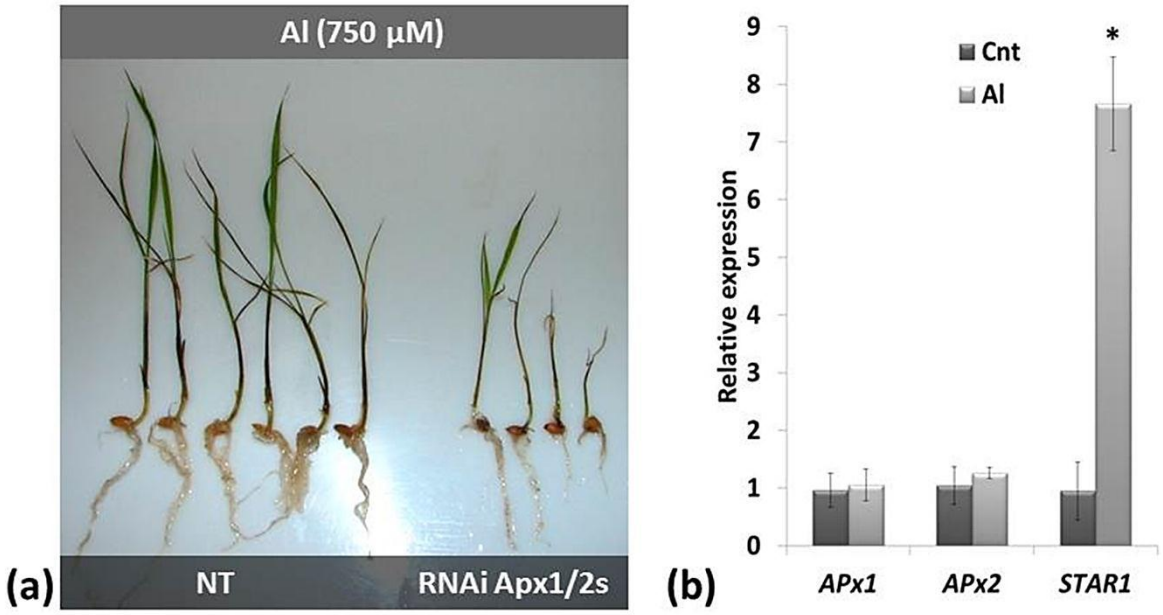

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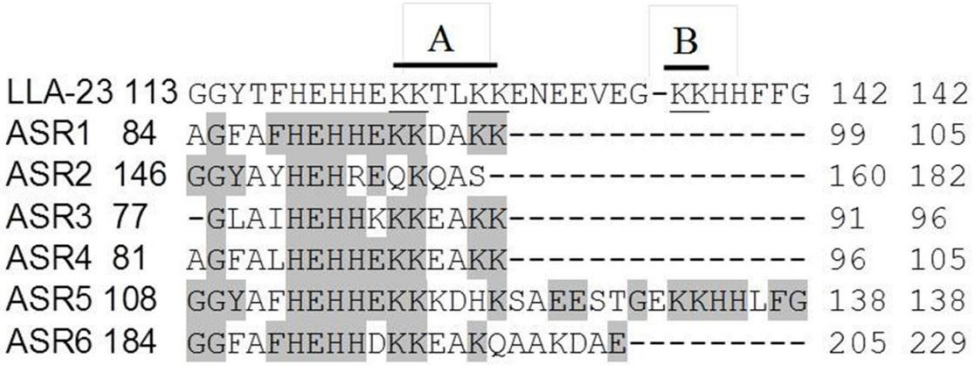

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CAPÍTULO 2

The rice ASR5 protein: a putative role in the response to aluminium photosynthesis disturbance

Este estudo resultou em um artigo intitulado: ‘‘**The rice ASR5 protein: a putative role in the response to aluminium photosynthesis disturbance**’’,
aceito para publicação ao periódico
Plant Signaling and Behavior.

Este artigo foi escrito a convite do editor chefe do periódico Dr. Frantisek Baluska.

Short Communication

The rice ASR5 protein: a putative role in the response to aluminium photosynthesis disturbance

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Key words: ASR, Chloroplast, Aluminium, Transcription Factor, GFP.

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Abstract

Under acidic soil conditions, aluminium (Al) becomes available to plants, which must cope with its toxicity by mechanisms involving both internal and external detoxification. Rice is the most Al-tolerant among the crop species, with Al detoxification being managed by both mechanisms. Recently, we focused on ASR (Abscisic acid, Stress and Ripening) gene expression analyses and observed increased ASR5 transcript levels in roots and shoots in response to Al. In addition, ASR5 RNAi knock down plants presented an Al-sensitive phenotype. A proteomic approach showed that ASR5 silencing affected several proteins related to photosynthesis in RNAi rice shoots. Furthermore, an ASR5-GFP fusion in rice protoplasts revealed for the first time a chloroplast localization of this protein. Because it is well known that Al induces photosynthetic dysfunction, here we discuss the hypothesis that ASR5 might be sequestered in the chloroplasts as an inactive transcription factor that could be released to the nucleus in response to Al to regulate genes related to photosynthesis.

Text

According to the FAO, aluminium (Al) toxicity is the most serious problem in the soil constraints on agriculture, second to erosion hazards.[1] Al is a very abundant metal in the earth's crust, but it becomes a problem under acidic conditions under which Al becomes soluble and is taken up by plants. Plants have developed strategies to cope with Al via internal and external detoxification systems. The release of organic acids that form complexes and prevent Al entry into cells has been well documented.[2-4] However, some plants have managed to mediate Al by chelation and storage in the vacuoles.[5,6] Among crops, rice is the most Al-tolerant species due to its capacity to cope with Al using both mechanisms.[7-8] Although rice can exclude Al from the roots, a portion of the Al can enter the cells and be transported to the shoots.[9]

In recent years, different studies aiming to decipher Al-tolerance in rice permitted the identification of a few genes,[10] and our previous work has added a new association between ASR genes (Abscisic acid, Stress and Ripening) and Al

tolerance.[11] The ASR genes are also involved in many abiotic and biotic stresses (for a review, see 12). The exact function, however, remains enigmatic as the possible roles of the ASR genes cannot be deduced by sequence homology with other known proteins.[12] Most of the ASR proteins reported to date are located in the nucleus and possess DNA-binding activities. ASR from grape, for example, was able to bind to the promoter of a hexose transporter gene and regulate its expression.[13] Selex-binding experiments showed that ASR1 from tomato exhibit DNA sequence-preferential binding.[14] Furthermore, ASR1 from tomato was able to compete for the transcription factor ABI4 binding motif when overexpressed in Arabidopsis.[15]

We have previously shown that ASR5 transcripts are increased in response to Al in roots and shoots.[11] ASR5_RNAi plants presented a high Al sensitivity and a trichome-less phenotype. In addition, a proteomic approach revealed several proteins of which the expression was affected in shoots due to the silencing of ASR5. Furthermore, we confirmed the nuclear localization of ASR5 using rice calli transformed with an ASR5_GFP fusion construct. However, when this construct was transformed in protoplasts, ASR5 was accumulated in the chloroplast precursors (etioplastids) of rice leaves protoplasts grown in dark conditions (Fig. 1a) and chloroplasts (Fig.1b) of rice leaves protoplasts grown under light conditions. GFP alone was used as positive control (Fig. 1c). This new and intriguing localization for ASR5 led us to speculate that ASR5 plays a role in this organelle, most likely linking the signaling between the chloroplasts and nucleus and regulating the expression of chloroplast proteins. Al can enter rice chloroplasts[16] and decrease the photosynthetic ratio.[17,18] The ASR5 silencing affected the expression of at least 41 proteins in rice leaves.[11] A total of 19 out of these 41 proteins contains the chloroplast transfer peptide (cTP) signal, as predicted by sequence analyses[19,20] and, were found to be differentially expressed in our RNAi line. The identified proteins are involved in such processes as photosynthesis, electron transport and stress responses (Table. 1). The overexpression of *ASR5* (named *ASR1* in the cited report) in rice, increased the tolerance to cold, and the plants presented approximately two-fold higher Fv/Fm

values for the photosynthetic efficiency when compared to non-transformed plants.[21] Out of the 19 proteins with a cTP signal found in our plants, 14 were decreased in the ASR5_RNAi line and might be helpful to explain the Al sensitivity of the RNAi plant. These genes may be regulated by ASR5 in the nucleus because most of the proteins with cTP signals are encoded in the nuclear genome, translated in the cytosol and post-translationally imported into the chloroplasts.[22]

Our hypothesis is that ASR5 is stored inside the chloroplast until specific conditions (such as Al stress) require their activity in the nucleus; a scenario proposed for certain other dual-target transcription factors.[23] For example, Whirly1 is localized in both the nucleus and plastids of the same cells in barley leaves.[24]

Curiously, the ASR5 N-terminal region lacks the expected cTP sequence required to target this protein to the chloroplast. Notwithstanding, in Arabidopsis chloroplast preparations, a total of 604 proteins encoded in the nuclear genome were found, and surprisingly, only 376 were predicted to have the cTP when analyzed by a chloroplast-targeting prediction tool.[25] Proteins with cTP signals are targeted to the chloroplast surface and imported across the double-membrane envelope by translocons in the outer and inner envelope membranes, termed TOC and TIC, respectively.[26] However, increasing evidence shows that some proteins can enter the chloroplast by a TOC-TIC independent pathway.[27-29] The transcriptional responses to abrupt environmental changes often have to occur rapidly and might require a release of pre-produced inactive transcription factors. A controlled sequestering of proteins at intracellular membranes seems to be an established way of controlling gene expression and intracellular communication.[23] Furthermore, a potential myristoylation site in rice ASR5 sequence is conserved among several ASR proteins at the same location when aligned.[30] The myristoyl residue may bind to a hydrophobic pocket and thus confer structural stability to a protein.[31] The most obvious function of the myristoyl moieties is to mediate membrane binding[32] and is frequently found in proteins shuttling across

membranes, where the myristoyl residue inserts into the inner lipid layer of the plasma membrane and facilitates membrane interaction. [30]

In tobacco, Al accumulated in the chloroplasts after entering the cells, reacted with or replaced the non-heme iron between the QA and QB binding sites and blocked PSII electron transport, resulting in PSII photochemical damage and the inhibition of photosynthesis.[33] Thus, Al could be the signal for ASR5 release from the chloroplasts to regulate photosynthesis related gene expression and cope with Al toxicity in rice shoots.

Material and Methods

The complete sequence of *ASR5* was amplified using cDNA library of rice Japonica cultivar, sequenced and cloned fusionated with YFP coding sequence at its N-terminus into the Gateway vector pART7-YFP.[34] Protoplast isolation was performed according to Chen *et al*. (2006)[35] and protoplast transformation according to Tao *et al*. (2002).[36] Transformed rice protoplasts grown under dark and under light conditions were incubated in the dark for 24–48 h at 27°C prior to imaging. Fluorescence microscopy was performed with an Olympus FluoView 1000 confocal laser-scanning microscope equipped with a set of filters capable of distinguishing green and yellow fluorescent proteins (EGFP and EYFP, respectively) and plastid autofluorescence. Images were captured with a high-sensitivity photomultiplier tube detector. A vector containing only GFP was used as positive control and an empty vector was used as a negative control. The experiment was performed in biological triplicate.

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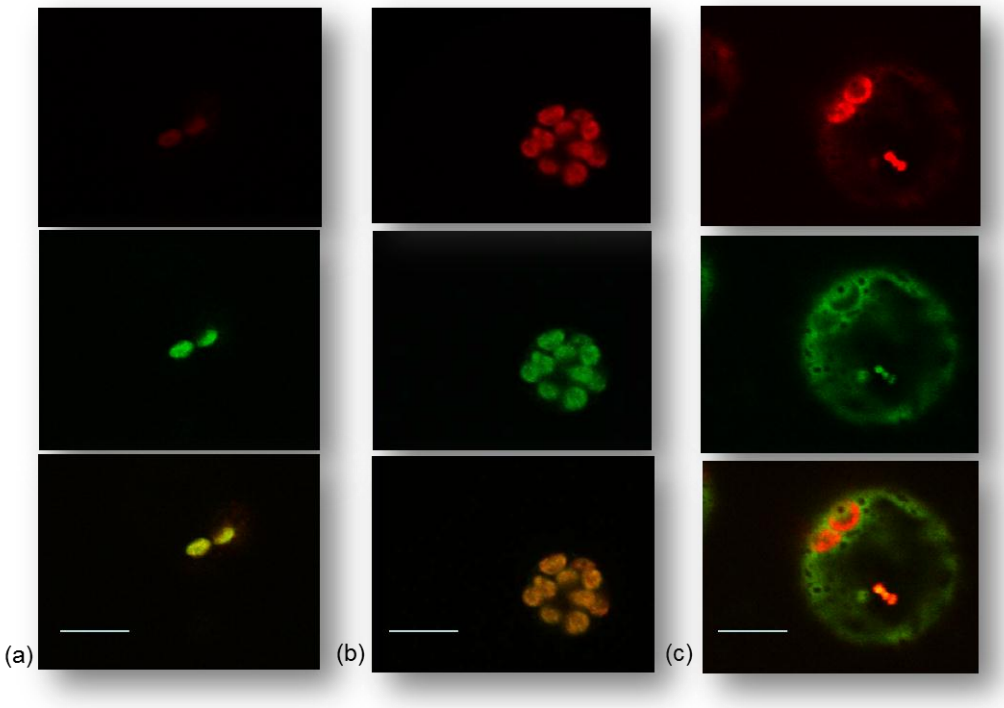

Figure Legend

Figure 1. Rice protoplast transformation with ASR5-YFP fusion. From upper to lower – The ref fluorescence of chlorophyll, ASR5-YFP fluorescence and merged images using a confocal laser scanning microscope. (a) Protoplast from rice leaves grown under dark conditions. (b) Protoplast from rice leaves grown under light conditions. (c) Vector with GFP only used as positive control. No green fluorescence was detected in negative control (data not shown). Bar = 10µM.

Table 1

Proteins differentially expressed in RNAi_ASR rice plants.

The proteins differentially expressed in transgenic rice shoots silenced for ASR5 with a chloroplast transfer peptide signal (cTP), as predicted by PCLR and ChloroP 1.1 software.

Function	*Expression*	*Protein name*	*Accession (NCBI)*
Protein degradation			
1	↓	Heat shock protein 70	gi|222631026
2	↓	Mitochondrial chaperonin	gi|115488160
Photosynthesis			
3	↓	ATP synthase gamma chain	gi|115472339
4	↑	Putative oxygen-evolving enhancer protein	gi|115436780
5	↓	Ferredoxin-NADP(H) oxidoreductase	gi|41052915
6	↑	PSI reaction center subunit IV	gi|34394725
Carbohydrate metabolism			
7	↓	Putative transketolase	gi|28190676
8	↓	Sedoheptulose 1-7 bisphosphatase	gi|27804768
9	↓	Putative uridylyltransferase-related	gi|187608845
10	↓	Ribulose-5-phosphate-3-epimerase	gi|4105561
Cellular component			
11	↓	Fibrillin-like protein	gi|29367475
12	↓	PAP fibrillin family domain protein	gi|115486133
Amino acid metabolism			
13	↓	Putative glycine cleavage system H protein	gi|115482934
Stress response			
14	↑	Class III peroxidase (OsPrx111)	gi|20286

15	↓	Chloroplastic lipocalin (Os04g0626400)	gi|115460690
16	↑	Putative superoxide dismutase [Cu-Zn]	gi|42408425
Transport of electrons			
17	↓	Oxidoreductase NAD-binding domain	gi|115445869
18	↑	2Fe-2S iron-sulfur cluster binding domain	gi|18698985
Nucleotide binding			
19	↓	Chloroplast elongation factor Tu	gi|218191089

CAPÍTULO 3

Through rice aluminium response: the role of ASR5 as a key regulator

Este estudo resultou em um artigo intitulado: ‘‘**Through rice aluminium response: the role of ASR5 as a key regulator”**, e será submetido ao periódico *Proceedings of the National Academy of Sciences - PNAS*.

Title: **Through rice aluminium response: the role of ASR5 as a key regulator**

BIOLOGICAL SCIENCES

PLANT BIOLOGY

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Abstract: Aluminium (Al) toxicity in plants is one of the main constraints in crop production. Al^{3+}, the most toxic form of Al, is released in soils under acidic conditions causing several damages in plants, especially in roots. Recent advances in molecular biology through large scale sequencing and microarray analyses, lead to the discovery of several Al-responsive genes in plants. Rice is considered one of the most Al-tolerant species among cereal crops, however, only a few genes were characterized until now. Recently, ASR5 was found to be one important gene related to Al-tolerance in rice. In this work, we used high throughput analyses and showed for the first time a large scale profile of Al-responsive genes in rice. Using ASR5_RNAi plants, a global transcriptome analysis was done comparing non-transformed and ASR silenced plants. The results depict several genes affected by ASR5 silencing. Chromatin imunoprecipitation followed by deep sequencing revealed the binding motif for ASR5 showing that ASR5 can act as transcription factor in rice regulating gene expression under Al stress.

Keywords: Aluminium, ChIP-Seq, RNA-Seq, Rice, ASR

Introduction

Abiotic stress is the major cause of crop failure worldwide, due to reduction in crop productivity, which threatens the agricultural sustainability (1). Aluminium (Al), an abundant metal from earth's crust is found as a component of soils clay but, under acidic conditions, the trivalent form Al^{3+} is solubilized in the soil solution and becomes highly toxic for plants (2). With about 30 - 50% of the arable land in the world showing acidic conditions, this is the primary limiting factor in crop productivity (3). Inhibition of root elongation is the primary and the first visual effect of Al toxicity. Inside the plants the effects are more dramatic since Al causes extensive root injury leading to poor ion and water uptake (4).

In the course of evolution, plants have developed mechanisms to deal with Al toxicity, classified as either external and or internal tolerance mechanisms (5). External mechanisms as root exudation of organic acids (OA), which bind to Al and prevent its entrance in cells, have been well characterized in several species as wheat, sorghum, maize among others (5). Internal mechanisms as compartmentalization of Al in the vacuole have been demonstrated in some species (6-8).

Rice (*Oryza sativa*) is the most Al resistant crop under field conditions (9). As a species, rice is two to five times more Al tolerant than wheat, sorghum, and maize (2). Multiple genes involved in rice Al detoxification are regulated by the transcription factor ART1 (10). At least four genes regulated by ART1 were characterized, *STAR1*, *STAR2*, *Nrat1* and *FRDL4*. *STAR1* and *STAR2* encode an ATP-binding and a transmembrane domain protein, respectively. The complex formed by STAR1 and STAR2 transports UDP-glucose, a substrate used to modify the cell wall masking Al binding sites (11). *Nrat1*, an Nramp family protein, encodes an Al transporter (12) and *FRDL4*, a multidrug and toxic compound extrusion (MATE) family protein, encodes an Al-induced citrate transporter involved in citrate secretion (13). Taken together, these studies indicate that rice may use a novel or deal with both types of Al mechanism (external and internal detoxification). Using deep cover analyses, several genes have been identified as Al responsive in species like *Arabidopsis* (14, 15), Aspen (16), *Medicago truncatula* (17), common bean (18), maize (19, 20), soybean (21, 22) and wheat

(23, 24). In rice, however, there is a lack of deep cover information and, as mentioned above, the high Al-tolerance and the ability of this species to deal with Al toxicity should require an elaborated machinery involving a complex transcriptional regulation and protein activity, and more candidate genes should be evaluated.

ASR (Absiscic Acid, Stress and Ripening) is a low molecular-weight and high hydrophilic plant-specific protein family. These proteins are involved in several processes such as fruit development (25, 26), abiotic stresses (27-30), biotic stress (31), chaperone activity (32) and more importantly, as regulators of transcription (25, 30, 33, 34).

We have previously shown that ASR5 (LOC_Os11g06720) protein is localized in both the cytoplasm and nucleus, the transcript levels increase in response to Al and ASR5 silenced plants becomes extremely sensitive to Al (35). In this study, we introduced a global analysis of rice transcripts in response to Al comparing Non-Transformed (NT) plants and ASR5_RNAi plants. Besides, a role of ASR5 as a transcription factor regulating Al-responsive genes such as *STAR1* is described.

Results and Discussion

A global analysis of rice transcripts in response to Al reveals a set of novel candidate genes for Al tolerance in Non-transformed and ASR5_RNAi plants

Roots of non-transformed (NT) and ASR5_RNAi plants (12 old days) previously obtained (35), were used to generated 4 RNA libraries cultivated in control conditions and submitted to $AlCl_3$ (450µM) for 8 hours, named as follows: 1) NT_Cnt (non-transformed in control conditions), 2) NT_Al (non-transformed in Al conditions), 3) ASR5_RNAi_Cnt (ASR5 silenced plants in control conditions), 4) ASR5_RNAi (ASR5_RNAi plants in Al conditions). Illumina RNA-Seq data from these libraries generated a total of 9.497.649 millions of reads mapped to the Rice Genome Annotation Version 7.0 in NT_Cnt, 11.257.398 million in NT_Al, 2.013.886 million in ASR5_RNAi_Cnt and 1.831.659 in ASR5_RNAi_Al. Digital gene expression (DGE) from counted reads were normalized using statistical

analysis performed by edgeR (36) for the four libraries and used to perform three comparisons: i) NT_Cnt vs NT_Al, ii) ASR5_RNAi _Cnt vs ASR5_RNAi _Al and iii) NT_Cnt vs ASR5_RNAi_Cnt. Using this statistical method, we were able to normalize the data despite the variation in the number of uniquely mapped reads (Fig S1 A-C). The robustness of this statistical approach is supported by the fact that known ASR5 reference gene is up regulated in response to Al in NT plants and is silenced in ASR5_RNAi plants (Table S1). Besides, a set of other important genes previously described in rice as Al responsive showed the same pattern when compared with our libraries (Table S1). Furthermore, most of these genes did not respond anymore to the Al treatment in ASR5_RNAi plants, being this the first clue of the high Al sensitivity displayed by these plants. The raw data of these experiments is available in Supplementary Information S2.

A hierarchical clustering and heat map analysis showed that the expression pattern between NT and ASR5_RNAi clustered separately in the presence of Al and reveals that hundreds of genes were affected by ASR5 silencing (Fig. S1D). A total of 961 genes were differentially expressed in rice roots in response to Al in NT plants (475 up and 486 down regulated), while in ASR5_RNAi plants, only 309 genes showed differentially expression patterns (234 up and 75 down regulated) (Fig. 1A). When compared the transcript levels of non-stressed conditions between NT and ASR5_RNAi plants, a total of 1756 genes were affected (632 up and 1124 down regulated) in ASR5_RNAi, indicating a complex disorder due to ASR silencing (Fig. 1B). Only 48 up-regulated genes are common between NT and ASR5_RNAi Al-treated plants while only 4 Al down regulated genes overlapped between the two groups (Fig. 1A). A qualitative analysis of the differences among these comparisons can be visualized in Fig. S1D, which clearly shows the difference in NT Al-responsive genes and ASR5_RNAi Al-responsive genes. Many genes failed to respond to Al or presented opposite patterns, suggesting that knock-down of ASR protein is important to Al response. When comparing ASR5_RNAi_Cnt vs NT_Cnt, many genes differentially expressed were found that, apparently, are not related with Al responses, suggesting that ASR can be involved in other cellular processes

Gene ontology of RNA-Seq data

A gene ontology (GO) analysis searching for biological processes using the Web sites software's AgriGO (42) and ReviGO (43) with the RNA-Seq data set reveals several enriched terms. A total of 83 GO terms were found in Up-regulated genes responsive to Al in NT plants. Programmed Cell Death (PCD) like apoptotic process, response to stress, signaling and ion transport were some of the enriched GOs (Fig. S2A). Al-PCD has been shown to play an important role in plants (44-46). This mechanism may separate the disordered root system from plant tissue and prevent the influx of excess ions into the shoot for plant survival (44).

For down-regulated genes, 131 Go terms were enriched, appearing in a wide range in the classes of primary metabolism, sugar pathways, cellulose biosynthesis (including glucan and carbohydrate biosynthetic process, glucose and hexose metabolic process) and cellular macromolecule complex assembly (including chromatin assembly, chromosome organization and DNA packing) (Fig. S2B). This set of classes is in accordance with data described by Chandran *et al.* (2008) in *Medicago truncatula* (17), which showed stress responsive genes up-regulated as well as primary metabolism and cell cycle genes being down-regulated in response to Al. Down regulation of primary metabolism might be necessary to cope with stressful conditions (17) and adaptive reprogramming require a larger portion of down-regulated genes than up-regulated genes (47). GO of genes up regulated in ASR5_RNAi in response to Al are shown on Supplementary Information S3. In the same experimental condition, no significant terms were found in the 75 ASR5_RNAi down-regulated genes, using the established threshold.

We also performed the gene ontology with the 632 up and the 1124 down regulated genes affected by ASR silencing. Because of the large amount of genes, we set these analyses using only GO terms that presented a P-Value Log10 lower than 0.0009. We retrieved 33 and 99 GO terms for Up and Down-regulated genes, respectively. We observed that genes up regulated due to ASR silencing were enriched in programmed cell death (apoptosis) when compared to reference genome (Fig. S2C). In NT plants, this mechanism could be a way to cope with Al

toxicity but ASR5_RNAi presented this pattern in normal condition, which can lead to a disorder in cells associated with pleiotropic developmental defects and lethality (48). Genes down regulated belonged to cell wall macromolecule catabolism, response to stress, oxidative stress, chemical stimulus and antioxidant activity among others, showing a relation with up-regulated genes in response to Al in NT plants (Fig. S2D).

ASR5 silencing affects a specific set of genes

The difference in gene ontology between NT and ASR5_RNAi plants lead us to search in the RNA-seq data, rice Al up-regulated genes, which orthologous and homologues were previously reported in the literature. First, we searched in our experiment for gene families that increased their transcript levels in response to Al in NT plants but not in ASR5_RNAi plants. Genes that fit on these criteria and were knock-down in ASR5_RNAi plant were also included. A summary of these data is exposed in Fig. S3. Complete set of genes can be found in Supplementary Information S2. Using this approach, we found 4 ABC transporters genes with increased transcript levels only in NT plants (LOC_Os01g50100, LOC_Os03g54790 and LOC_Os04g49900). The fourth ABC transporter, *STAR1* (LOC_Os06g48060), increased transcripts levels only in NT and is a knock-down gene in ASR5_RNAi plants (Fig. S3A). ABC family genes transport toxic chemicals from the cytoplasm into the vacuoles and are well documented in rice and *Arabidopsis* in Al resistance experiments (11, 14, 49). A total of 14 Cytochrome P450 genes were increased only in NT plants in response to Al (LOC_Os12g32850, LOC_Os07g11739, LOC_Os02g09390, LOC_Os02g47470, LOC_Os06g43410, LOC_Os06g19070, LOC_Os07g33560, LOC_Os02g09240, LOC_Os01g43750, LOC_Os01g43720 LOC_Os01g43710 and LOC_Os07g45000, LOC_Os10g38090, LOC_Os09g26960) (Fig. S3B). LOC_Os07g45000, LOC_Os10g38090, LOC_Os09g26960 are knock-down in ASR5_RNAi plants. These classes of genes were up-regulated in response to Al also in soybean (21, 23), common bean (18) and *Arabidopsis* (15). Cytochrome P450s may serve as monooxygenases in the biosynthetic pathways for lignin, defense compounds and in the detoxification pathway catalyzing numerous

endogenous and exogenous toxic compounds encountered in the environment (50). For Glycosyl hydrolases, 6 genes (LOC_Os01g71820, LOC_Os10g28080, LOC_Os10g28120, LOC_Os01g47070, LOC_Os04g40490 and LOC_Os01g71350) were up regulated only in NT plants under Al treatment (Fig. S3 C) and from those, only LOC_Os01g71820 is not knock-down in ASR5_RNAi plants. They were also up-regulated in *Arabidopsis* and *Populus* (14-16). Root growth inhibition upon exposure to Al is the earliest symptom of Al toxicity and occurs as a result of a rapid inhibition of cell elongation (51). Plants are believed to employ several different mechanisms to loose the cell wall during cell extension and for this, glycosyl hydrolases might play a crucial role dealing with Al cell wall stiffening effect. For Metallothionein and Metal transporters, 9 genes (LOC_Os05g39540, LOC_Os03g38970, LOC_Os02g50730, LOC_Os12g38290, LOC_Os12g38270, LOC_Os12g38010, LOC_Os12g38040, LOC_Os12g38051 and LOC_Os12g38300) were up regulated in NT plants (Fig. S3 D), with homologs showing same pattern (37, 40). LOC_Os12g38051 and LOC_Os12g38300 are knock-down in ASR5_RNAi plants. Plants have evolved a range of potential cellular mechanisms that may be involved in the detoxification of heavy metals by chelation with high-affinity ligands such as Metallothioneins contributing to metal detoxification by buffering cytosolic metal concentration (52). For MYB transcription factors genes, 4 genes (LOC_Os03g55590, LOC_Os01g41900, LOC_Os04g56990 and LOC_Os01g74410↓) were up-regulated in NT plants under Al treatment (Fig. S3E). LOC_Os01g74410 is knock-down in ASR5_RNAi plants. This gene family was also up-regulated in response to Al in *Arabidopsis* (14), soybean (21) and rice (53). For class III Peroxidases, 5 genes had their expression increased in NT plants in response to Al and 1 of them was knock-down in ASR5_RNAi plants (LOC_Os04g59190, LOC_Os06g16350, LOC_Os08g02110, LOC_Os10g02070 and LOC_Os05g06970) (Fig. S3 F). Class III peroxidases are involved in diverse functions including the cross-linking of cell wall constituents, and catabolism of lignin and certain hormones (54). These class of genes are not usually considered to be part of the ROS scavenging network and may have more diverse roles including generating active oxygen species (AOS) for cell wall modification (55).

We also searched for genes that were Al-down regulated only in NT plants. We found 9 Cellulose synthase encoding genes (Supplementary Information S2). Cellulose is the main constituent of cell wall in plants and it was shown that cell wall polysaccharides levels are inversely related to plant Al tolerance (56, 57). Many genes related to cell wall and plasma membrane composition reported as Al up-regulated genes in *Arabidopsis* (15) and soybean (57) did not respond to Al in the NT plants or either in ASR5_RNAi plants, but a high number of them are affected by ASR silencing (knock-down) in ASR5_RNAi plants in control conditions. Using this approach, we found 4 genes coding for Annexins, which constitutes a family of calcium-dependent membrane-binding proteins and possess a diverse array of functions including Golgi mediated secretion of newly synthesized plasma membrane and wall materials, nucleotide phosphodiesterase activity, peroxidase activity, vacuole biogenesis and cell expansion, DNA replication, and Ca channel activity (58). Nine genes coding for Glutathione S-transferase (GST) were also affected by ASR silencing. GSTs are involved in cellular detoxification and antioxidation system by transferring glutathione to such toxic substances forming s-gluthionaylated reaction products that are nontoxic to the cell (59). Furthermore, reduced glutathione has an antioxidant property capable of scavenging Al-triggered ROS (60).

Identification of *in vivo* ASR5 binding sites

The sensitivity to Al in ASR5_RNAi plants and the role of ASR as transcription factor in Grape (25), led us to look for the *in vivo* binding sites of ASR5. For this, multiple chromatin-immunoprecipitation (ChIP) experiments were performed and then combined the DNA to perform ChIP-Seq analysis in roots of NT rice (12 days-old) submitted to $AlCl_3$ (450µM) for 8 hours. As a control, pre-Serum was used in the same conditions. Specificity of the α-ASR antibody and increase of ASR5 protein in response to Al can be visualized in Fig. 2A. Two ChIP DNA libraries were generated, one for α-ASR and one for the Pre-Serum. A total of 6.0 million reads and 1.74 million reads, respectively (35 bp/read with unique and up to 2 mismatches), were mapped to the Rice Genome Annotation Version 7.0, using the model base (MACS) software (61). When the binding site was

located in the gene body region (5kb upstream to 5'UTR and 1kb downstream 3'UTR), the gene was assumed as a potential ASR5 target gene. If the binding site were not inside a gene body region, the upstream nearest gene and the downstream nearest gene of this binding site were defined as target genes. For the binding site located in the 5000-bp region upstream of its target gene, it was classified as the promoter region group; when the binding site was located in the 1000-bp region downstream of its target genes, it was classified as the downstream region group; for those located inside 5'UTR, CDS or 3'UTR they were classified as CDS region group, and finally, the binding sites located in either of these parts of the genes were classified as others (Fig. 2B). Using this approach, a total of 1086 loci were found in the ChIP-Seq binding peak analyses, 420 (38.7%) in the promoter region, 148 (13.62%) in CDS region, 103 (9.48%) and 415 (38.21%) in others (Fig. 2C). The binding central peak distribution of the raw data is showed in Fig. 2D.

In order to refine our search for ASR5 target genes in response to Al, we searched for genes in RNA-Seq that responded to Al in NT plants and did not respond in ASR5_RNAi plants, but showed differential expression pattern due ASR silencing in ASR5_RNAi plants. Using this approach, a total of 836 genes (469 + 367) was found (Fig. 3 A). After comparison of the loci of these genes with the ChIP-Seq loci data, a total of 36 genes (21 + 15) were found to be directly regulated by ASR5 in response to Al (Fig. 3 B). The complete gene list of this analysis is described in Table S4. One of the target genes of ASR5 named *STAR1* called our attention because of its role in Al resistance in rice (11). STAR1 protein belongs to an ATP-binding cassette (ABC) transporter and together with STAR2, mediates the transport of UDP-glucose to the cell wall, playing a role in masking Al binding sites. It has been shown that STAR1 is regulated by the transcription factor ART1 (10). However, ART1 protein is not affected by Al treatment and the authors suggested that other factors and/or signals might be required to activate ART1 and their target genes.

We also compared the set of 1024 genes in RNA-Seq that were not responsive to Al in NT and ASR5_RNAi but were affected by ASR5 silencing. Sixty eight genes overlapped between RNA and ChIP-Seq data (Fig. 3C and Table S4). These

genes may be regulated by ASR5 in other conditions and are not altered by Al stress. In order to confirm the RNA-Seq and ChIP-Seq data, we performed real time RT-qPCR to validate the expression pattern of 19 genes (including genes responsive to Al in literature as *ASR5, STAR1, MATE and metallothioneins)* in NT and ASR5_RNAi plants submitted to $AlCl_3$ (450µM) for 8 hours (Fig. S4).

Identification of the ASR5 DNA binding motif

Previous studies of Kalifa *et al*. 2004 (62) suggested that tomato ASR1 binds in a DNA specific sequence (C2-3 (C/G) A). In order to investigate potential ASR5 binding motifs, we used the DREME (Discriminative DNA Motif Discovery) tool (63) to search statistically overrepresented motifs in the ASR5 binding regions of the ChIP-Seq data. First, 649 binding sequences, which included all the binding sites of ChIP-Seq, were used. DREME analysis was able to find 10 enriched possible motifs sequences (Fig. 4A). Interestingly, 3 of them showed a good similarity with the binding SELEX experiment from Kalifa *et al.* 2004 (62) represented by the sequences (1- A(C/A)(G/A)GCCCA, 2- (G/A)GCCCAT and 3- GGCCCA(A/C)). To narrow our search by the ASR5 motif, we used only the binding peaks found in the promoter region (5kb – 322 sequences) and using this approach a consensus sequence was found (GGCCCA(T/A)) (Fig. 4B). We also tested if the sequences CCCA, GCCCA and GGCCCA were enriched in our data comparing to rice genome. For this, 200-bp sequences (100 bp upstream and 100 bp downstream) surrounding the peak of each binding sites were extracted and searched for perfect match to the consensus transcription factor binding motifs, detected by DREME. The frequencies of these consensus binding motifs per 1000-bp sequences were richer than those in the whole rice genome (Fig. 4C). ASR1 from tomato was previously shown to bind and compete with ABI4 transcription factor motif in *Arabidopsis* (64). However, the motif (CACCG) was not found enriched using DREME and frequencies analyses (Fig 4C).

ChIP-qPCR confirmed the enrichment of STAR1 promoter region when compared to control (Fig. 5A) and this promoter region was used for *in vitro* interaction analysis (65). Four hundred forty-two base pairs around the peak of ASR5 DNA binding site of the STAR1 promoter region were divided into three segments

(referred to as F1, F2 and F3) and amplified by PCR using rice genomic DNA (Fig. 5 B). Two forward primers located in the same position (biotinylated and non-biotinylated primer) were used to amplify the three different fragments (Fig. 5C). Polydeoxynucleotides (Di-DC) was used as a non-specific competitor. ASR5 protein fused to GST and GST-α antibody were used in the analysis. Pull Down analyses showed that GST protein alone was not capable of binding to biotinylated DNA. Instead, GST-ASR5 is strongly linked to the biotinylated DNA fragment F1. Di-DC 25x concentrated was unable to compete with biotinylated F1. Besides, the fragment F1 concentrated 25x was able to compete strongly with F1 DNA biotinylated whereas F2 and F3 were not (Fig. 5D). The F1 fragment contains a motif identified by DREME (AGCCCAT) duplicated and separately by 58bp, indicating that rice ASR5 is able to act as a transcription fator interacting with cis elements in the STAR1 promoter region.

ASR5 promoter analysis

To characterize the tissue expression conferred by ASR5 promoter, a construction with 2kb before start codon of rice ASR5 fusioned with GUS was used to transform rice plants. Non transformed plants were used as a negative control. The GUS activity was detected in the vascular tissue of the root (Fig. 6A-B), root cap (Fig. 6C-D), root cap of lateral root (Fig. 6E-F), and not structured cells from root cap (Root Border Cells) (Fig. 6G). The transversal section reveals the GUS expression also in cells of exodermis layer and cortex, at the vascular tissue of parenchimatics cells of xylem and the companions cells of phloem (Fig. 6H). The emergence of the lateral roots damage mechanically the cortex cells beside the apical zone and the GUS expression is stronger than the other cortex cells (Fig. 6F).

We showed previously ASR transcripts in rice roots and an increase pattern in response to Al, especially on root apex (35). Interestingly, a QTL for root thickness, which contains *ASR5* gene, was found in rice (66). The GUS expression driven by ASR5 promoter in root apex, more specifically on root border cells, raise new insights about Al resistance in rice. Root Border Cells (RBC) is a population of mucilage-secreting cells surrounding the root cap (67). Border cells

provide means of packaging exudates in living cells, which act as a chemical, physical, and biological interface between roots and soil (68). These cells also function to protect the root tip from Al toxicity by secreting mucilage (68), producing reactive oxygen species to induce programmed cell death (69) and by immobilizing Al in their cell-wall pectin (70). In a recent work, Al inhibited root elongation and increased Al accumulation in the rice root tips. Physical removal of RBCs from root tips resulted in a more severe inhibition of root elongation and a higher Al accumulation in the root tips. These effects were more pronounced in an Al-sensitive than in an Al-tolerant cultivar (71).

GUS analyses in leaves also reveal the GUS expression in vascular tissues (Fig. S5A-B), trichomes (Fig. S5A) and also in response to mechanical damage (Fig. S5B).

In floral tissues, *GUS* expression was detected in vascular tissue of anther (Fig. S5C), in the stigma (Fig. S5D), palea and lemma (Fig. S5E) and trichomes of palea and lemma (Fig. S5F). In rice, ASR5 was expressed also in leaves and parenchyma cells of palea and lemma in plants subjected to cold stress (30). In *Pinus*, the ASR-ortholog LP3 promoter region, driven the expression of GUS in tobacco root meristematic regions, shoot meristematic regions, most cell types in leaves, trichomes, root hairs, stems, pistils of developing floral buds, developing ovary and embryos, placenta and developing seeds (72). Also in tobacco, ASR promoter region from grape driven the expression of GUS in petioles and shoots, young primary roots and on thin layer slices from stems (73). Trichomes are specialized unicellular or multicellular structures derived from the epidermal cell layer. Glutathione and metallothioneins, both involved in responses to toxins and heavy metal stress are also presented at high levels in trichomes of *Arabidopsis* (74). Glutathiones are precursor of phytochelatins and metallothioneins, and high glutathione concentrations may allow trichomes to play a role in relieving heavy metal stress in plants (75). In tobacco, trichomes have been shown to secrete metal in a complex with calcium crystals when grown in medium with toxic levels of Zinc (76). We suggest that ASR5 is acting on trichomes development and might help rice to deal with toxic levels of Al since it was showed that Al can accumulate

in shoots of rice (2). Besides, Al-susceptible ASR5_RNAi plants present a reduced content of trichomes in leaves and in palea and lemma (35).

To our knowledge, this is the first report of a large scale data analyses on gene expression modulation under Al stress in rice. We provide new keys that will help to the comprehension of Al-resistance mechanism in rice since it seems that both mechanisms (exclusion and internal detoxification) are acting in concert. All together our results suggest that under Al stress, ASR5 protein binds to STAR1 promoter and other target genes modulating their expression (Fig. S6). However, we cannot rule out that Al can also directly bind to ASR5 (since in tomato, metal zinc ions can bind in ASR1) permitting this protein to act as chaperone protecting proteins from Al cellular toxicity. This binding could also produce homodimerization allowing ASR5 to bind to the DNA and regulate different gene targets.

MATERIAL and METHODS

Aluminium Treatment and Sample preparation for Transcriptome Sequencing

Rice seeds were germinated on filter paper for 4 d in the dark at 28 °C.The seedlings were grown in a hydroponic Baier's solution (77) for 12 d in a growth chamber at 28 °C under 12 hours of light. The hydroponic solution was replaced every 4 d. After 12 days, root samples of non-transformed (NT) and ASR5 silenced plants (ASR5_RNAi) rice plants (ssp Japonica cv Nipponbare) in control conditions and under Al treatment (8 hours – $AlCl_3$ 450µM) were collected and immediately frozen in liquid nitrogen; total RNA was then extracted with Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (> 10 µg) was sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland) for sample preparation to cDNA and shotgun sequencing using Illumina Hiseq 2000 (Ilumina CO). The polyadenylated transcript sequencing (mRNA-seq) was performed using the following successive steps: Poli-A purification, cDNA synthesis using Poli-T primer shotgun to generate inserts of 300 to 500 nucleotides, 3p and 5p adapters ligations, pre-amplification, colony generation and sequencing. cDNA sequencing was performed using single-end and 100 nucleotide read length.

Transcriptome Sequencing Data Analysis

Mapping Method: Reads were aligned with Bowtie v 0.12.7 using the default parameters, with the first seed alignment > 28 nucleotide in size, allowing zero mismatches and unique mapped reads. As reference sequence, rice genome sequence RGAP v7 (http://rice.plantbiology.msu.edu/) was used. The SAM files from Bowtie were then processed using Python scripts to assign the counted reads mapped on each gene region.

Statistics Methods: Scaling normalization method was used for data normalization according to Robinson and Oshlack A (2010) (78). Evaluation of differential gene expression was measured by R package EdgeR (36). Briefly, EdgeR uses a negative binomial model to estimate overdispersion from gene count. The dispersion parameter of each gene was estimated by the tagwise dispersion. Then, differential expression is assessed for each gene using an adapted exact test for overdispersed data.

Western blot

12-day-old rice roots of control and Al treatment (8 hours – $AlCl_3$ 450µM) were macerated and homogenized in 0.5 M Tris-HCl (pH 8.3), 2% Triton X-100, 20 mM MgCl2, 2% β-mercaptoethanol, 1 mM PMSF, 2.5% PEG and 1 mM EDTA; and incubated at 4°C for 1 hour. Samples of each sample were loaded and separated by SDS-PAGE in a 15% gel. ASR5 protein was detected with a rabbit polyclonal ASR5 antibody (1:500 dilution). To prepare the antibody, rice ASR5 full length cDNA was cloned into a pGEX KG (GE) vector; the recombinant clone was introduced into *Escherichia coli* (BL21 codonplus(DE3)-RIL). ASR5 expression was induced by 0.5 mM IPTG, and the protein was purified with Glutathione Sepharose 4B (GE). GST fusion tag was removed incubating thrombin (3U - Sigma) overnight at room temperature. The purified protein was injected into a rabbit and the serum obtained from that animal contained the antibody. Goat anti-rabbit IgG (1:1000) conjugated to alkaline phosphatase was used as the secondary antibody. The bands were detected with a premixed BCIP/NBT substrate solution (Sigma) and recorded on X-ray film.

ChIP-seq Data Analysis

Mapping method: ChIP-Seq libraries were sequenced using an Illumina GenomeAnalyzer IIx. Thirty-six cycles of sequencing were performed, generating a total of more than 40 million sequence reads per sample. Then sequencing reads were mapped to Released RGAP v7 Rice Genome Annotation using SOAPaligner2.21 software (79) allowing for up to two mismatching nucleotides and no gaps. Only unique mapped reads were filtered out for further analyses.

Binding sites detection method: MACS software (61) with the default parameters was used to search for binding peaks. All the binding sites were detected based on the following criteria: (1) Binding sites detection using only our sample data (α-ASR5); (2) Binding sites detection adding our control (Pre-Serum) data set to MACS software and (3) Comparison of these two criteria in order to filter the binding sites overlapped in both conditions. Also, binding sites that presented less than 50% of overlap with the control binding sites were included and the binding sites resulting were considered the real aluminium binding sites in rice.

ChIP qPCR

Chromatin-immunoprecipitation (ChIP) experiments were performed using protocol previously reported (80) using plants in the same conditions of ChIP-Seq in a triplicate biological repeat. The ChIP products were analyzed by quantitative real-time PCR (primer sequences are listed in Table S5), and enrichment was calculated as ratio between the control sample and α-ASR5 sample. Ubiquitin (LOC_Os04g57220) was used as reference gene.

ASR5 binding motif identification

For motif search, 200-bp sequences (100 bp upstream and 100 bp downstream) surrounding the peak of each binding sites were extracted and used to search consensus transcription factor binding motifs using bioinformatics tool DREME (63). The enrichment of motifs found by DREME was calculated by the frequencies of these consensus binding motifs per 1000-bp sequences in the whole rice genome.

Pull Down assay

Pull down assay was performed as described (65). The biotin-labeled and non-labeled forward primer and reverse primers were used to amplify the fragments by PCR using rice genomic DNA. The amplified products were bound to streptavidin-agarose beads and used for precipitation of ASR5 protein and a western blot assay. Primers are listed in Table S5.

Real Time RT-qPCR

Plant material and Al conditions were the same as described in above item (Aluminium Treatment and Sample preparation for Transcriptome Sequencing).

RNA was extracted with Trizol (Invitrogen) according to the manufacter's instructions. cDNA synthesis was carried out using the M-MLV RT reverse transcriptase enzyme (Promega). The reverse transcription reaction was performed in a final volume of 50 µl. A 1:10 solution of total cDNA was prepared (stock solution). For real time RT-qPCR reactions, the stock solution was diluted 10x. The protocol for real time RT-qPCR is summarized as follows: an initial step of 5 minutes at 94ºC followed by 40 cycles of 10 seconds at 94ºC, 15 seconds at 60º C and 15 seconds at 72ºC. Samples were maintained for 2 minutes at 40ºC to promote re-annealing and were then warmed from 55ºC to 99º C to generate relative denaturing curve data for the amplification products. Real time RT-qPCR was carried out with 10 µL of the diluted cDNA (1:100), 2,0 µL of 10X PCR buffer, (Tris/HCL at 100 mM at pH 8,0, KCl at 500 mM), 1,2 µL MgCl2 50 mM, 0,1 µL 5 mM dNTPs, 0,2 µL of each individual 10 µM primer solution, 4,25 µL H2O, 2,0 µL SYBR GREEN (1:100,000), and 0,05 µL Platinum Taq DNA Polymerase (5 U/µL, Invitrogen), in a final volume of 20 µl. Relative changes in gene expression levels were performed using the $2^{-\Delta\Delta Ct}$ method (81). All reactions were performed in 4 technical replications. Quantitative PCR was performed using specific primer pairs listed in Table S5. Real time RT-qPCR was conducted in StepOne Applied Biosystem real-time cyclerTM. The genes *FDH* (LOC_Os02g57040) and *Actin2* (LOC_Os08g29650) were used as reference genes.

GUS expression

The GUS histochemical assay was performed on the different organs of transgenic rice according to the method described (82). Approximately 2kb before start codon of ASR5 gene was amplified using specific primers listed in Table S5. The amplified product was cloned in pENTR-D TOPO vector and recombined, via LR reaction into pHGWFS7 (83) vector. The resulting plasmid was used to transform rice calli (84). Regenerated plants were incubated in X-Gluc 1 mM, phosphate buffer 100 mM (pH 7,0), KH2Fe 2 mM and Triton X-100 0.5%. Samples were incubated for 16 hours/37C. After the reaction, green tissues were incubated in ethanol 70% for chlorophyll removal. Tissues were fixed in solution of formaldehyde 4%, glutaraldehyde 1% in sodium phosphate buffer (pH 7.2) (85), dehydrated in ascending ethanol solutions and included in hydroxyethylmethacrylate (86). Sections with 10µm were obtained in rotative microtome and the analysis was performed using the Leica DMR-HC microscope equipped with Leica DFC500 camera.

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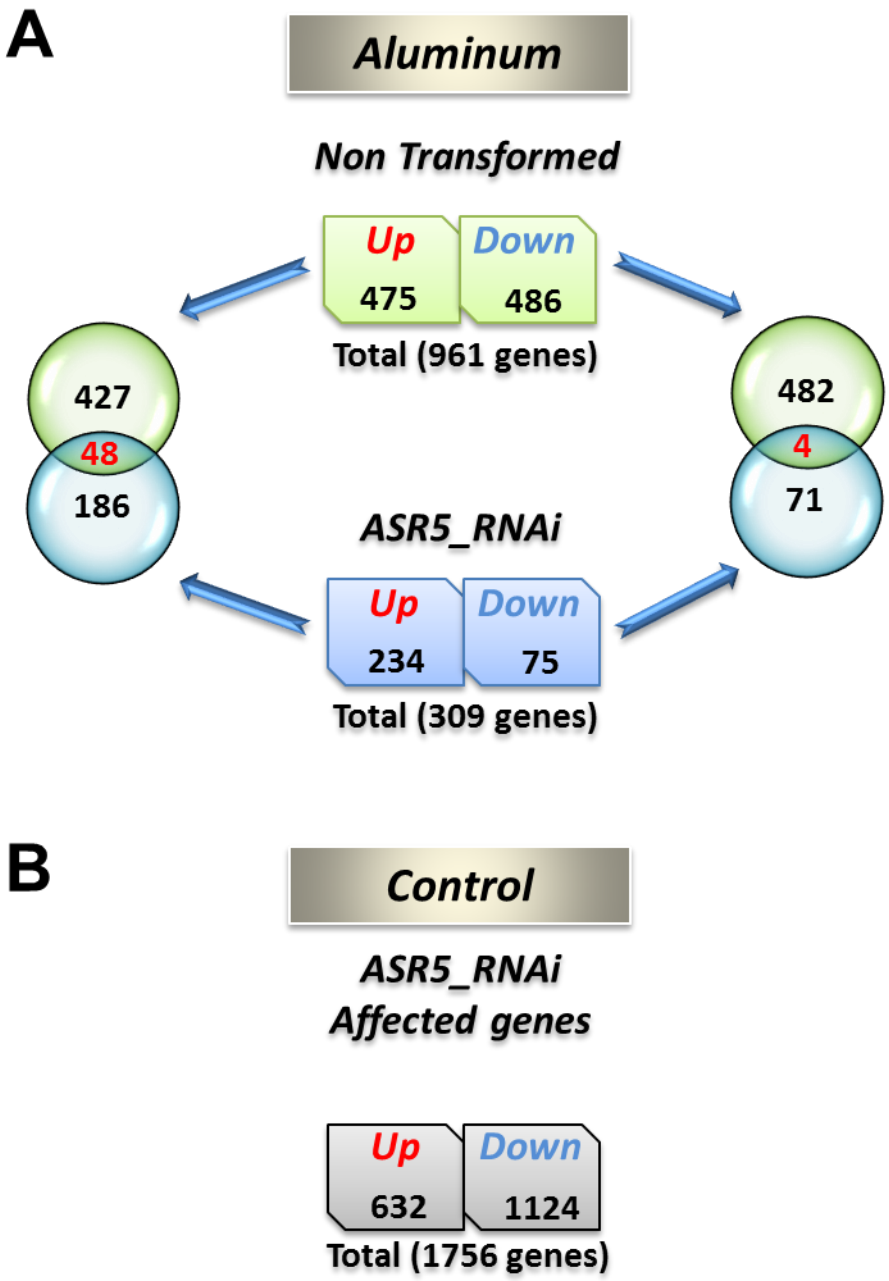

Figure 1. Responsive genes to Al in Non-transformed (NT) and ASR5_RNAi plants. (A) Venn diagram showing overlap of Al up-regulated and Al down-regulated genes between NT and ASR5_RNAi plants. (B) Number of genes affected by ASR5 silencing in ASR5_RNAi plants.

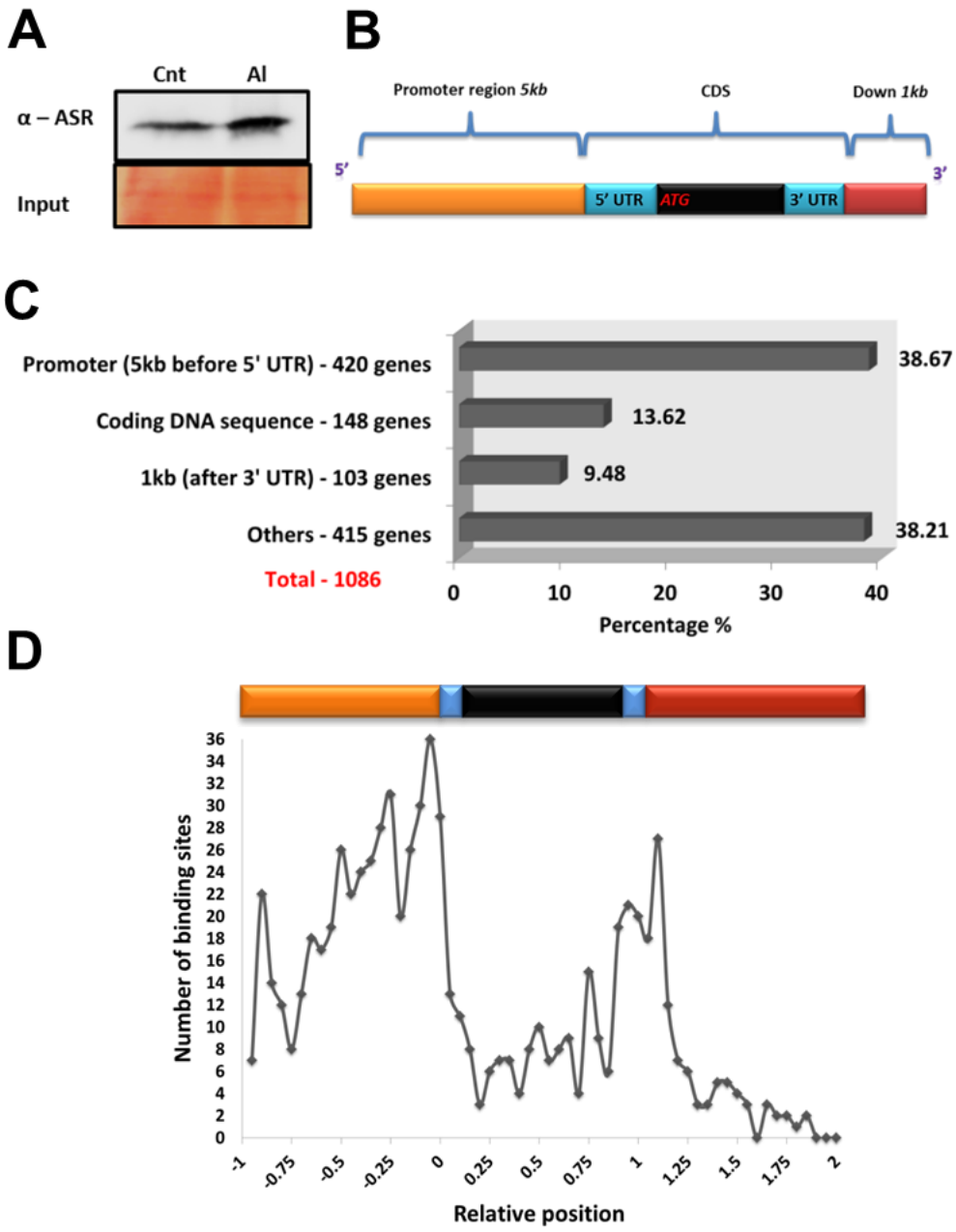

Figure 2. ChIP-Seq analysis of rice plants subjected to Al. (A) Western blot showing increased ASR5 protein levels in rice plants in response to Al. Total protein extracts were detected with anti-ASR5. (Cnt) indicates control untreated plants whereas Al indicates plants treated with $AlCl_3$ 450µM for 8 h. (B) Scheme showing parameters used to classify binding peaks regions in the rice genome. (C) Number and percentage of Loci found in each binding region. (D) Distribution of binding sites. X axis means the relative distance, stands for the promoter region group indicated in top yellow bar, coding region group, indicated by top blue and black bar and downstream region group indicated by top red bar. Y axis means the numbers of binding sites located at different groups.

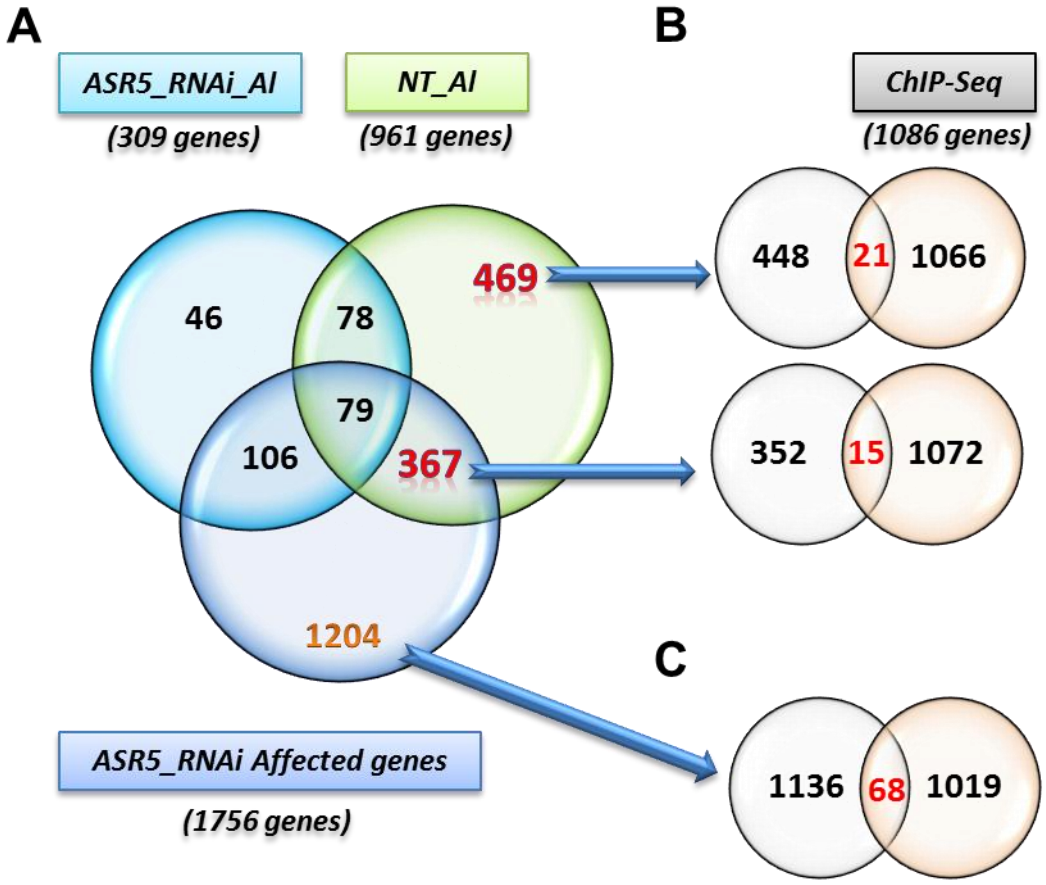

Figure 3. Overlap between ASR5_RNAi affected genes, Al-responsive genes and ASR5 ChIPseq loci. (A) Venn diagram showing overlap of Al-responsive genes between Non-transformed (NT) and ASR5_RNAi plants, and ASR5_RNAi affected genes due to ASR silencing. (B) Venn diagram showing overlap between the 469 genes responsive to Al in NT and ChIP-Seq loci. Venn diagram showing overlap between the 367 genes responsive to Al in NT and affected in ASR5_RNAi due to ASR silencing, and ChIP-Seq loci. (C) Venn diagram showing overlap between the 1204 affected genes due to ASR5 silencing but not responsive to Al.

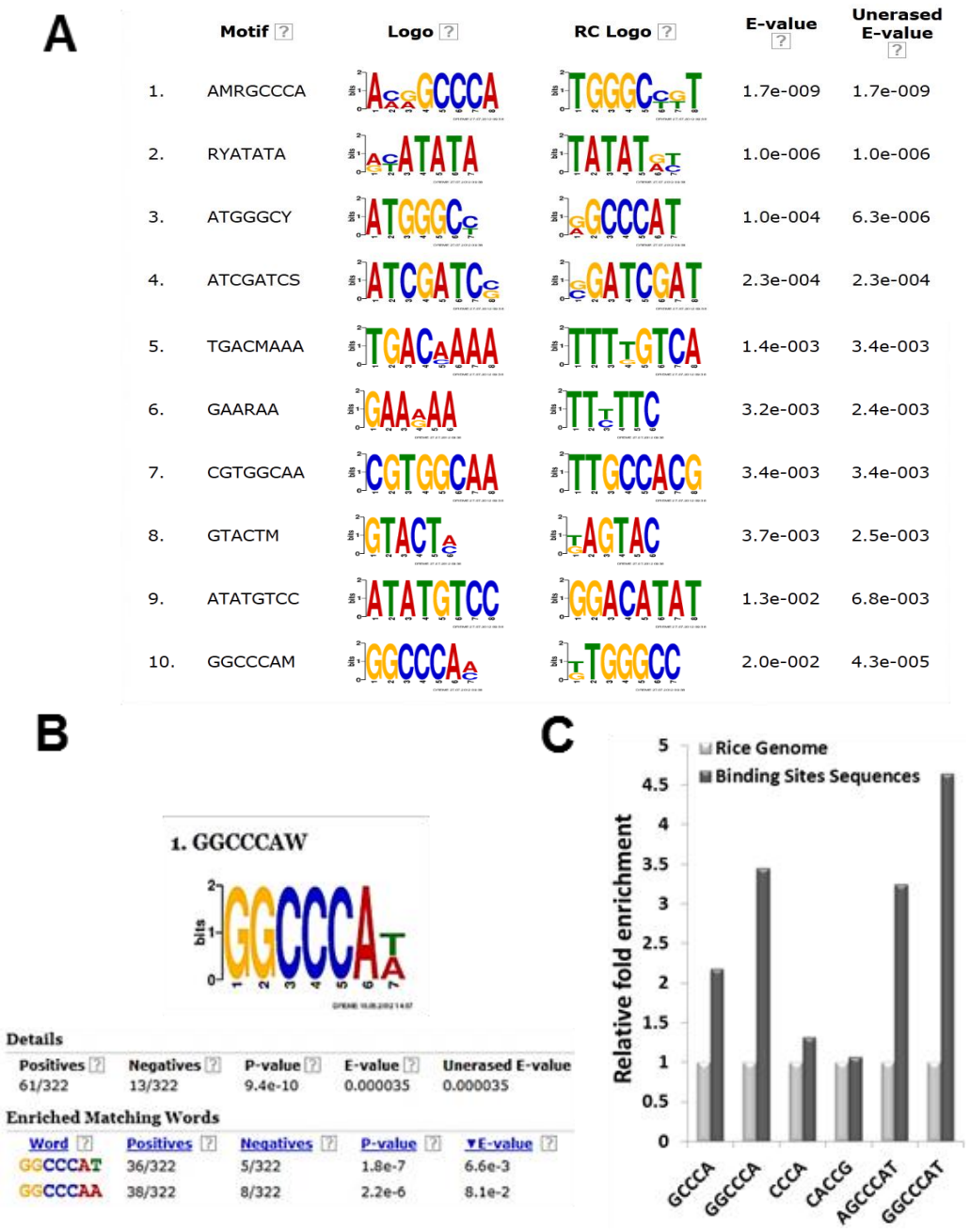

Figure 4. Discriminative motif discovery in ASR5 ChIP-Seq data. (A) shows the logo of the binding motifs discovered by DREME using all ChIP-Seq binding peaks data. (B) The most significant motif found using only promoter regions of binding peaks from ChIP-Seq. (C) Enrichment of possible motifs, found by DREME, in comparison to rice genome as reference.

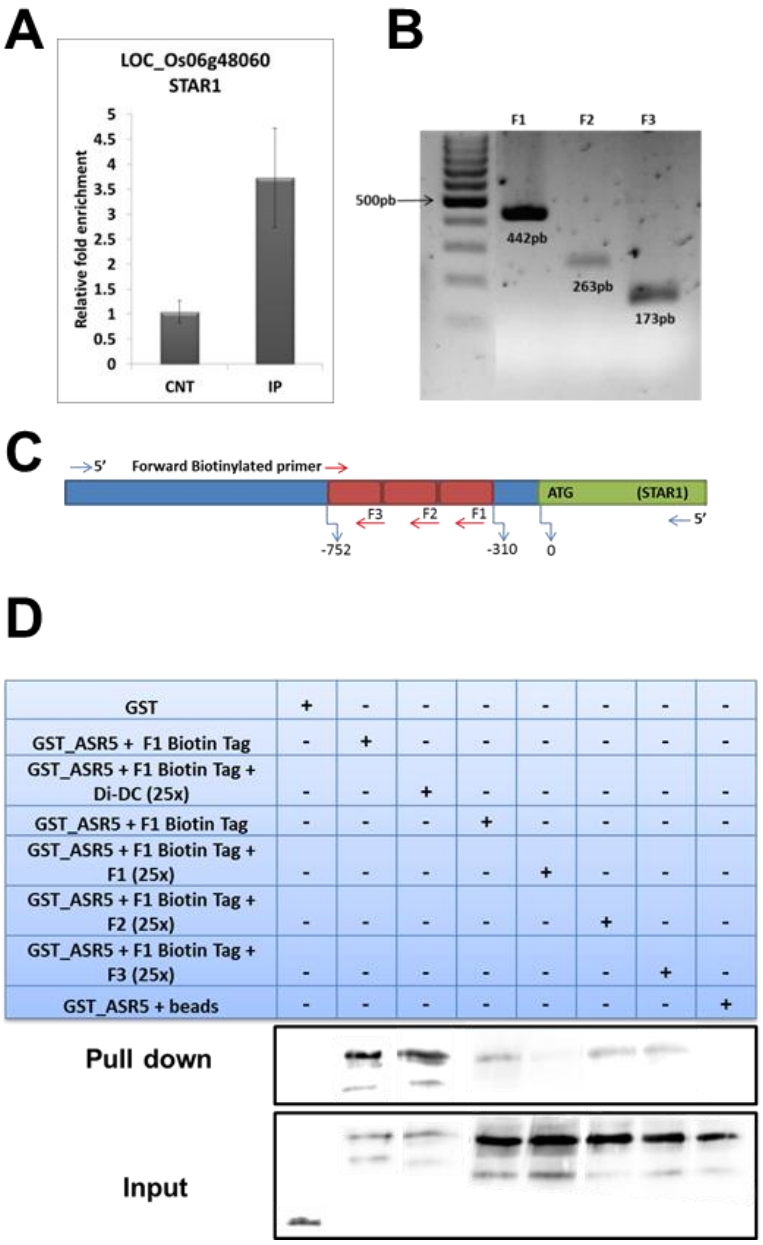

Figure 5. ASR5 binds to STAR1 promoter *in vivo.* (A) ChIP qPCR showing enrichment of STAR1 promoter region using α-ASR5 as antibody. (B) PCR amplification of fragments F1, F2 and F3 of STAR1 promoter region using rice genomic DNA. (C) Scheme showing the amplification sites for STAR1 promoter. (D) SAPA Pull down system showing ASR5-GST binding to DNA F1 biotinylated fragment. Fragments F1, F2, F3 and DiDc were used as competitors. GST alone was used as negative control.

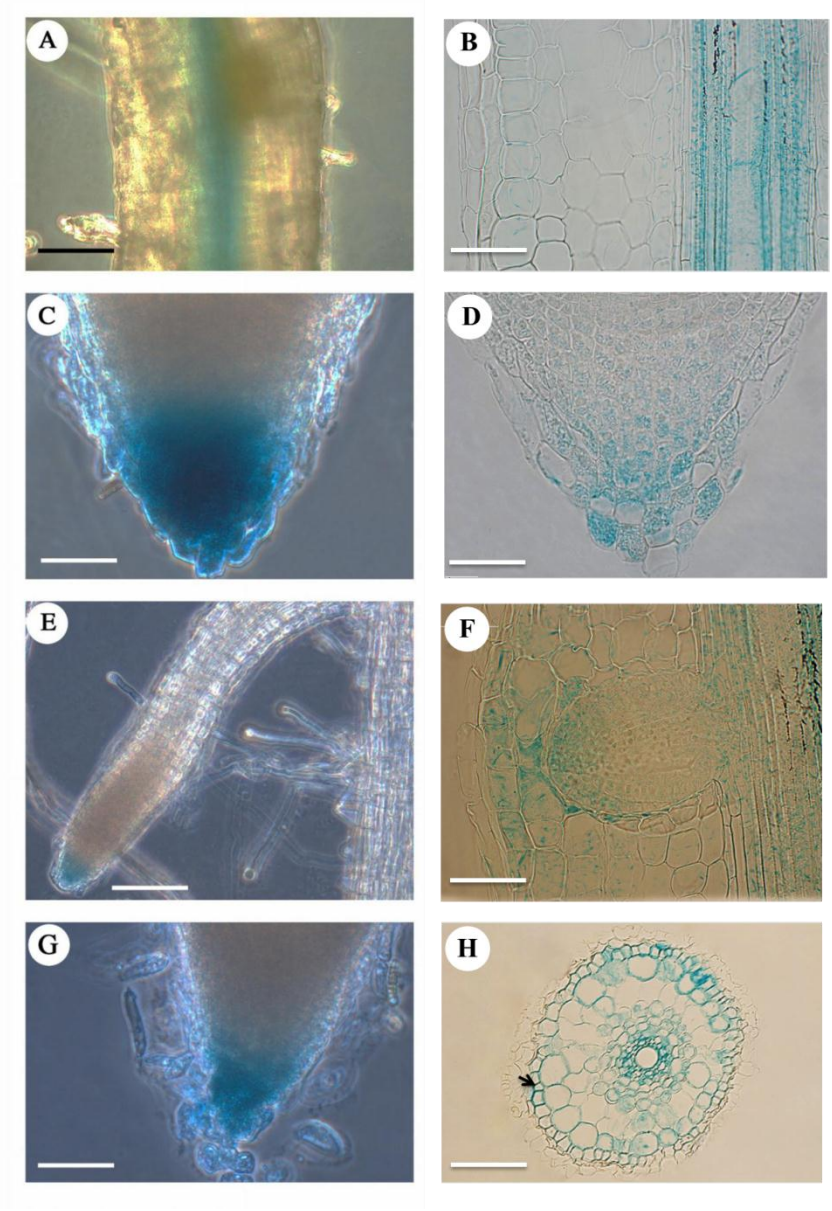

Figure 6. Expression pattern of ASR5 prom:GUS in rice plants. (A) Root elongation zone in macroscopic view. (B) Longitudinal section of root elongation zone. (C) Macroscopic view of the root cap. (D) Longitudinal section of root cap. (E) Root cap of the lateral root and (F) the mechanical damage at the cortex cells. (G) Root cap with not structured cells (Root Border Cells). (H) Transversal section of the root elongation zone showing GUS positive reaction in exodermis cells (arrow), cortex, pericycle, parenchimatic cells of the xylem and companion cells of the phloem. Bars in A=150µm, B=50µm, C=50µm, D=50µm, E=100µm, F=100µm and G=100µm.

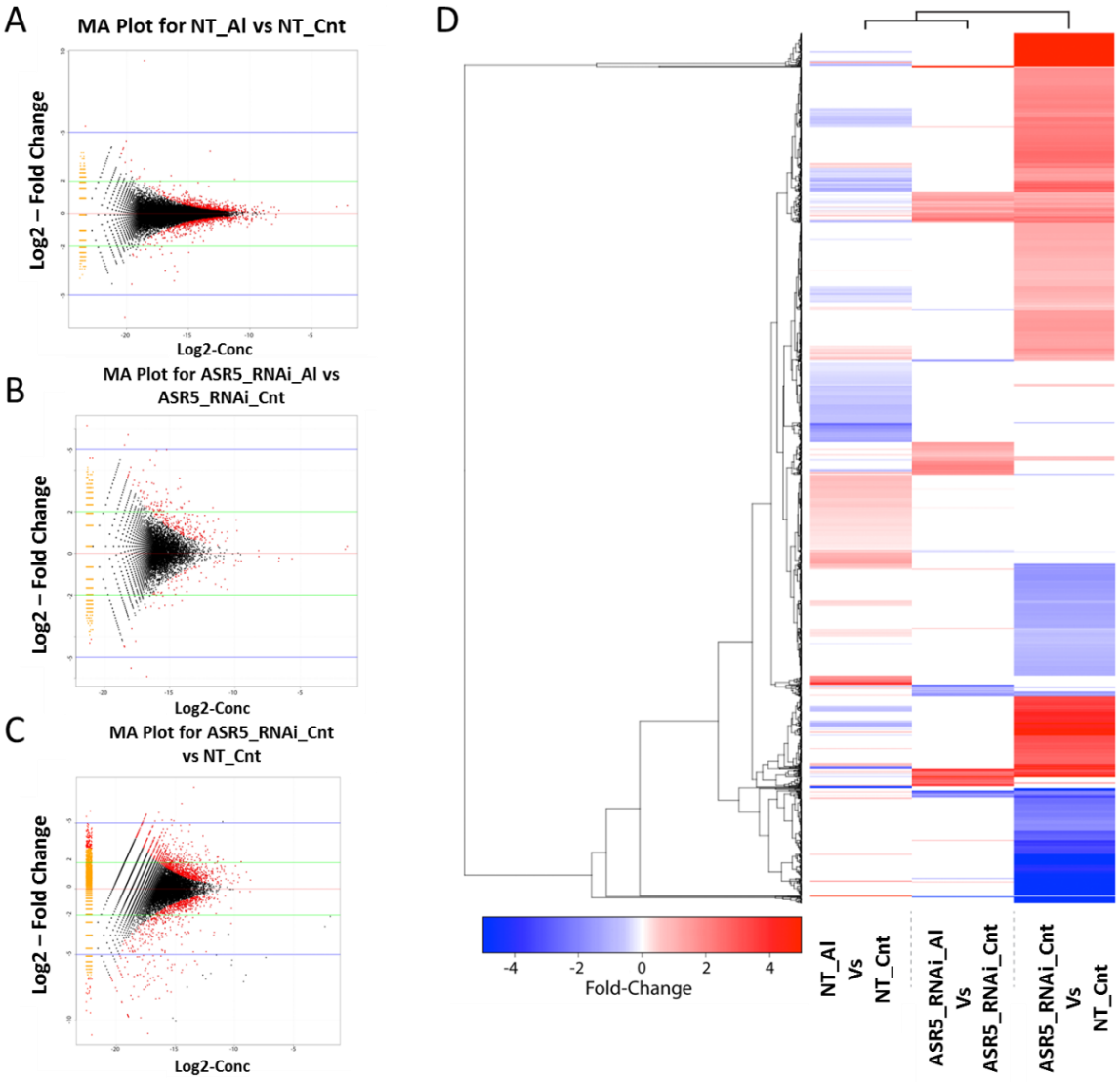

Figure S1. Normalization plots and heatmap for RNA-Seq genes. (A) Normalization plot for Al-responsive genes in Non-transformed plants. (B) Normalization plot for Al-responsive genes in ASR5_RNAi plants. (C) Normalization plot for ASR5_RNAi affected genes. (D) Heatmap showing the three groups analyzed. *NT= Non-transformed plants; ASR5_RNAi= ASR5 silenced plants; Cnt= control conditions; Al= Aluminium conditions.*

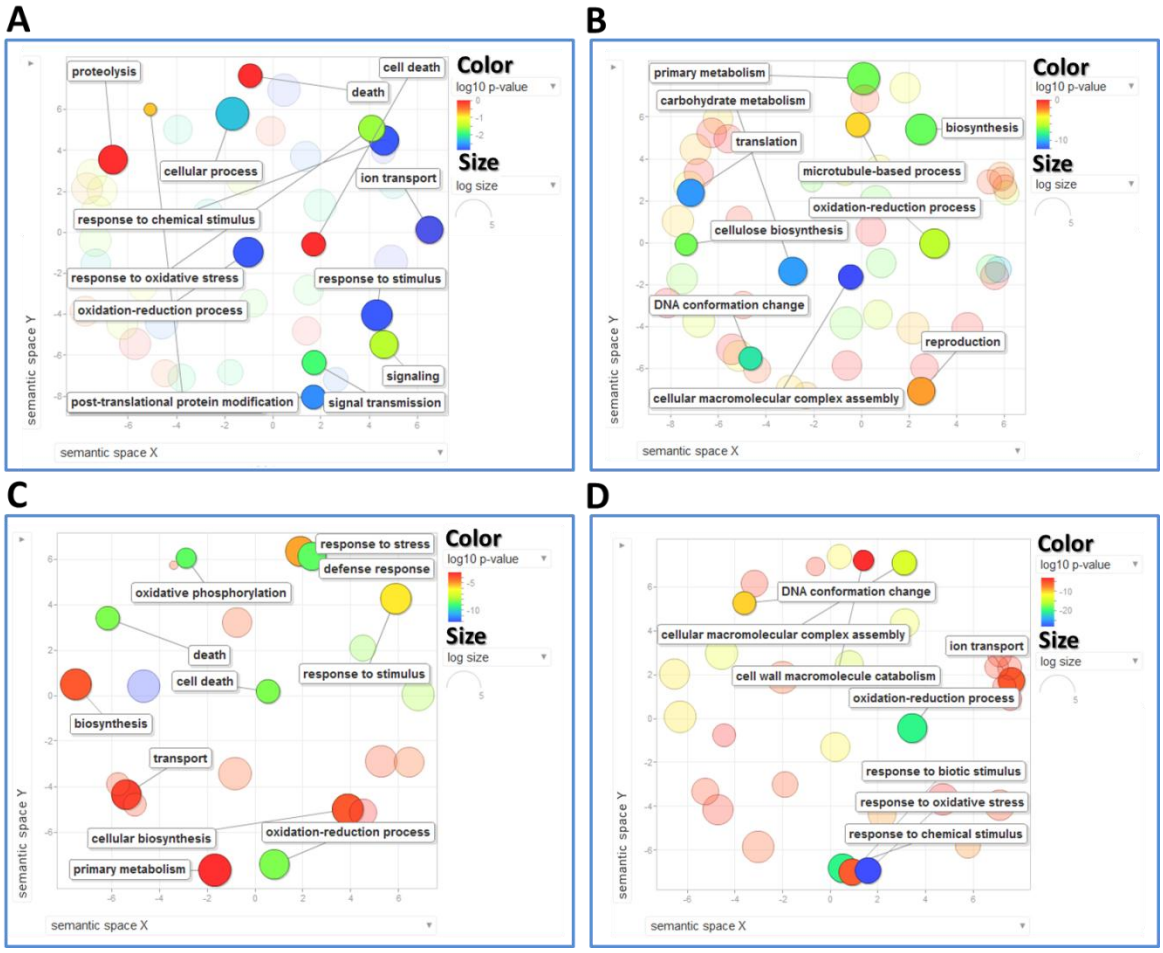

Figure S2. The gene ontology for RNA-Seq genes. The scatterplot shows the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Bubble color indicates the AgriGO-provided p-value (legend in upper right-hand corner); size indicates the frequency of the GO term in the underlying GO. A database (bubbles of more general terms are larger). Enriched terms for Al up-regulated genes in Non-transformed (NT) plants (A), Al down-regulated genes in Non-transformed plants (B), ASR5_RNAi plants affected genes (up-regulated in comparison with NT) (C), and ASR5_RNAi plants affected genes (down-regulated in comparison with NT) in (D).

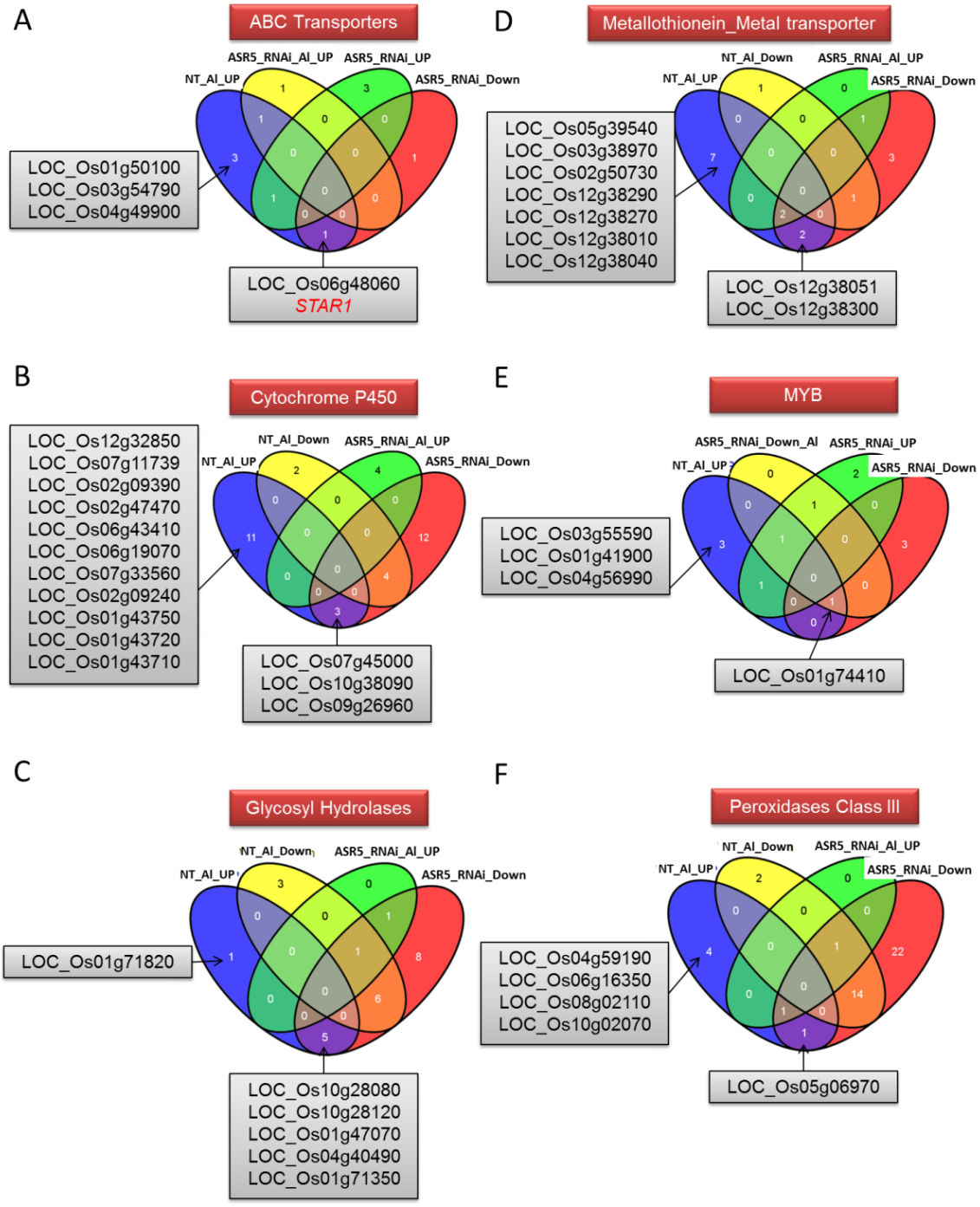

Figure S3. Families of Al responsive genes in Non-transformed (NT) and ASR5_RNAi plants. Six genes families were chosen and compared using venn diagram. (A-F) Venn diagrams showing a comparison between genes in NT plants and ASR5_RNAi plants. *NT= non-transformed plants; ASR5_RNAi= silenced plants for ASR5 gene; Al_UP= Al up-regulated genes; Al_Down= Al down-regulated genes; ASR5_RNAi_UP= up-regulated genes due to ASR silencing in silenced plants; ASR5_RNAi_Down= down-regulated genes due to ASR silencing in silenced plants.*

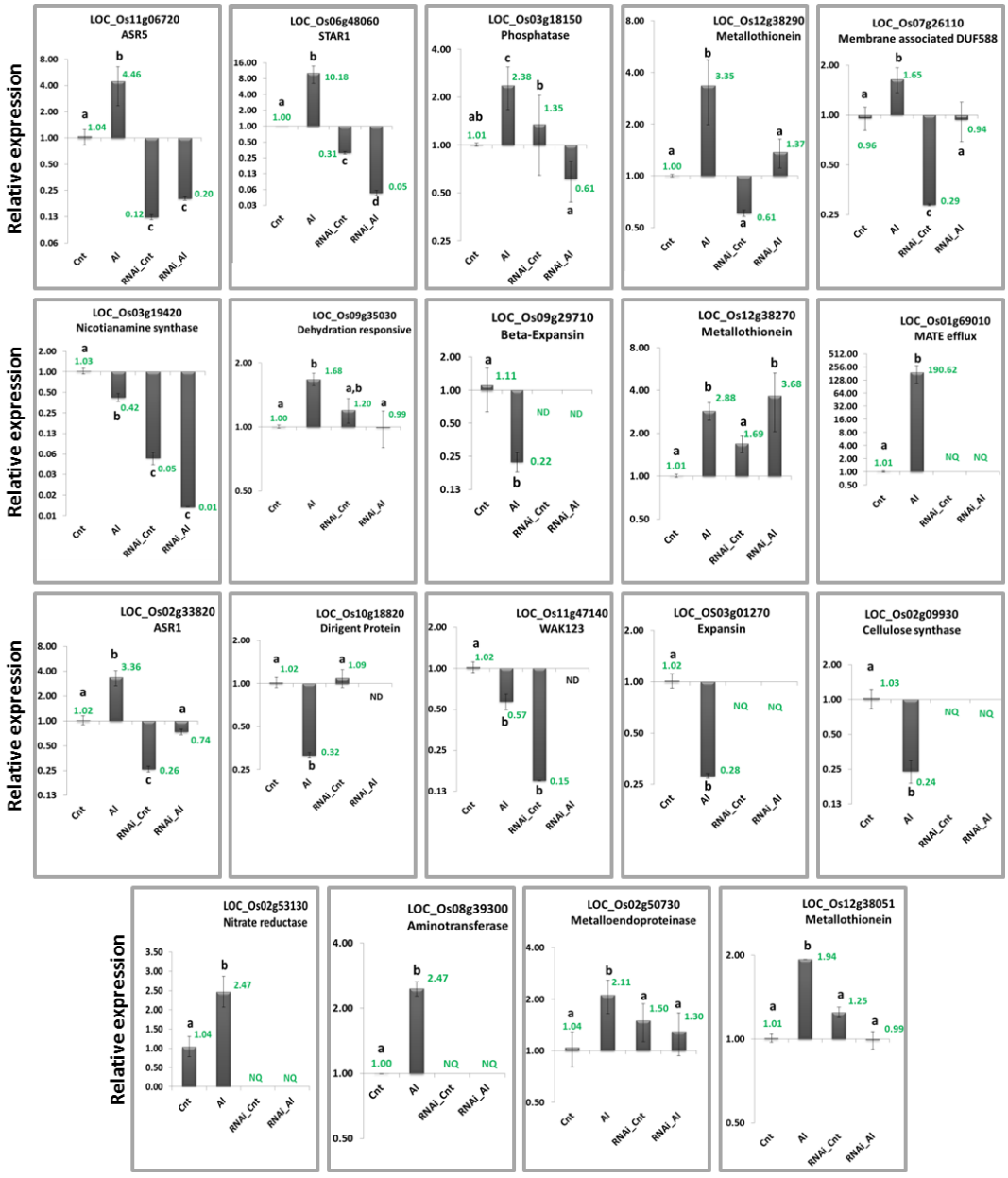

Figure S4. Quantitative Real Time PCR of nineteen selected genes in the RNA-Seq analysis. Total RNA was extracted from roots and used for cDNA synthesis. Relative expression was plotted using *FDH* and *Actin 2* expression levels as Reference genes. Roots of Nipponbare cultivar were collected after 8 hours of treatment with $AlCl_3$ (450 µM). *Bars with different letters are significantly different (ANOVA,* $P < 0.05$*).*

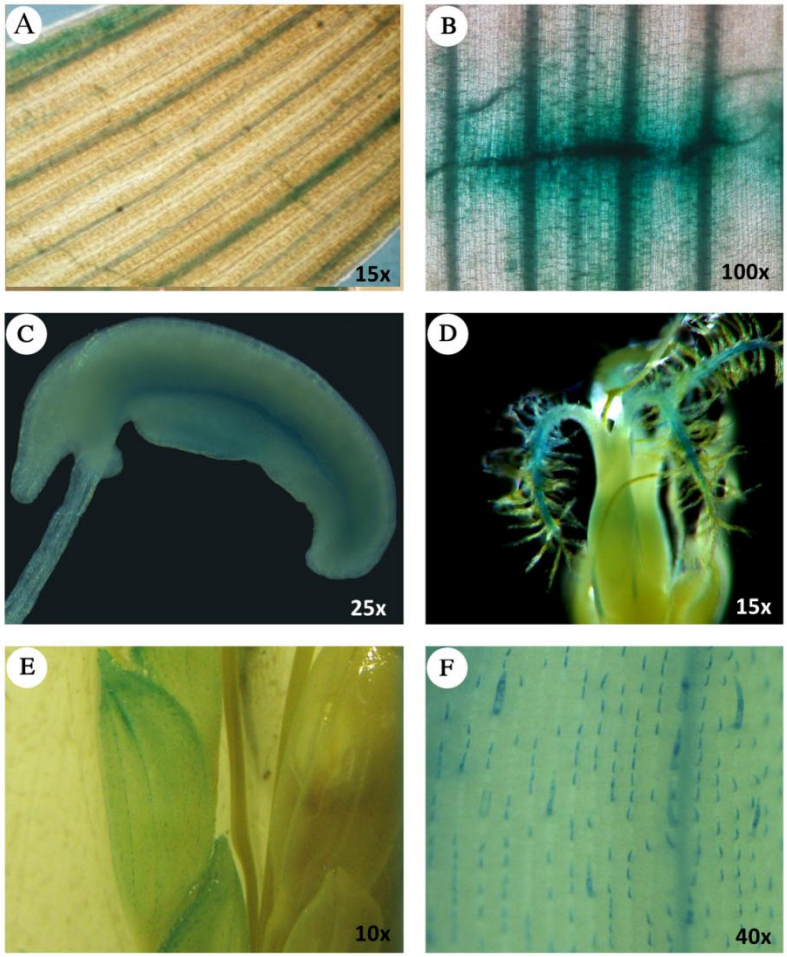

Figure S5. Expression pattern of ASR5 prom:GUS in rice plants. (A) Leaf vascular tissues. (B) Response to mechanical damage in leafs. (C) Anther. (D) Stigma. (E) Vascular tissue of Palea and Lemma. (F) Trichomes of Palea and Lemma.

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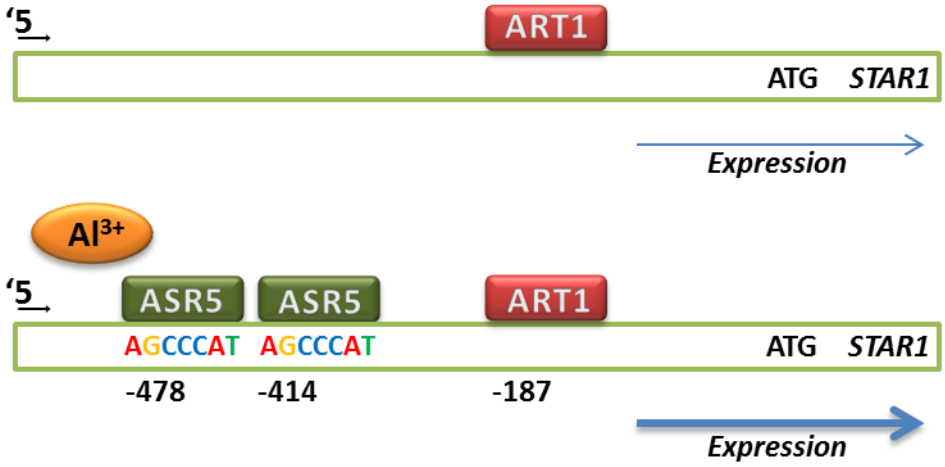

Figure S6. A proposed model for ASR5-STAR1 promoter interaction. ART1 do not respond to Al in rice but maintain a housekeeping expression of *STAR1* under control conditions. In response to Al, ASR5 interact as a homodimer in *STAR1* promoter enhancing its expression.

Table S1. List of genes Al-responsive in rice and comparison with RNA-Seq data. ↓= Genes repressed under Al treatment; ↑ Genes increased under Al treatment; - = Genes not responsive or not detected under Al treatment.

(Annotation)		Our data			
		Expression in Reference papers	NT Al	RNAi Al	Expression in RNAi control compared with NT control
	(36)*Ref*				
LOC_Os01g49290	Guanine nucleotide binding protein beta	↓	↓	-	↓
LOC_Os12g01760	F-box/LRR domain containing protein	↑	↑	-	-
LOC_Os11g01780	F-box/LRR-repeat protein	↑	↑	-	-
LOC_Os07g14270	Calreticulin precursor protein	↓	↓	-	-
LOC_Os12g38290	Metallothionein	↑	↑	↓	-
	(37) *Ref*				
LOC_Os03g13540	Ser/Thr protein phosphatase	↑	↑	↑	-
LOC_Os03g05390	Citrate transporter protein	↑	↑	-	-
	(10) *Ref*				
LOC_Os03g55290	GASR3 - Gibberellin-regulated	↑	↑	-	-
LOC_Os10g38080	Putative Subtilisin homologue	↑	↑	-	↓
LOC_Os03g54790	ABC transporter	↑	↑	↑	-
LOC_Os10g13940	MATE efflux protein	↑	↑	-	-
LOC_Os10g42780	LrgB-like family protein	↑	↑	-	-
LOC_Os02g09390	Cytochrome P450	↑	↑	-	-
LOC_Os02g53130	Nitrate reductase	↑	↑	-	-
LOC_Os04g41750	expressed protein	↑	↑	-	-
	(10, 12, 38) *Ref*				
LOC_Os02g03900	(Nrat1) Metal transporter Nramp6	↑	↑	-	↓
	(10, 11) *Ref*				
LOC_Os06g48060	ABC transporter, STAR1	↑	↑	-	↓
	(39) *Ref*				
LOC_Os12g38051	metallothionein	↑	↑	-	↓
	(40) *Ref*				
LOC_Os05g09440	malic enzyme	↑	↑	-	-
	(13) *Ref*				
LOC_Os01g69010	MATE or FDR4	↑	↑	-	-
	(35) *Ref*				
LOC_Os02g33820	ASR1	↑	↑	-	↓
LOC_Os01g72910	ASR4	↑	↑	-	↓
LOC_Os01g06720	ASR5	↑	↑	-	↓

Supplementary Information S2 e S3 não foram inseridas na tese devido ao tamanho – Aproximadamente 4000 páginas.

Table S4. Table showing gene list overlapped between RNA-Seq and ChIP-Seq. (A) Overlapp between ChIP-Seq and the 469 genes from RNA-Seq responsive to Al only in Non-transformed plants. (B) Overlapp between ChIP-Seq and the 367 genes from RNA-Seq responsive to Al in Non-transformed plants, not responsive to Al in ASR5_RNAi plants but, affected due to ASR5 silencing. (C) Overlapp between ChIP-Seq and the 1204 genes from RNA-Seq not responsive to Al but affected due to ASR silencing.

A	Gene Locus	Annotation	Binding peak region	Strand	Distance from Start codon in Bp	Number of reads in Al	Number of reads in Control	Fold Change log2 in WT plants in response to Al
1	LOC_Os01g28790	PRAS-rich protein putative	5 kb	-+	1530	324	193	0.674350934
2	LOC_Os01g46370	lipase class 3 family protein	CDS	-		502	336	0.506184099
3	LOC_Os01g65900	chitin-inducible gibberellin-responsive protein	5kb	--	3314	1997	1454	0.384765031
4	LOC_Os01g71820	glycosyl hydrolases family 17 putative	5 kb	-+	4212	205	98	0.991728223
5	LOC_Os02g01220	rhodanese-like domain containing protein	CDS	+		328	442	-0.503392587
6	LOC_Os02g50730	metalloendoproteinase 1 precursor	CDS/5'UTR	-		156	82	0.854808182
7	LOC_Os02g53130	nitrate reductase,	5kb	--	3549	563	376	0.509360228
8	LOC_Os03g05530	nodulin, putative	down 1kb	--		1537	1130	0.37075236
9	LOC_Os03g55590	MYB family transcription factor	down 1kb	++		1198	904	0.333191198
10	LOC_Os04g54190	cysteine-rich receptor-like protein kinase 8 precursor	5kb	-+	1984	542	336	0.616789586
11	LOC_Os05g42424	ubiquitin family protein	CDS	+		1875	1471	0.277051317
12	LOC_Os06g03810	protein	CDS	-		90	33	1.374416945
13	LOC_Os08g06170	berberine and berberine like domain containing protein	other			90	33	1.374416945
14	LOC_Os08g24310	receptor-like protein kinase precursor	CDS	+		237	115	0.970211166
15	LOC_Os08g37370	mitochondrial carrier protein,	down 1kb	--		1221	781	0.571626715
16	LOC_Os08g39300	aminotransferase putative	5 kb	-+	225	614	365	0.677300159
17	LOC_Os09g35030	dehydration-responsive element-binding protein	5 kb	-+	1766	134	32	1.993047158
18	LOC_Os11g01780	F-box 2FLRR-repeat protein 3 putative	5 kb	-+	406	843	461	0.797723848
19	LOC_Os11g39530	jacalin-like lectin domain containing protein	5kb	--	3506	46	112	-1.356834998
20	LOC_Os12g33946	cytochrome c oxidase subunit 1	down 1kb	++		377	250	0.519594397
21	LOC_Os01g64470	harpin-induced protein 1 domain containing protein	CDS	-		120	34	1.746385722

B	Gene Locus	Annotation	Binding peak region	Strand	Distance from Start codon in Bp	Number of reads in Al	Number of reads in Control	Fold Change log2 in WT plants in response to Al	RNAi Expression in Control (Fold Change log2)
1	LOC_Os01g18170	Cupin domain containing protein,	5kb	-+	3547	5054	4340	0.14668859	-2.188908303
2	LOC_Os01g49370	protein	5kb	--	113	374	247	0.525485196	4.800540083
3	LOC_Os01g58890	cysteine proteinase inhibitor precursor protein	5kb	++	157	2857	2392	0.183241615	-1.439055141
4	LOC_Os02g33820	ASR1	CDS/5'UTR	-		8318	5756	0.458128061	-4.866369015
5	LOC_Os02g44108	expansin precursor,	other			230	437	-0.999041451	-2.427115527
6	LOC_Os02g50620	dehydrogenase E1 component domain containing protein	other			872	1080	-0.553862333	-1.330343211
7	LOC_Os03g18150	protein phosphatase putative	5kb	++	1289	424	227	0.828329935	1.325180377
8	LOC_Os04g55600	protein	down 1kb	--		2635	2142	0.22580245	-2.772836242
9	LOC_Os07g26110	membrane associated DUF588 domain containing protein	5kb	-+	2622	1565	824	0.680215354	-1.149481507
10	LOC_Os07g26690	aquaporin protein putative	other			2931	3728	-0.592239953	-1.079239611
11	LOC_Os11g47140	OsWAK123 - OsWAK receptor-like protein kinase	CDS	+	2632	315	504	-0.923302966	-2.632905981
12	LOC_Os12g25690	UDP-glucose 6-dehydrogenase, putative,	5kb	++	458	3220	3668	-0.260974983	-0.828025472
13	LOC_Os12g34094	NADH-ubiquinone oxidoreductase chain 4	other			404	215	0.664787573	5.48056969
14	LOC_Os03g35340	protein	other			1555	1232	0.262870292	0.696497234
15	LOC_Os06g48060	STAR1 - ABC transporter	5kb		467	914	416	1.062568605	-2.841492603

C	Gene Locus	Annotation	Binding peak region	RNAi Expression in Control (Fold Change log2)
1	IOC_Os12g41090	CAMK includes calcium 2Fcalmodulin depedent	other	-2.754
2	IOC_Os01g09030	2-aminoethanethiol dioxygenase putative	other	-2.608
3	IOC_Os12g42280	9-cis-epoxycarotenoid dioxygenase 1 chloroplast precursor	other	3.122
4	IOC_Os03g20420	alpha-N-arabinofuranosidase A putative	cds	-0.963
5	IOC_Os07g42510	AP2 domain containing protein	5kb	-1.135
6	IOC_Os01g65902	apocytochrome f precursor putative	other	5.274
7	IOC_Os01g58010	ATP synthase A subunit family protein putative	other	6.353
8	IOC_Os12g23610	ATP synthase A subunit family protein putative	other	5.768
9	IOC_Os10g38274	ATP synthase C chain putative	5kb	5.537
10	IOC_Os12g28590	ATPase 2 putative	cds	1.098
11	IOC_Os01g03330	BBTI3 - Bowman-Birk type bran trypsin inhibitor precursor	5kb	-34.136
12	IOC_Os10g21236	chloroplast ATP synthase a chain precursor putative	cds	5.122
13	IOC_Os01g05900	Core histone H2A 2FH2B 2FH3 2FH4 domain	cds	-0.991
14	IOC_Os02g21920	CPuORF24 - conserved peptide uORF-containing transcript	5kb	-1.831
15	IOC_Os11g40690	dehydrogenase putative	cds	-2.895
16	IOC_Os07g42520	dirigent putative	5kb	-3.038
17	IOC_Os06g04540	DNA binding protein putative	5kb	-2.496
18	IOC_Os02g24614	DNA-directed RNA polymerase subunit beta putative	5kb	4.641
19	IOC_Os04g16830	DNA-directed RNA polymerase subunit beta putative	cds	4.699
20	IOC_Os02g42600	double-stranded RNA binding motif containing protein	CDS	1.094
21	IOC_Os02g30270	protein	other	3.090
22	IOC_Os03g48626	protein	cds	-1.637
23	IOC_Os05g10414	protein	5kb	-3.692
24	IOC_Os07g26100	protein	other	34.724
25	IOC_Os09g17360	protein	cds	-32.874
26	IOC_Os11g47640	protein	5kb	-33.082
27	IOC_Os12g14939	protein	other	7.029
28	IOC_Os12g23570	protein	5kb	6.537
29	IOC_Os12g33928	protein	cds	6.746
30	IOC_Os12g34096	protein	down 1kb	6.573
31	IOC_Os03g03034	flavonol synthase 2Fflavanone 3-hydroxylase putative	other	1.689
32	IOC_Os05g05620	glutathione S-transferase putative	5kb	2.269
33	IOC_Os02g07340	HEAT repeat family protein putative	cds	1.623
34	IOC_Os06g04030	histone H3 putative	cds	-1.472
35	IOC_Os12g34120	hypothetical protein	5kb	6.225
36	IOC_Os10g04270	jacalin-like lectin domain containing protein	other	-1.488
37	IOC_Os12g15680	laccase precursor protein putative	cds	-4.142
38	IOC_Os12g33956	maturase putative	other	5.403
39	IOC_Os02g42040	MIF4G domain containing protein putative	other	1.011
40	IOC_Os09g08890	mitochondrial 60S ribosomal protein l5 putative	other	6.018
41	IOC_Os12g34054	mitochondrial ribosomal protein S3 putative	5kb	5.759
42	IOC_Os12g34124	mitochondrial ribosomal protein S3 putative	5kb	5.891
43	IOC_Os12g34045	mitochondrial ribosomal protein S7 putative	other	5.759
44	IOC_Os08g43550	MYB family transcription factor putative	other	-1.107
45	IOC_Os07g29820	NBS-IRR disease resistance protein putative	5kb	1.094
46	IOC_Os11g31890	NII interacting factor-like phosphatase putative	cds	0.954
47	IOC_Os03g21380	OsCML27 - Calmodulin-related calcium sensor protein	5kb	-3.107
48	IOC_Os07g07410	oxidoreductase 2OG-Fe oxygenase family protein putative	5kb	-1.770
49	IOC_Os08g37250	patatin putative	other	-1.834
50	IOC_Os11g39990	patatin putative	other	-3.251
51	IOC_Os01g65380	patellin protein putative	other	-1.285
52	IOC_Os06g49250	peptide transporter PTR2 putative	other	-2.542
53	IOC_Os09g39780	peptidyl-prolyl cis-trans isomerase putative	5kb	3.447
54	IOC_Os03g27230	phospho-2-dehydro-3-deoxyheptonate aldolase chloroplast	5kb	-1.134
55	IOC_Os01g58049	photosystem I assembly protein ycf4 putative	other	4.817
56	IOC_Os04g33390	prephenate dehydratase domain containing protein	cds	-3.491
57	IOC_Os01g63960	retrotransposon protein putative Ty1-copia subclass	down 1kb	6.996
58	IOC_Os01g67364	retrotransposon protein putative Ty3-gypsy subclass	other	8.370
59	IOC_Os12g23590	retrotransposon protein putative unclassified	5kb	6.200
60	IOC_Os01g63970	sialyltransferase family domain containing protein	5kb	-1.244
61	IOC_Os01g60860	spotted leaf 11 putative	other	-1.353
62	IOC_Os01g64750	sterol 3-beta-glucosyltransferase putative	5kb	1.952
63	IOC_Os06g43790	transcription initiation factor TFIID subunit 1 putative	down 1kb	1.018
64	IOC_Os05g39790	transposon protein putative unclassified	other	33.631
65	IOC_Os02g52550	uncharacterized Cys-rich domain containing	other	-2.335
66	IOC_Os03g62370	WD40-like Beta Propeller Repeat family protein	5kb	-2.893
67	IOC_Os02g45780	zinc finger C3HC4 type domain containing protein	5kb	2.537
68	IOC_Os02g46610	zinc-binding protein putative	down 1kb	-1.553

Table S5. List of primers used for PCR, Real Time PCR, ChIP_qPCR, Promoter analyses and Pull Down assay. *Italic= Reference genes primers.*

	Gene ID	Primers
	Real Time RT_qPCR	
1	LOC_Os09g35030_F	GGATCAAGCAGGAGATGAGC
2	LOC_Os09g35030_R	GCCTCGTCTCCCTGAACTT
3	LOC_Os02g50730_F	GCGTACCAGCGCAACTTC
4	LOC_Os02g50730_R	AGTTCCTGTCCATGGTGGAT
5	LOC_Os07g26110_F	GCCAAGTACACGCACTCACC
6	LOC_Os07g26110_R	GAGCTGGAGAGCAACCTCAC
7	LOC_Os03g18150_F	GAAGGGTGGAGATGGAGGAC
8	LOC_Os03g18150_R	TCTCCTCGGCCATGAACTT
9	LOC_Os11g47140_F	AGAGCTGAATTGGGGGAACT
10	LOC_Os11g47140_R	AATCTCCACGGCTGAATTTG
11	LOC_Os01g69010_F	GTCTGCTCCAGGCCGTATT
12	LOC_Os01g69010_R	TACCAGGTATCGCAGTGCAG
13	LOC_Os12g38290_F	ATGTCGTGCGGTGGAAGT
14	LOC_Os12g38290_R	CACCGAGGATCATGGTATCTG
15	LOC_Os12g38051_F	GTACCCTGACCTGGCTGAGA
16	LOC_Os12g38051_R	GCCAACCATCACCTCAAAGT
17	LOC_Os12g38270_F	CACTTCAGCAACTATGGTCCTC
18	LOC_Os12g38270_R	CAGTTGCAGGGATTGCATTT
19	LOC_Os09g29710_F	GGCACTGCGACCTTCTACAC
20	LOC_Os09g29710_R	GCACGTCACCACGTACTGC
21	LOC_Os03g19420_F	GCGGTTCCAGTGGTGAGC
22	LOC_Os03g19420_R	TCAGACGGATAGCCTCTTGG
23	LOC_Os10g18820_F	AAGCTCTACCAGCGAGGATG
24	LOC_Os10g18820_R	TGGCAGACGAGTAGCATTTG
25	LOC_Os02g53130_F	ATGAGGTGCTCCCCATCAAT
26	LOC_Os02g53130_R	CAGGGTCACCTCTACCCTTG
27	LOC_Os08g39300_F	CTCGAGGACGTCAAGCAGAT
28	LOC_Os08g39300_R	CGATCAGGAACGACACGAT
29	LOC_Os02g09930_F	AGATCCCCATGTTCAACGAG
30	LOC_Os02g09930_R	CTTGATGACGGGGTCAGTG
31	LOC_Os03g01270_F	CTCTCTTCAAGGACGGCAAG
32	LOC_Os03g01270_R	GTCGAAGTGGTACAGCGACA
33	LOC_Os02g33820_F	TGGTGGACTACGACAAGGAGA
34	LOC_Os02g33820_R	GCCACCTCCTCCTTCACC
35	LOC_Os11g06720_F	CCAGGACGAGTACGAGAGGT
36	LOC_Os11g07620_R	CGATCTCCTCCGTGATCTTG
37	LOC_Os06g48060_F	TCGCATTGGCTCGCACCCT

38	LOC_Os06g48060_R	TCGTCTTCTTCAGCCGCACGAT
39	*LOC_Os02g57040_F*	TTCCAATGCATTCAAAGCTG
40	*LOC_Os02g57040_R*	CAAAATCAGCTGGTGCTTCTC
41	*LOC_Os08g29650_F*	GGGGACATCCGATAAAATTG
42	*LOC_Os08g29650_R*	AGGGGCCATGCTAATCTTCT
	ChIP_qPCR	
1	LOC_Os06g48060_F	TCAAGTACTCGCAGCAATGTT
2	LOC_Os06g48060_R	CTTTTGGGCCATGGAGAAG
3	*LOC_Os04g57220_F*	TATCCAACATGAATGCCACA
4	*LOC_Os04g57220_R*	CAGCACGAGATGAGTAAAACAA
	Pull Down Assay	
1	Forward Primer	TAGGAAAAAGGAAGCGGGAT
2	Reverse F1	CTGGTGGCGTAGCTATCCAT
3	Reverse F2	GAAGGAATGGACAGAAGGCC
4	Reverse F3	CAAGGGCATCAGATTAGCAG
	ASR5 promoter	
1	Forward	CACCGGACATACTTGCAATATCCTTCTT
2	Reverse	AGCTAGAAGCTAGTGATGACAATTAGG

DISCUSSÃO GERAL

Estresses primários provocados por salinidade, seca, frio e excesso de Al são geralmente interconectados e afetam dois grupos de proteínas: o primeiro grupo contendo proteínas cuja atividade permite as plantas sobreviverem (proteínas para controle da homeostase, controle de espécies reativas de oxigênio, etc) e um segundo grupo contendo proteínas envolvidas na sinalização e transdução de sinais como fatores de transcrição, kinases e fosfatases (Saumonneau *et al*., 2011), sendo as proteínas ASR exemplos de proteínas que podem atuar nos dois grupos citados acima (Yang *et al*., 2005).

De um lado, alguns autores classificam as ASRs como proteínas do tipo LEA (Late Embryogenesis Abundant) (Battaglia *et al*., 2008). Sua afiliação com proteínas do tipo LEA ocorre primariamente devido a suas características físico-químicas bem como sua alta hidrofilicidade. Sua exata classificação ainda é controversa uma vez que ASRs não contém assinaturas específicas de proteínas do tipo LEA (Garay-Arroyo *et al*., 2000). De outro lado, sua habilidade de ligação ao DNA em uma sequência específica e sua transição de uma estrutura desordenada para uma estrututra ordenada dependente de zinco são consistentes com seu papel como fatores de transcrição (Goldgur *et al*., 2007). Finalmente, a localização subcelular das ASRs no citoplasma e núcleo enfatizam seu papel duplo (Çakir *et al*., 2003) em plantas.

Desde a descoberta dos genes *ASR*, diversos trabalhos têm mostrado o acúmulo dessas proteínas em processos ligados ao desenvolvimento da planta, tais como desenvolvimento de frutos, maturação do pólen e metabolismo do açúcar (Iusem *et al.,* 1993; Wang *et al*., 1998; Çakir *et al*., 2003; Frankel *et al*., 2007; Yang *et al*., 2008), e em resposta a diversos estresses como déficit de água, excesso de sal, frio e ataque de patógenos (Schneider *et al*., 1997; Zivy *et al*., 1998; Vaidyanathan *et al*., 1999; Maskin *et al*., 2001; Kalifa *et al*., 2004b; Liu *et al*., 2010; Philippe *et al*., 2010; Henry *et al*., 2011). Sua exata função, no entanto, permanece enigmática uma vez que os mecanismos moleculares e funções biológicas das ASRs não podem ser preditas por homologia de sequências com outras proteínas conhecidas (Virlouvet *et al*., 2011). Tentativas de cristalizar a

proteína ASR1 de tomate usando mais de 600 condições não foram bem sucedidas (Goldgur *et al*., 2007). Além disso, os genes ASR não estão presentes em *Arabidopsis* (Saumonneau *et al*., 2011). Dessecação, ligação a zinco e homodimerização podem ser induzidos na proteína ASR1 de tomate (Goldgur *et al*., 2007) que possui atividade de ligação a DNA dependente de zinco (Rom *et al*., 2006).

O arroz possui 6 cópias do gene *ASR* (Frankel *et al*., 2006). O gene *ASR5* foi o foco central nesta tese por ser o gene mais expresso e por codificar a proteína mais similar às ASR encontradas em outras espécies. O gene *ASR5* foi caracterizado pela primeira vez em arroz por Vaidyanathan *et al* (1999) que demonstraram que o mesmo tem sua expressão regulada em resposta ao estresse hídrico. Em nosso trabalho, *ASR5* aumentou seus níveis de transcritos em resposta a seca confirmando os dados obtidos por Vaidyanathan *et al*., (1999). Além disso, todos os genes *ASR* apresentaram níveis aumentados de transcritos em resposta ao Al. Yang *et al* (2007) mostraram que os níveis da proteína ASR5 aumentaram em relação ao controle em plantas de arroz sob estresse por Al. O trabalho de Yang *et al* (2007) foi o ponto de partida para o nosso estudo, uma vez que descrevia uma proteína inédita na resposta da planta ao estresse por Al. Utilizando milho como modelo, Cançado *et al* em 2008 (Cançado *et al* 2008) também encontraram um gene *ASR* aumentado em resposta ao Al.

A expressão do gene *ASR5* foi avaliada também em dois segmentos da raiz (ápice e base) e foi encontrado um aumento evidente no ápice em resposta ao Al. A região do ápice da raiz, que abrange a coifa, o meristema e a zona de elongação, é altamente sensível ao Al e o acumula facilmente (Panda *et al*., 2009). Os eventos seguintes resultam em danos físicos da região madura dos tecidos radiculares. O efeito tóxico primário do Al ocorre na zona de transição distal do ápice da raiz (Sivaguru and Horst, 1998).

O arroz é relativamente tolerante ao Al comparado a outras espécies (Famoso *et al*., 2010), porém existe alta variabilidade dos genótipos encontrados. De maneira geral, cultivares da ssp Japonica são mais resistentes que as cultivares da ssp Indica (Ma *et al*., 2002). Em nosso trabalho, verificamos que o

gene *ASR5* não respondeu ao Al em plantas da ssp Indica cv Taim. A fim de obter maiores informações a respeito da função do gene *ASR5*, plantas transgênicas de arroz silenciadas para o gene *ASR5* foram obtidas. Essas plantas apresentaram fenótipo semelhante a mutantes sensíveis ao Al (Ma *et al*., 2005; Huang *et al*., 2009) quando expostas ao Al, sendo a inibição da elongação a raiz o aspecto mais evidente. Devido a similaridade das ASRs de arroz, foi constatado que a construção ASR5_RNAi afetou também a expressão de *ASR1*. Não se pode descartar porém, a possibilidade do fenótipo das plantas ASR5_RNAi ser devido em parte ao silenciamento de *ASR1*.

Além disso, utilizando análises proteômicas verificamos que o silenciamento dos genes *ASR* produziu mudanças significativas no padrão de expressão de várias proteínas em folhas de arroz. As proteínas foram associadas funcionalmente ao desenvolvimento, degradação, transporte de elétrons, metabolismo de carboidratos, resposta a estresses entre outros. A grande maioria, no entanto, foi classificada como proteínas envolvidas na fotossíntese. A toxicidade ao Al em plantas afeta a absorção de nutrientes e diversos processos metabólicos, especialmente a fotossíntese (Chen *et al*., 2005a). Além do mais, a assimilação de CO_2 é reduzida em folhas suplementadas com Al (Chen *et al*., 2005b). Recentemente, Li *et al*., (2012) mostraram que o tratamento com Al inibiu a taxa fotossintética e a transferência de elétrons, além de reduzir a atividade fotoquímica do fotossistema II de uma maneira tempo-concentração depedente em folhas de tabaco.

Para definir a localização subcelular da proteína codificada pelo gene *ASR5* foi construído um vetor binário de transformação de plantas capaz de permitir a expressão de uma proteína da fusão ASR5-GFP. A proteína ASR5 localizou-se no citoplasma e núcleo em calos trangênicos de arroz. A introdução dessa construção em protoplastos obtidos de folhas de plantas de arroz crescidas na presença e na ausência de luz revelou pela primeira vez a localização de ASR5 em cloroplastos e/ou protoplastídeos. A localização nuclear das ASRs já havia sido relatada anteriormente (Çakir *et al*., 2003, Kalifa *et al*., 2004, Yang *et al*., 2005). A localização cloroplastídica de ASR5 no entanto, é inédita para esta família gênica.

O cloroplasto de plantas possui seu próprio genoma mas muitos de seus genes foram transferidos ao núcleo durante a evolução. Essas proteínas cloroplastídicas codificadas no núcleo, são direcionadas ao cloroplasto com a ajuda do peptídeo sinal de transferência localizado na região N-terminal da proteína (Robinson *et al*., 1997). Das 41 proteínas diferencialmente expressas nas plantas ASR5-RNAi, 19 contém o peptídeo sinal de transferência ao cloroplasto, sugerindo uma possível ligação dos genes *ASR* na regulação das respostas do cloroplasto ao tratamento com Al. A proteína ASR5 não contém o peptídeo sinal de transferencia ao cloroplasto porém, contém um sinal putativo de miristoilação na posição glicina-68 conservada entre diversas proteínas ASR (Vaidyanathan *et al*., 1999), o que sugere um importante papel na função da proteína e uma possível explicação para a localização cloroplastídica de ASR5. A miristoilação de proteínas em eucariotos consiste da transferência de uma cadeia lipídica (o miristato) através de enzimas específicas, para o aminoácido glicina da parte amino-terminal de uma determinada seqüência na proteína. Esta reação de transferência promove a associação de proteínas específicas entre si, bem como com domínios na membrana (Farazi *et al*., 2001).

No presente trabalho, objetivou-se também a busca de novos genes responsivos ao Al em arroz, bem como a busca de genes diferencialmente expressos em plantas ASR5_RNAi em condições controle e tratadas com Al.

Para isso, o sequenciamento total dos transcritos foi realizado, permitindo avaliar de uma maneira ampla, quais os tipos de genes responsivos ao Al em arroz ssp Japonica, cultivar Nipponbare não transformado. A confidência e o grau de acurácia desta ánalise é suportado pelo fato do gene *ASR5*, bem como diversos outros genes já relatados na literatura como *FRDL4*, *Nrat1* e *STAR1* responderem aumentando seus níveis de transcritos em resposta ao Al. Um total de 961 genes (475 aumentados e 486 reprimidos) responderam ao Al em nossas análises. A ontologia dos genes mostrou que parte dos genes aumentados estão enriquecidos nas classes de resposta a estresses, sinalização, transporte de íons e morte celular programada. Para genes reprimidos, a ontologia revelou genes enriquecidos nas classes de metabolismo primário, regulação da biossíntese e organização da cromatina. Estas classes enriquecidas estão em acordo com

trabalho utlizando *Medicago truncatula* tratadas com Al (Chandran *et al*., 2008). O sequenciamento total dos transcritos das plantas ASR5_RNAi revelou distintos padrões de expressão quando comparadas a plantas não transformadas. Apenas 309 genes (234 aumentados e 75 reprimidos) responderam ao Al. A baixa sobreposição dos genes responsivos ao Al nas plantas ASR5_RNAi com plantas não transformadas, mostrou o grau de desordem causado pelo silenciamento de *ASR5*, e diversos genes responsivos ao Al em plantas não transformadas não responderam nas plantas ASR5_RNAi como *Nrat1*, *FRDL4* e *STAR1*. Quando comparados os transcritos na situação controle entre ASR5_RNAi e plantas não transformadas, 1756 genes (632 aumentados e 1124 reprimidos) foram afetados devido ao silenciamento de *ASR5*. Estes resultados mostram a clara importância do gene *ASR5* na regulação gênica. Ao nosso conhecimento, este é o primeiro trabalho de sequenciamento em larga escala do RNA em plantas de arroz submetidas ao tratamento com Al. Além disso, o sequenciamento dos transcritos das plantas ASR5_RNAi nos permitiu mostrar potenciais genes regulados por ASR5.

Para a confirmação da atividade de ASR5 como fator de transcrição em arroz, foi realizado o sequenciamento total do DNA imunoprecipitado pela proteína ASR5 de arroz em resposta ao Al. A técnica de imunoprecipitação da cromatina (ChIP) seguida do sequenciamento total (ChIP-Seq) foi utilizada para determinar a localização no genoma de sítios de ligação de ASR5. O complexo DNA-ASR5 foi imunoprecipitado usando anticorpos específicos (anticorpo anti ASR5 produzido através da expressão da proteína fusionada a proteína GST em *E. coli*, purificação, clivagem com trombina e injeção da proteína ASR5 pura em coelho). A cobertura e a alta resolução combinada com a habilidade de seqüenciar diversos milhões de bases em curto espaço de tempo nos permitiu mapear as interações ASR5/DNA em resposta ao Al. Utilizando esta abordagem, um total de 1086 genes possivelmente regulados por ASR5 foram encontrados. Uma busca discriminativa por motivos de ligação enriquecidos nos dados do ChIP-Seq revelou a sequência (A/G)GCCCA(A/T). Este motivo possui semelhança ao encontrado por Kalifa *et al*., (2004b) C2-3(C/G)A utilizando ASR1 de tomate. A grande quantidade de genes encontrados no ChIP e a grande

quantidade de genes diferencialmente expressos nas plantas ASR5_RNAi, sugerem que ASR5 regula diversos genes não relacionados a resposta ao Al. No intuito de refinar nossa busca por genes regulados por ASR5 em resposta ao Al, foram sobrepostos os genes encontrados no ChIP-Seq com os genes do RNA-Seq que responderam ao Al somente nas plantas não transformadas, e com genes que além de não respoderem mais ao Al, encontraram-se diferencialmente expressos nas plantas ASR5_RNAi (reprimidos ou aumentados devido ao silenciamento de *ASR5*). Nesta análise, um total de 36 genes foram identificados sendo regulados diretamente por ASR5 em resposta ao Al. Destes 36 genes, o gene *STAR1* foi escolhido para interações *in vivo*.

A proteína STAR1 pertence à classe dos transportadores do tipo ABC (ATP-Binding Cassette) e juntamente com a proteína STAR2, formam um complexo que media o transporte de UDPglicose a parede celular, mascarando os sítios de ligação do Al na célula (Huang *et al*., 2009). O gene *STAR1* é regulado pelo fator de transcrição ART1 (Yamaji *et al*., 2009) que por sua vez, não é afetado pelo tratamento com Al, sugerindo que outros sinais e ou/fatores atuem na ativação dos genes alvo de ART1.

A região do promotor de *STAR1* enriquecida no ChIP foi então analisada e um motivo similar ao encontrado por análises *in silico* (AGCCCAT) duplicado e separado por 58 pares de base foi encontrado indicando que ASR5 atua como fator de transcrição interagindo com elementos “cis” do promotor do gene *STAR1* regulando sua expressão em resposta ao Al uma vez que, 1- Os transcritos de *ASR5* e *STAR1* aumentam em resposta ao Al em plantas não transformadas; 2- *STAR1* encontra-se reprimido nas plantas ASR5_RNAi e 3- STAR1 não responde ao Al nas plantas ASR5_RNAi.

Como fator de transcrição, o ortólogo de *ASR* em videira é capaz de ligar-se ao promotor de um gene relacionado ao metabolismo de açúcar (Çakir et al., 2003) e pode formar heterodímeros no núcleo (Saumonneau *et al*., 2008). ASR1 de tomate superexpresso em *Arabidopsis* compete com o motivo de ligação do fator de transcrição ABI4, regulando negativamente diversos genes (Shkolnik and Bar-zvi, 2008). Pela primeira vez em arroz entretanto, mostramos que ASR5 liga-

se a um motivo específico do promotor de genes relacionados com a tolerância ao Al.

A obtenção de plantas de arroz transgênicas transformadas com a construção contendo o promotor de *ASR5* fusionado a GUS revelaram a ampla gama de tecidos onde ASR5 é expresso. Entre eles está o ápice das raízes. Estudos indicaram que o ápice da raiz, mais especificamente a parte distal da zona de transição do ápice, é o alvo primário da toxicidade causada pelo Al (Sivaguru and Horst, 1998). O Al pode entrar no citosol das células em minutos e a exposição prolongada pode levar a interação do Al no núcleo, resultando na interrupção da divisão celular e ruptura do citoesqueleto (Silva *et al*., 2000).

Com base nos resultados deste trabalho, um modelo hipotético para o mecanismo de ação de ASR5 foi desenvolvido. Em células da raiz, o Al que entra é percebido pela célula, ativando ASR5 que em conjunto com ART1 regula a expressão do complexo STAR1-STAR2, que transportam UDP-glicose a parede celular mascarando assim os sítios de ligação do Al, prevenindo seu acúmulo nas células (Figura 1a). Em células da parte aérea, o Al que penetrou na raiz e foi transportado, ativa a liberação de ASR5 dos cloroplastos que por sua vez, regula genes cloroplastídicos no núcleo responsáveis por mitigar o efeito do Al e pela manutenção da fotossíntese (Figura 1b).

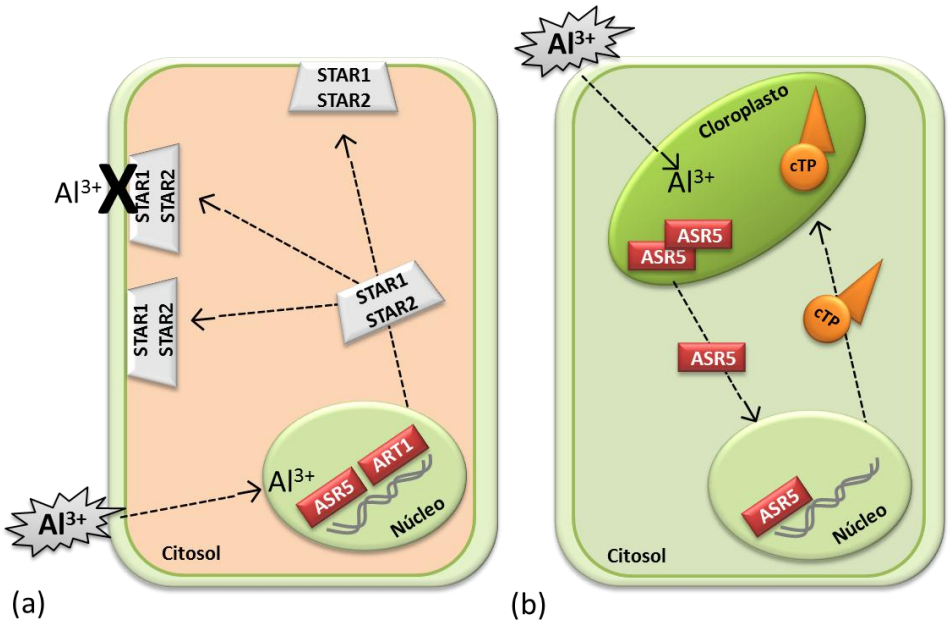

Figura 1. Modelo hipotético do mecanismo de ação de ASR5 no controle da regulação gênica por ASR5 em células de arroz em resposta ao alumínio. (a) em células da raiz, o Al ativa ASR5 que, juntamente com ART1, regula a expressão do complexo STAR1-STAR2. Este complexo transporta UDP-glicose à parede celular e mascara os sítios de ligação do Al, prevenindo seu acúmulo. (b) em células da parte aérea, o Al que entra nos cloroplastos, libera ASR5 que, por sua vez, regula a expressão de genes cloroplastídicos no núcleo responsáveis por reduzir o efeito tóxico do Al na fotossíntese.

Em conclusão, a família gênica *ASR* de arroz, em especial *ASR5*, desempenha um papel fundamental na proteção da planta em resposta ao Al, regulando direta e ou indiretamente uma gama de genes culminando na maior resistência a toxicidade causada por Al, sendo um promissor fator de transcrição para a utilização e produção de plantas com maior tolerância a Al e particularmente, a solos ácidos. Com base nos dados gerados neste trabalho, um pedido de patente foi emitido (ver anexo) no intuito de regular e proteger o uso dos genes *ASR* visando aumento da tolerância ao Al.

Como perspectivas, pretende-se agora construir vetores binários de transformação de plantas e obter plantas transgênicas superexpressando a proteína ASR5 para determinar os efeitos da super-expressão em plantas de arroz, especialmente as variedades sensíveis, frente a diversos estresses como Al, seca e salinidade.

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Anexos: Pedido de Patente

Relatório Descritivo de Patente de Invenção

CONSTRUÇÃO GÊNICA, PROCESSO PARA MODULAR A RESPOSTA OXIDATIVA EM ARROZ, PROCESSO PARA MODULAR A TOLERÂNCIA DE PLANTAS AO ALUMÍNIO

Campo da Invenção

A presente invenção está no campo de Biotecnologia e Biologia Molecular. Mais especificamente, a presente invenção proporciona: construções gênicas; processos para modular resposta oxidativa em arroz; e processos para modular a tolerância de plantas ao alumínio. Os objetos da invenção têm como conceito inventivo comum o uso de ASR de forma indireta para modular a atividade de outros materiais biológicos que contribuem para a proteção celular contra metais, especialmente contra Alumínio (Al).

Reivindicações

CONSTRUÇÃO GÊNICA, PROCESSO PARA MODULAR A RESPOSTA OXIDATIVA EM ARROZ, PROCESSO PARA MODULAR A TOLERÂNCIA DE PLANTAS AO ALUMÍNIO

A presente invenção proporciona: construções gênicas; processos para modular resposta oxidativa em arroz; e processos para modular a tolerância de plantas ao alumínio. Os objetos da invenção têm como conceito inventivo comum o uso de ASR de forma indireta para modular a atividade de outros materiais biológicos que contribuem para a proteção celular contra metais, especialmente contra Alumínio (Al).

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Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Razoavelmente.

Espanhol Compreende Razoavelmente, Fala Pouco, Lê Razoavelmente, Escreve Pouco.

Produção em C,T & A

Produção bibliográfica

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